Functional analysis of *Dlx* intergenic enhancers in the developing mouse forebrain

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Abstract:

The *Distal-less homeobox* (*Dlx*) genes encode a group of transcription factors that are involved in various developmental processes including forebrain development. *Dlx* genes are arranged in convergently transcribed bigene clusters with enhancer sequences located in the intergenic region of each cluster. The expression patterns of *Dlx1/Dlx2* and of *Dlx5/Dlx6* are attributed in part to the activity of I12a/I12b and I56i/I56ii intergenic enhancers, respectively. In an effort to determine how *Dlx* intergenic enhancers interact with the promoter regions of each cluster, I employed the Chromosome Conformation Capture (3C) technique on developing forebrain at E13.5 and E15.5. My 3C analysis provided potential enhancer-promoter interaction, in *cis*, that are consistent with previously known regulatory mechanisms. Furthermore, *trans* interactions may exist between *Dlx1/Dlx2* and *Dlx5/Dlx6* clusters in the developing forebrain at E13.5, thus providing a possible novel cross-regulatory mechanism between these two loci. I have also investigated the phenotypic consequences of *Dlx* enhancer deletion(s) on forebrain development by characterizing mice with I56ii and I56ii/I12b enhancer deletions. Enhancer deletions significantly impair *Dlx* expression as well as that of *Evf2, Gad2* and of the striatal markers *Islet1* and *Meis2*. Enhancer deletion(s) also reduce the expression of ISLET1 and CTIP2 proteins and Semaphorin 3A, Slit1 and Ephrin A5 that are thought to provide guidance cues in the corridor cells. Overall, these changes may disrupt the guidance of the thalamocortical axons. The data presented here further our understanding of the interactions between *Dlx* intergenic enhancers and promoter regions. Enhancer deletion(s) furthers our understanding of *Dlx* regulatory networks necessary that ensure proper *Dlx* expression, which, in turn may be involved in a genetic pathway underlying the synthesis of GABA, which may be further essential in maintaining the GABAergic phenotype.

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Abbreviations and nomenclature:

BAC: bacterial artificial chromosome

bp: base pairs

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

MGE: Medial Ganglionic Eminence

CGE: Caudal Ganglionic Eminence

Mash1: Mammalian achaete scute homolog-1

mI56ii: mouse I56ii enhancer

VZ: Ventricular Zone

SVZ: Subventricular Zone

MZ: Mantle Zone

ncRNA: non-coding ribonucleic acid

PBS: phosphate buffered saline

PFA: paraformaldehyde

qRT- PCR: Quantitative Real-Time PCR

RNA: ribonucleic acid

RT: room temperature

SNP: single nucleotide polymorphism

trucRNA: transcription-regulating ultraconserved non-coding ribonucleic acid

URE2: upstream regulatory element 2

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

Statement of Contributions:

The thesis, "Functional analysis of *Dlx* intergenic enhancers in the developing mouse forebrain", is composed of three related studies, all of which were collective efforts.

Chapter 2 is the result of the experiments that were primarily carried out by myself. I designed the 3C primers and generated various libraries. Our lab technician, Gary Hatch assisted me with the trouble-shooting aspect of the project. Dr. Josee Dostie at McGill University helped us with the initial design of the experiments and interpreting the data. I wrote the initial manuscript.

Chapter 3 is the results of the experiments that were performed by a group of graduate students to characterize the consequence of enhancer deletion on *Dlx* activity. I performed qRT-PCR on I56ii mutant line as well as all the in situ hybridization and immunohistochemistry experiments on I56i, vI56i and I56ii mutant mice. I also wrote the original manuscript. Crystal Esau performed qRT-PCR on I56i mutant mice and performed behavioural studies on the I56i mutant mice. Cindy Lesage-Pelletier and Man Yu performed qRT-PCR analysis of vI56i and I12b mutant mice respectively. Dr. Luc Poitras designed and contributed to the production of all mutant lines.

Chapter 4 is the results of the experiments that were designed and performed primarily by myself and a group of undergraduate students who assisted me and worked under my supervision. Meina Roufaiel helped with the qRT-PCR analysis of I56ii mutant, while Ema Allemano helped with the qRT-PCR analysis of the I56ii-I12b mutant mice. William Nicola and Samuel McDonnell assisted me with the *in situ* hybridization portion of this project. Lastly, Tanya Plaoude assisted me with sectioning and immunohistochemistry experiments. I wrote the initial manuscript for this project.

Chapter 1: Introduction

1.1 Chromosome territory as a measure of genomic organization:

The largest genomic unit of organization within the eukaryotic cell is the chromosome. Chromosomes are arranged according to the chromosome territory which includes the preferred location of chromosomes with respect to each other and to the center or the periphery of the nucleus (Cremer et al., 2006; Fraser, 2006). Therefore, the positioning of the chromosomes as well as their neighbouring in the nucleus is not random (Fraser & Bickmore, 2007). This precise genomic organization has a direct impact on the chromosome's ability to interact with other parts of the genome, whether in *cis* or *trans*, as it has been demonstrated in studies investigating the frequency of chromosome translocation within the nucleus (Parada et al., 2004; Parada & Misteli, 2002). Such spatial organization within the nucleus plays a very important role on regulating gene expression (Fraser & Bickmore, 2007; Parada & Misteli, 2002). Several studies over the past decade have investigated this phenomenon. Kurz et al. (1995) demonstrated that the activity or inactivity of certain genes is directly correlated to their genomic localization relative to their chromosome territory (Kurz et al., 1995). *Hoxb* studies utilizing Circularized Chromosome Conformation Capture (4C) analyses demonstrated that a gene's nuclear environment is determined by the two-dimensional structure of the genome and the three-dimensional organization of the chromosome territory (Würtele & Chartrand, 2006).

Studies have shown that genes have the ability to relocate outside of the chromosome territory (Biran & Meshorer, 2012). Such events can occur in regions with considerably high gene expression (Mahy et al., 2002) or when the expression is induced experimentally (Volpi et al., 2000). For instance, the *Hoxb* cluster is activated once it has relocated outside of or looped out from the chromosome territory (Bickmore & Chubb, 2003; Würtele & Chartrand, 2006). Other

studies have shown that when the activity of RNA polymerase II was experimentally inhibited, it reduced the rate of chromosome relocation outside of the chromosome territory, suggesting that this process is in fact induced by the process of gene transcription itself (Mahy et al., 2002; Volpi et al., 2000). A recent study on the *Hoxd* cluster in developing mouse embryos revealed that the activation of *Hoxd* along the anterior-posterior axis is initiated when *Hoxd* is looped out from the chromosome territory. However, initiation of *Hoxd* expression in the limb bud, at the same developmental stage, did not require the gene to loop out of its chromosome territory (Morey, Da Silva, Perry, & Bickmore, 2007). These findings imply that gene relocation out of chromosome territory depends on the way gene transcription was activated (Morey et al., 2007). However, whether the "looping-out" mechanism is a bi-product of transcriptional activity or whether it plays a role in regulating transcription processes, has not been fully understood.

Lastly, nuclear co-localization of the active genes and regulatory elements within the nuclear space is another means by which chromosome territory can influence gene activity. Actively transcribed genes are usually localized within regions referred to as transcription factories (Osborne et al., 2004; Osborne & Eskiw, 2008). It has been suggested that multiple actively transcribed genes may share the same factory since the number of transcription factories are lower than the number of expressed genes within the nucleus (Osborne et al., 2004). This is further supported by the study of β-globin gene in fetal liver tissue, where the β-globin gene was associated with the several other actively transcribed genes (Simonis et al., 2006).

It is evident that the genes within the nucleus have some independence in their nuclear localization, but do not function in isolation from one another. These genomic environments such as transcription factories are frequently shared by different genes (Osborne & Eskiw, 2008). As a result, multiple active genes are often located together at the places within the nucleus where there

are high concentrations of transcriptional proteins in order to maintain efficient transcriptional processing (Chambeyron & Bickmore, 2004; Mahy et al., 2002; Osborne et al., 2004). The ability to detect interactions between regulatory elements has provided further supporting evidence to such studies (Simonis et al., 2006; Simonis, Kooren, & de Laat, 2007). Thus, genomic organization reflects the processes that regulate the genome's functions (Chakalova et al., 2005). As a result, genome structure is not a rigid conformation, but a dynamic organization, which changes during development and differentiation (Chakalova et al., 2005; Osborne et al., 2004).

1.2 Modification of Enhancer Chromatin

Functional *cis*-regulatory elements mediate the interpretation of genomic information at the chromatin level. Amongst the *cis*-regulatory elements, enhancers are capable of stimulating transcription initiation of their target at a great distance and also play an essential role in cell-type-specific gene expression (Ong & Corces, 2011). Current understanding of the chromatin features associated with the enhancer region allowed researchers to be able to identify and annotate enhancers at a whole-genome level (Buecker & Wysocka, 2012; Heintzman et al., 2009). Current work in the field of epigenomic profiling has provided strong evidence that enhancers are the most dynamic part of the genome with a central feature; their ability to serve as a binding platform for transcription factors (TFs) (Spitz & Furlong, 2012). Enhancer DNA is usually 200 – 500 bp in length and mainly contains the recognition sites for variety of TFs (Weake & Workman, 2010).

Enhancers are generally activated prior to transcription initiation via the presence of several TFs and the recruitment of co-activator proteins (Heintzman et al., 2007). Co-activators usually stimulate enhancer function through histone modifications (Natoli & Andrau, 2012). Enhancers play an important role in the formation of the pre-initiation complex or the transition from initiation

to elongation by delivering the regulatory factors to the promoter of the target gene (Bulger & Groudine, 2011). Therefore, two major models, "looping" and "tracking" have been proposed in order to explain the mechanisms underlying such long-range communication between enhancer and promoter (Bulger & Groudine, 2011). According to the looping model, the enhancer and associated regulatory factors are brought within close physical proximity to the promoter by a direct interaction between the two regulatory regions while "looping out" the intervening DNA sequence. However, the tracking model proposes that the enhancer region initiates transcription by "tracking" RNA polymerase II along with other factors down the intervening DNA to interact with the promoter (Bulger & Groudine, 2011). Of the two models, the looping model has been supported extensively with the emergence of the chromosome conformation capture technique and its derivatives (de Wit & de Laat, 2012; Dekker, 2003, 2008; Dekker, Rippe, Dekker, & Kleckner, 2002; Gibcus & Dekker, 2013; Miele & Dekker, 2008). However, the tracking model requires further investigation and systematic analysis.

Active enhancers are functionally defined as DNA *cis*-regulatory elements that are capable of acting on and regulating the activity of their target independent of their genomic position and orientation (Ong & Corces, 2011). The flexible genomic localization of enhancers with respect to their target gene complicates the genome-wide identification of these enhancers. It was not until recently due to technological advancements including microarray-based (ChIP-on-ChIP) and high throughput sequencing (ChIP-seq) that researchers were able to identify enhancers at the genome-wide scale (Barski et al., 2007; Guenther et al., 2007; Mendenhall & Bernstein, 2008). Heintzman and colleagues (2007) investigated the chromatin signature of transcriptional promoters and enhancers using components of the transcription machinery including RNA polymerase II, Transcription factor Binding Protein-associated factor 1 (TAF1) and transcriptional co-activator

P300 (Heintzman et al., 2007). It was demonstrated that the mono-methylation of histone H3 lysine 4 (H3K4me1) was one hallmark of enhancers in HeLa cells (Heintzman et al., 2007). This finding, however, has several caveats since H3K4me1 enriched regions are usually a lot larger than the associated enhancer, which complicates the identification of the exact location of the enhancer region (Barski et al., 2007; Pekowska et al., 2011). Additionally, the presence of H3K4me1 does not necessarily correlate with the functional activity of the enhancer (Mercer et al., 2011). Another study conducted by Wang and colleagues (2008) demonstrated that putative active enhancer regions in human $CD4^+$ T cells are highly enriched in a large set of histone modifications including H3K4me1, H3K4me2, H3K4me3, and H3K27 acetylation (H3K27ac) (Wang et al., 2008). This discovery was further supported by comparative studies of epigenetic profiling of ES cells and various differentiated tissues (Bernstein et al., 2006; Cui et al., 2009; Zentner et al., 2011). It was confirmed that H3K27ac was associated with active enhancers (Bernstein et al., 2006; Cui et al., 2009; Zentner et al., 2011).

It has been suggested that the abundance of H3K9me3 is a chromatin signature of active promoters (Heintzman et al., 2007). However, Pekowska and colleagues (2011), through their work on T cells from adult mouse thymus, demonstrated that active enhancers were generally associated with both H3K4me2 and H3K4me3; whereas, H3K4me1 was associated with enhancers regardless of their functional state (Pekowska et al., 2011). Thus, it is important to mention that H3K4me1 is not unique to enhancers since it is ubiquitously distributed and covers a much greater genomic region than the underlying enhancer elements (Barski et al., 2007). It has been suggested that H3K4me1 perhaps presents a window of opportunity for the activation of enhancer chromatin. Further specification of the spatiotemporal activity of the enhancer is determined by a combinatorial assembly of other *cis* or *trans* signals during this window of opportunity (Calo &

Wysocka, 2013). Indeed, Bonn and colleagues (2012) demonstrated that H3K27ac modification associated with active enhancers is acquired in the context of pre-existing H3K4me1 (Bonn et al., 2012). Collectively, it has been debated that distal H3K4me1 domains are highly enriched at lineage specific enhancers, whereas the activity of such enhancers can be further affected by the presence of other histone modifications (Ernst et al., 2011; Heintzman et al., 2009; Mikkelsen et al., 2007). Therefore, active enhancers could be more accurately identified by the presence of H3K4me1, H3K4me2, H2K27ac, and for a small group of active enhancers, H3K4me3 and H3K9ac (Ernst et al., 2011).

1.3 Conservation and divergence of *cis*-regulatory information

Cis-regulatory information encoded in elements such as enhancers and promoters are responsible for regulating gene expression. Mutations within *cis*-regulatory elements that affect the activity of these regulatory elements and as a result the regulation of gene expression is thought to be one source of evolutionary and phenotypic divergence (Carroll, 2008). A recent study by Savinkova and colleagues (2009) demonstrated that even though mutations in the promoter region are often associated with human disease, they do not appear to be the main cause of *cis*-regulatory divergence (Savinkova et al., 2009). This could be explained by the fact that promoters bind to a core group of commonly used and highly conserved regulatory factors controlling transcription within the cell and that promoters alone produce very low levels of mRNA during eukaryotic transcription (Brown & Feder, 2005). On the other hand, since enhancers are highly variable between different species, enhancers are thought to be the *cis*-regulatory element more often responsible for *cis*-regulatory divergence (Wray, 2007). A recent study by Hong and colleagues (2008) has shown that pairs or groups of enhancers that have a large functional overlap tend to

quench the effect of a mutation in one enhancer on the expression of the target gene and contribute to the phenotypic stability (Hong et al., 2008). Recently, Wittkopp and colleagues (2004) by measuring allele-specific expression, were able to detect the differences in cis-regulatory activity and showed that *cis*-regulatory divergence is a common phenomenon between species (Wittkopp et al., 2004). However the molecular mechanisms underlying such divergence remains mainly unknown due to the complex task of identifying the functionally divergent sites within each *cis*-regulatory element and further elucidating how these changes would affect interactions with other regulatory factors that control the expression of the target gene (Wittkopp et al., 2004).

It has been widely debated what kind of mutations underlie *cis*-regulatory divergence during evolutionary processes. However, three different mechanisms have been proposed to play an important role in *cis*-regulatory divergence (Frankel et al., 2011; Jeong et al., 2008; Williams et al., 2008). First, nucleotide substitutions that represent from point mutations are often considered to be sufficient to alter the activity of the *cis*-regulatory elements between species (Jeong et al., 2008; Williams et al., 2008). For instance, a study conducted by Frankel and colleagues (2011) showed that the difference between the *cis*-regulatory activity of *Drosophila* species associated with the loss of trichomes is simply due to thirteen nucleotide substitutions within each divergent enhancer region (Frankel et al., 2011). Deletions are the second mode of mutation that can change the activity of the *cis*-regulatory elements (Chan et al., 2010). Current investigations by Chan and colleagues (2010) corroborated the second proposed mechanism, where the fragile DNA sequence in the enhancer region of the three-spine stickleback appears to be prone to deletion mutations. These deletion mutations within the regulatory regions disrupt the activity of the enhancer, which results in the formation of alleles that contribute to the loss of pelvic structure in these fish (Chan et al., 2010). In this case, the deletion reduced the activity of the *cis*-regulatory elements in the

stickleback. However, another study in *D. melanogaster* demonstrated that the deletions within the enhancer region can increase the activity of the target gene by either removing a repressor binding site or by creating a novel binding site for transcriptional activators (Shirangi et al., 2009). Lastly, insertions are thought to be the third mode of *cis*-regulatory divergence (Chung et al., 2007; Kidwell & Lisch, 1997; Williams et al., 2008). Insertions can impact enhancer activity by introducing new transcription factor binding sites or by disrupting the genomic distances between transcription factor binding sites (Williams et al., 2008). In addition to affecting transcription factor binding, it has been well documented that insertions of transposable elements within the enhancer region can also contribute to *cis*-regulatory divergence by recruiting new regulatory elements to the target gene (Chung et al., 2007; Kidwell & Lisch, 1997). These insertions are shown to be a predominant source of phenotypic variation amongst different species (Kidwell & Lisch, 1997).

Overall, *cis*-regulatory divergence is considered to be one of the most important sources of evolutionary innovations (Carroll, 2008). This process is further facilitated by genomic redundancies that can reduce the impact of loss-of-function mutations (Hong et al., 2008). This buffering mechanism during the evolution of *cis*-regulatory elements is proposed to be achieved through "shadow enhancers"; where multiple enhancers share a functional overlapping activity pattern for the target gene (Hong et al., 2008). Additionally, the pilot project by the ENCODE consortium demonstrated that neutral binding sites might serve as a *de novo* source of novel enhancers from non-coding regions or regions of low genomic complexity (Consortium et al., 2007). Such *de novo* processes could have happened throughout evolution. This hypothesis is supported by a study on *Ciona* muscle enhancers, which demonstrated the presence of paralogous enhancers with vastly different architecture that can achieve the same function (Brown et al.,

2007). However, the exact mechanisms that maintain the *cis*-regulatory elements of developmental genes over the course of evolution is not fully understood. The high complexity of the regulation of transcription during embryonic development, where spatial and temporal information are combined, requires the assembly of very sophisticated multi-protein complexes (Plessy et al., 2005). Formation of such complex machinery at the enhancer region could prevent the accumulation of mutations within enhancers that would otherwise lead to loss of transcription factor binding sites, which would destabilize the entire complex. Such destabilization may disrupt the expression pattern of the target gene which could possibly result in embryonic lethality (Plessy et al., 2005). One set of such developmentally crucial gene family that plays a significant role during embryonic development are known as the *Dlx* homeobox genes.

1.4 *Dlx* genes

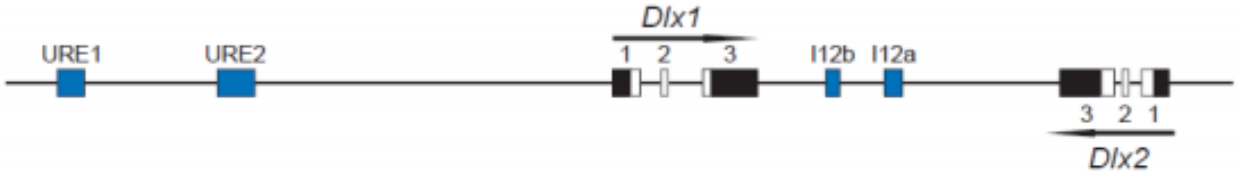
Dlx genes are mammalian homologs of the *Drosophila Distal-less* (*Dll*) gene that plays an important role in the development of the fly's appendages (Zerucha & Ekker, 2000). In *Drosophila*, the *Dll* gene is predominantly expressed during early embryogenesis and is responsible for the development of limbs, optic lobe and the glia cells of the ventral nerve cord (Kaphingst & Kunes, 1994). Prior to divergence of vertebrates from their ancestor and eventual diversification, the ancestral *Dll* gene underwent a series of duplication events, which led to the presence of up to three gene clusters in vertebrate species known as the *distal-less homeobox* gene clusters (*Dlx)*; namely the *Dlx*1/*Dlx*2, *Dlx*5/*Dlx*6, and *Dlx*3/*Dlx4* clusters (Fig. 1.1) (Zerucha et al., 2000). In the mouse, the *Dlx1/Dlx2* cluster is located on chromosome 2, whereas *Dlx5/Dlx6* and *Dlx3/Dlx4* are located on chromosome 6 and 11, respectively. In mice and humans, each *Dlx* bigene cluster is linked to a *Hox* cluster (Panganiban & Rubenstein, 2002). For instance, *Dlx1* and

Dlx2 are linked to the *Hoxd* cluster, whereas, *Dlx3* and *Dlx4* are linked to *Hoxb*, and *Dlx5* and *Dlx6* are linked to *Hoxa* (Panganiban & Rubenstein, 2002).

Dlx homeobox gene clusters are generally arranged in a convergent configuration with some of their *cis*-regulatory elements located in the intergenic region (which range in size from 3.5-16 Kb) (Zerucha & Ekker, 2000; Zerucha et al., 2000). Each *Dlx* gene is comprised of three exons, in which the homeobox region is spread over the second and third exons (Liu et al., 1997). Due to sequence similarities between different *Dlx* genes, mammalian *Dlx* genes are subcategorized into two groups comprising *Dlx1*, *Dlx6* and *Dlx4* in one group, while *Dlx2*, *Dlx3* and *Dlx5* form a second group (Stock et al., 1996). The expression patterns of *Dlx* genes overlap significantly despite their distinct overall identity (Ghanem et al., 2007). In addition to their role in appendage development, *Dlx* genes are thought to be involved in the development of the forebrain (including ventral telencephalon and diencephalon), branchial arches, neural crest, jaw and craniofacial bones, and sensory organs (Panganiban, 2000; Panganiban & Rubenstein, 2002). Since *Dll* functions have been conserved throughout evolution, including in the nervous system of *Caenorhabditis elegans*, it has been proposed that *Dll* functionality in the development of the nervous system predates the evolution of limbs (Panganiban, 2000).

Figure 1.1: Schematic representation of the genomic organization of the vertebrate *Dlx* genes. Exons of the *Dlx* genes are numbered with black boxes representing untranslated 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*).

Dlx1/Dlx2



Dlx3/Dlx4

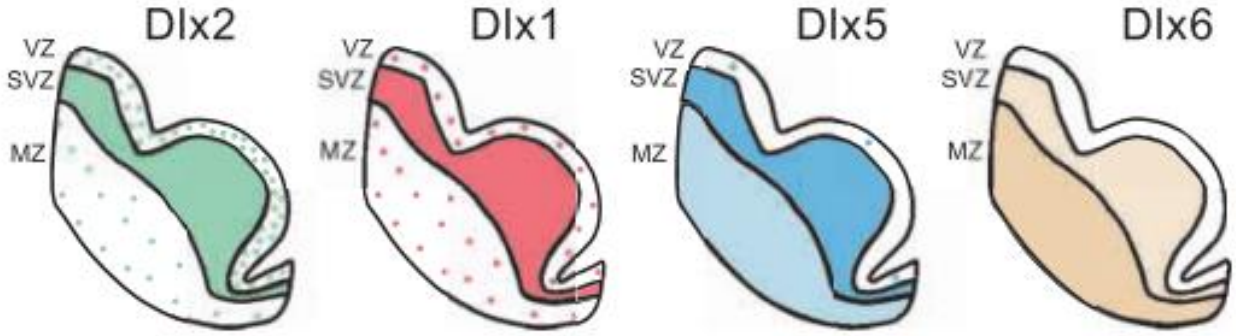
Dlx5/Dlx6

1.5 Expression patterns of the *Dlx* genes

As mentioned previously, *Dlx* genes play an important role during forebrain development. More specifically, four of the six *Dlx* genes (*Dlx*1/*Dlx*2 and *Dlx*5/*Dlx*6) are expressed in the forebrain, particularly in the majority of neural progenitor cells of the ventral telencephalon (Zerucha & Ekker, 2000). These *Dlx*-expressing progenitor cells migrate from the ventricular zone (VZ) to the subventricular zone (SVZ) and up to the cortex via tangential migration where they differentiate into GABAergic interneurons (Eisenstat et al., 1999). *Dlx2* is the first *Dlx* gene to be expressed in the immature cells of the ventricular and subventricular zone of the medial (MGE) and lateral ganglionic eminences (LGE) (Figure 1.2). The activity of *Dlx1* precedes *Dlx5*/6 expression in the vast majority of *Dlx2*-expressing cells of the SVZ and in the mantle zones (MZ) of the MGE and LGE (Eisenstat et al., 1999). Following *Dlx5* expression, *Dlx6* is also activated. Both *Dlx5* and *Dlx6* are expressed in the post-mitotic differentiating neurons of the SVZ and MZ (Wang et al., 2011). Merlo and colleagues (2000) demonstrated that later during development, *Dlx5* and *Dlx6* are expressed in the SVZ of the olfactory bulb. *Dlx5* and *Dlx6* expression persists at birth in the olfactory tuberculum and in the neocortex (Figure 1.2) (Merlo et al., 2000). It is clear that forebrain expression of *Dlx* paralogs overlaps in the telencephalon and the genes are activated one after the other, which suggests that there might be a partial redundancy in the function of the forebrain *Dlx* genes and their participation in a regulatory cascade (Zerucha & Ekker, 2000).

Figure 1.2: (A) Schematic representation of the *Dlx* expression pattern in the ventral telencephalon of E12.5 developing mouse embryo. Green (*Dlx2*), Red (*Dlx1*), Blue (*Dlx5*) and Beige (*Dlx6*). (B) Hypothesized *Dlx* genetic pathway showing the proposed order in which *Dlx* genes are activated. (Adapted from Panganiban and Rubenstein 2002).

A)



B)

Model:

Dlx2 → Dlx1 → Dlx5 → Dlx6

Dlx expression however, is not limited to the forebrain and the nervous system in vertebrates. *Dlx* genes are also active in the surface ectoderm including the branchial arches, apical ectodermal ridge of limb buds and in mesodermally derived cells such as in the otic vesicles, teeth and skeletal tissues (Merlo et al., 2000). The expression patterns of the *Dlx* genes, however, vary much more markedly in the branchial arches when compared to the forebrain expression patterns. In the branchial arches *Dlx1/Dlx2* are expressed all along the proximodistal axis, whereas the other *Dlx* genes are expressed more distally (Sumiyama & Ruddle, 2003). Sumiyama and Ruddle (2003) proposed that, considering the distinct *Dlx* expression patterns in the branchial arches, *Dlx* genes may play an essential role in jaw patterning of higher vertebrates (Sumiyama & Ruddle, 2003). In addition to their activity in the forebrain and branchial arches, *Dlx5* and *Dlx6* are expressed in the perichondrium and osteoblasts of endochondral bones (Panganiban & Rubenstein, 2002). It has been shown that during vertebrate limb development, all *Dlx* genes are responsible for regulating limb outgrowth and play an essential role in forming the apical ectodermal ridge of the limb bud (Panganiban & Rubenstein, 2002).

1.6 *Dlx* null phenotypes

Since *Dlx* genes are highly involved in the developmental processes, different studies have tried to characterize the phenotypic consequence of *Dlx* null mutations on the developing embryo, at birth and beyond. Anderson and colleagues (1997) demonstrated the importance of *Dlx* genes in the early patterning of the embryo by generating *Dlx1* and *Dlx2* null mice by homologous recombination in embryonic stem (ES) cells. Mutant mice lacking *Dlx*2 and *Dlx1/Dlx2* are not viable and die within hours of birth; however, *Dlx1* single mutant mice live up to one month after birth (P30) (Anderson et al., 1997a). In this study the main cause of death in the *Dlx*2 and

Dlx1/Dlx2 mutants was thought to be due to defects in the craniofacial and enteric nervous system (Anderson et al., 1997a). Phenotypically, mutant mice appeared to be normal, but the internal structures showed alterations in the craniofacial bones including the first and second arches (Qiu et al., 1997). Further examination demonstrated that *Dlx1/Dlx2* double-mutants lacked all maxillary upper molars, whereas the single *Dlx1* and *Dlx2* mutants exhibited normal phenotype with all incisors and molars in their correct position (Qiu et al., 1997). Further characterization of the impact of *Dlx1* and *Dlx2* null mutations on forebrain development demonstrated predominant disruptions in cell differentiation and migration in the neo-cortex of the mutant mice. These disruptions resulted in a significant reduction of GABAergic interneurons (Anderson et al., 1997b). At the molecular level, *Dlx5* and *Dlx6* transcript levels are severely reduced, eliminating almost all *Dlx* gene (*Dlx1*, *Dlx2*, *Dlx5*, and *Dlx6*) expression in the forebrain (Anderson et al., 1997b). Likewise, a separate study conducted by Qiu and colleagues (1995) showed that *Dlx1* mutant mice exhibited no phenotypic abnormalities in their forebrain, whereas *Dlx2* mutants exhibited reduction of interneurons in the olfactory bulb (Qiu et al., 1995).

It has been shown that *Dlx3* null mice exhibit a severe phenotype and die before E9.5 due to reduced placental vascularization as well as reduced secretion of placental growth factor (Clark et al., 2012). Interestingly, other *Dlx* genes do not seem to compensate for the loss of *Dlx3* function during placental formation and development (Clark et al., 2012). Thus far, *Dlx4* null mice have not been characterized and as a result, the impact of *Dlx4* is yet to be identified. A study by Depew and colleagues (2005) demonstrated that *Dlx5* null mice exhibit a severe phenotype with a multitude of craniofacial defects, malformations in the olfactory pit, associated skeletal elements and the inner ear (Depew et al., 2005). This study also demonstrated that the *Dlx5/Dlx6* knockout mice exhibited exencephaly, which is identified by the lack of closure of the neural tube leading

to the development of the brain outside of the skull. Additionally, this mutation is also associated with severe mandibular arch development in which the lower jaw structure is transformed into an upper jaw (Depew et al., 1999; Depew et al., 2005). According to these studies, it has been shown that the phenotypic effects observed in double mutants are much more severe than the ones observed in the single mutant, which further supports a functional redundancy between *Dlx* genes. Panganiban and colleagues (2002) suggested that, in the branchial arches, the *Dlx5/Dlx6* genes that are expressed more distally may play a compensatory role for the loss of proximally expressed *Dlx1/Dlx2* genes (Panganiban & Rubenstein, 2002). This hypothesis is supported by the evidence observed from the *Dlx1/Dlx2* double-mutant where the phenotypic consequence of these mutations are only seen in the proximal structure; however, in the case of *Dlx5/Dlx6* double mutants, *Dlx1/Dlx2* and *Dlx3/Dlx4* do not show any compensatory functions for the loss-of-function of *Dlx5/Dlx6*, exhibiting a severe phenotype and disruption of the proximodistal axis patterning in the developing branchial arches (Panganiban & Rubenstein, 2002).

1.7 Neuronal Migration

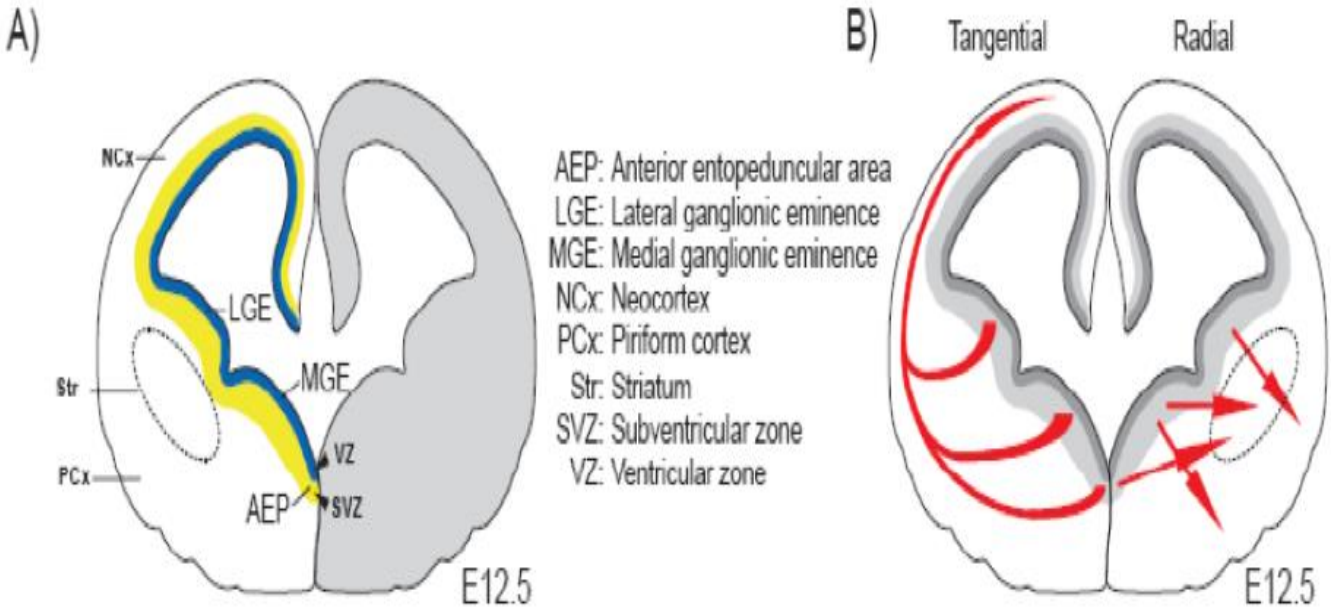
Proper brain development and function is dependent upon the proper neuronal migration to their correct final destination and subsequent differentiation (Wichterle et al., 2003). Guidance molecules located in the extracellular matrix aid in such axonal pathfinding (Wichterle et al., 2003). In addition to guidance cues, patterning information also provides very crucial information in directing the migrating neurons along their paths. It has been suggested that during brain development, post-mitotic neurons display guidance cues as the neuronal growth cones find their way (López-Bendito & Molnár, 2003). This patterning information is transferred to the post-mitotic cells from neuronal progenitors via radial migration. Radial migration is a mode of

neuronal migration in which the newly born neurons occupy adjacent regions of the mantle zone (Lopez-Bendito et al., 2006). Thus, it has been suggested that the main role of radial migration is to convey the patterning information from progenitor cells to post-mitotic neurons.

Another essential mode of neuronal translocation within the developing brain is referred to as tangential migration (Figure 1.3) (Lopez-Bendito et al., 2006; López-Bendito & Molnár, 2003; Wichterle et al., 2003). During tangential migration, neurons migrate parallel to the surface of the brain from the ventral telencephalon to the developing neocortex (Parnavelas, 2000). It has been thought that tangential migration has evolved to allow for a much higher level of complexity in the neuronal circuitry, since tangential migration allows the neurons born from different progenitor zones to have the same final destination (Marin & Rubenstein, 2001). Tangential migration occurs frequently in the nervous system but is much more prominent in the developing ventral telencephalon since various thalamocortical connections navigate through towards their final destination (Figure 1.3) (Lopez-Bendito et al., 2006; Marin & Rubenstein, 2001). Tangentially migrating thalamocortical axons are responsible for transferring sensory and motor information to the cortex, where an appropriate response is generated (Lopez-Bendito et al., 2006; Marin & Rubenstein, 2001). It has been shown that the development of the thalamocortical projections in forebrain depends on the early tangential migration of a population of neurons derived from the ventral telencephalon (Poitras et al., 2007). The tangential migration of the thalamocortical axons are facilitated via the establishment of a permissive corridor (referred to as corridor cells), essential for thalamocortical pathfinding (Lopez-Bendito et al., 2006). A recent study by Lopez-Bendito and colleagues (2006) using a GFP-expressing tissue transplant onto telencephalic slice cultures suggested that corridor cells play an essential role in tangential migration which allows the enhancement of the functional complexity of the brain by mixing of neuronal subtypes from

different progenitor zones. In addition, corridor cells also provide support and guidance for the axonal projections that are migrating towards the neocortex (Lopez-Bendito et al., 2006). Other studies have suggested that corridor cells express Gad, responsible for GABA synthesis, which suggests that corridor cells are GABA-containing (GABAergic) neurons (Marin, 2003; Marín et al., 2002).

Figure 1.3: Schematic representation of the tangential and radial neuronal migration within the coronal section of the ventral telencephalon of the developing E12.5 mouse embryo. (Adapted from Panganiban and Rubenstein 2002).


A)
NCx
LGE
MGE
Str
PCx
VZ
AEP
SVZ
E12.5
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)
Tangential
Radial
E12.5

1.8 *Dlx* function and GABAergic Interneurons

GABAergic interneurons are a group of inhibitory interneurons that utilize γ-aminobutyric acid (GABA) as their neurotransmitter (Panganiban & Rubenstein, 2002). It has been demonstrated that the endogenous expression pattern of forebrain *Dlx* genes (namely *Dlx1, Dlx2, Dlx5* and *Dlx6*) overlap with the activity of GABAergic interneurons in the telencephalon (Stühmer, Anderson, Ekker, & Rubenstein, 2002). As mentioned in section 1.6, *Dlx1/Dlx2* double-mutant mice showed a severe defects in neuronal migration, maturation and a significant decrease in number of GABAergic projections and interneuron; further supporting the essential role of DLX1 and DLX2 proteins in the development of GABAergic neurons (Anderson et al., 1997a; Eisenstat et al., 1999). The *Dlx1/Dlx2* double mutant mice also exhibited a decrease in *Gad* expression. *Gad* is the enzyme responsible for synthesizing GABA from glutamic acid, which is mainly expressed in the axon terminals and the dendritic regions (Anderson et al., 1997b; Anderson et al., 1997a; Benes & Berretta, 2001). The two *Gad* isoforms, namely *Gad1* and *Gad2*, are co-localized in approximately 95% of GABA expressing cells within the hippocampus.

GABAergic interneurons are born in the subpallial lateral ganglionic eminence (LGE) and migrate tangentially to the olfactory bulb, hippocampus and neocortex (López-Bendito & Molnár, 2003; Potter et al., 2009). Even though it has been suggested that GABAergic interneurons are inhibitory in nature, Ben-Ari (2002) showed that during the early stages of forebrain development, GABAergic interneurons play an excitatory role in order to initiate the cortical network and then become inhibitory once the developing neurons have been excited (Ben-Ari, 2002). Potter and colleagues (2009) suggested that 20% of all neurons found in the cortex and hippocampus are GABAergic interneurons. Additionally, 95% of all the neurons in the striatum are GABAergic (Potter et al., 2009).

A study conducted by Benes and Berretta (2001) subdivided GABAergic interneurons into different groups according to their synaptic connectivity, morphology, and electrophysiological properties (Benes & Berretta, 2001). The first group of GABAergic interneurons is referred to as 'basket cells', which comprise the most commonly encountered GABAergic interneurons. One of the unique characteristics of the 'basket cells' is the presence of an axo-somatic inhibitory synapse. The second group of GABAergic interneurons includes 'chandelier cells'. The characteristic feature of this subgroup of GABAergic interneuron is that the axonal branches extend at right angles forming "candles" in a vertical orientation. Additionally, the second GABAergic subgroup does not possess any axonal inhibitory synapses. The third group of GABAergic interneurons, referred to as 'bouquet' and 'tuft cells', are capable of distributing within the narrow columns of the cortical mantle zone. In addition to their unique distribution, this group possesses axo-dendritic inhibitory and excitatory synapses (Benes & Berretta, 2001). The various GABAergic interneuron subtypes are characterized by their unique expression profiling of neurochemical markers, especially calcium binding proteins including calbindin, parvalbumin, calretinin, somatostatin, and neuropeptide-Y (Potter et al., 2009). Since GABAergic interneurons are mostly inhibitory, it uses GABA to regulate the activity of the other excitatory neurons via a negative feedback loop. GABA, upon being released from the synapse, activates the chloride receptors. This activation results in an influx of chloride, which initiates an inhibitory postsynaptic potential in the postsynaptic neurons (Sun et al., 2012).

1.9 *Cis* and *Trans* Regulation of the *Dlx* genes

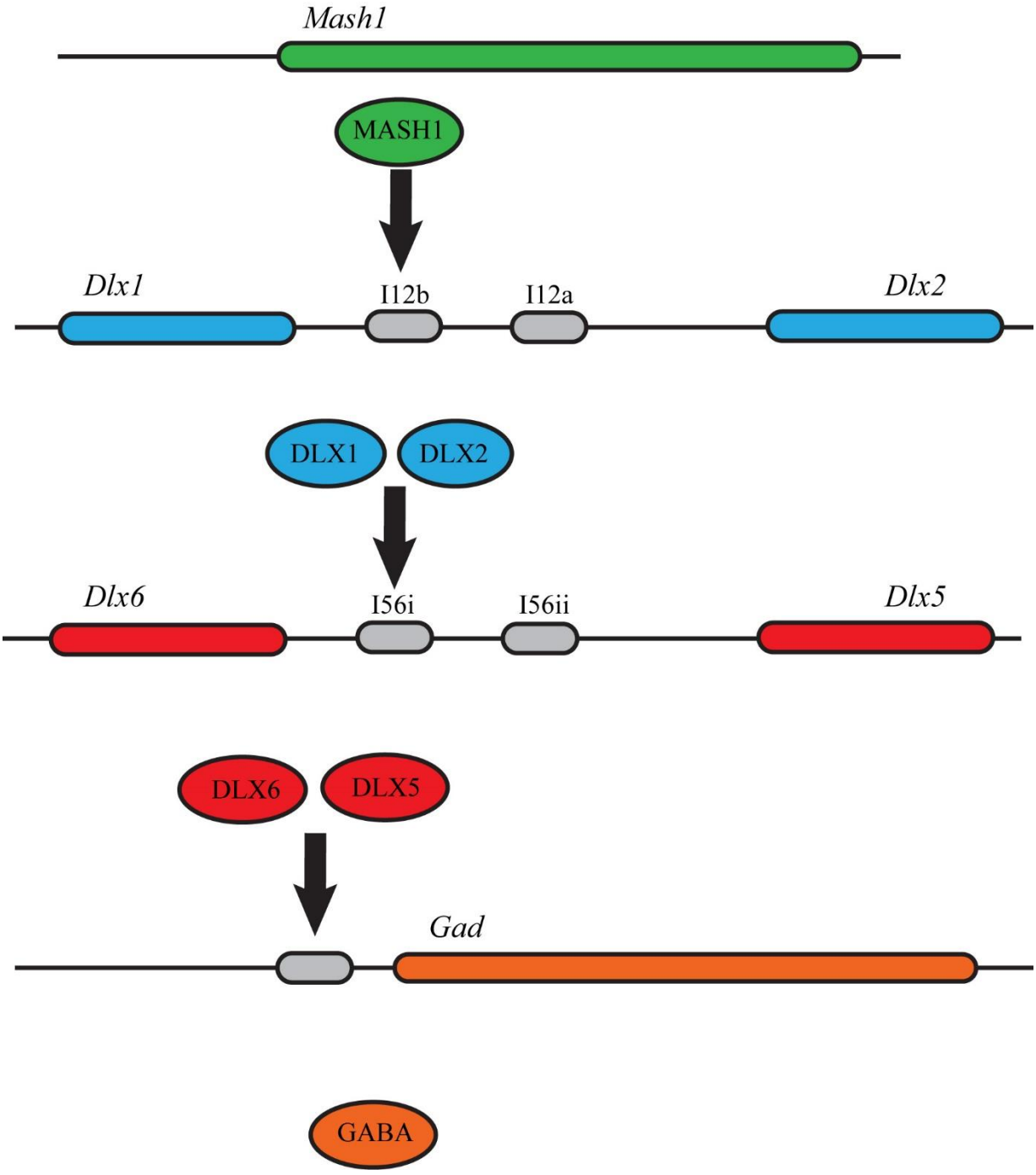
Cis-regulatory elements regulate the activity of the developmental genes by attracting a specific group of regulatory factors including transcription factors, co-activators, and other extrinsic signals received from neighbouring cells that allows the gene to be turned on and off appropriately during development. Transcription factors, amongst other cofactors, are regulatory

proteins that are responsible for activating and repressing transcription (Wolpert & Tickle, 2011). Transcription factors harbor a DNA binding domain, which allows them to differentiate between regulatory sequences and between target genes. *Homeobox* genes are one of the important classes of transcription factors responsible for regulating genes during development (Wolpert & Tickle, 2011). *Homeobox* genes are usually characterized by the presence of a homeodomain that contains a structural domain of helix-turn-helix configuration that interacts directly with DNA (Alberts et al., 2002). Vertebrate *Dlx* genes contain a DNA binding homeodomain, which ensures that transcription of the target genes is regulated at the appropriate time and place during developmental stages. As discussed in section 1.5, due to the overlapping expression patterns of *Dlx* genes and to their bigene cluster arrangement, the idea of enhancer sharing has been proposed in which the *cis*-regulatory elements located within the intergenic regions of each *Dlx* cluster interact with one another and with the *Dlx* genes (Zerucha & Ekker, 2000; Zhou et al., 2004).

In addition to *cis*-regulatory elements, several different *trans*-acting factors have also been identified as key regulators of the *Dlx* genes. Several studies have demonstrated that *Mammalian achaete scute homolog-1* (*Mash1),* which is an upstream regulator of *Dlx1/Dlx2* interacts with the I12b enhancer located in the intergenic region of *Dlx1/Dlx2*, and maintains the transcription of *Dlx1/Dlx2* (Figure 1.4) (Poitras, Ghanem, Hatch, & Ekker, 2007; Porteus et al., 1994). In addition to *Mash1,* it has also been demonstrated that DLX1 and DLX2 proteins have the ability to interact in *trans* with the I56i enhancer located in the intergenic region of *Dlx5/6*; resulting in the activation of transcription of *Dlx5* and *Dlx6* (Anderson et al., 1997b; Eisenstat et al., 1999; Ghanem et al., 2007). Upon activation of *Dlx5* and *Dlx6,* DLX5/DLX6 proteins activate downstream targets including glutamic acid decarboxylase (Gad) via interactions with the GAD enhancer region. GAD

is the enzyme responsible for the synthesis of the γ–aminobutyric acid (GABA), which functions as an inhibitory neurotransmitter in the GABAergic interneurons (Anderson et al., 1997b).

Figure 1.4: Proposed schematic representation of the *Dlx* Gene Regulatory Network in neurons. *Mash1* located upstream of *Dlx1/Dlx2* regulates the activity of *Dlx1/Dlx2* through interaction with the I12b enhancer, resulting in the initiation of transcription of *Dlx1/Dlx2*. The DLX1/DLX2 proteins bind to the I56i enhancer, initiating transcription of *Dlx5/Dlx6*. DLX5/DLX6 proteins subsequently bind to a Gad enhancer and activate *Gad* transcription and consequently GABA synthesis. Gray boxes represent conserved intergenic *cis*-regulatory elements.


Mash1
MASH1
Dlx1
I12b
I12a
Dlx2
DLX1
DLX2
Dlx6
I56i
I56ii
Dlx5
DLX6
DLX5
Gad
GABA

Other factors contribute to *Dlx* regulation. One family of such factors, known as fibroblast growth factors (FGF's) can induce or maintain *Dlx* activity (Panganiban & Rubenstein, 2002). A study conducted by Ferrari and colleagues (1999) suggested that FGF2 can induce *Dlx5* activity in the nascent chick limb (Ferrari et al., 1999). Several other studies have investigated the importance of bone morphogenetic proteins (BMPs) on *Dlx* regulation including BMP2 and BMP4 (Bei & Maas, 1998; Feledy et al., 1999; Miyama et al., 1999; Xu et al., 2001). It has been demonstrated that BMP2 can induce *Dlx2* expression in chondrocytes (Xu et al., 2001); whereas, BMP4 is capable of inducing *Dlx5* expression in osteoblasts (Miyama et al., 1999) and *Dlx1/Dlx2* expression in dental mesenchyme (Bei & Maas, 1998). It has been suggested that BMP4 can induce *Dlx3* expression in embryonic ectoderm (Feledy et al., 1999). Ellies and colleagues (1997) demonstrated that administration of retinoic acid to zebrafish embryos reduced *Dlx* expression in the ectomesenchymal cells (Ellies et al., 1997). Lastly, long non-coding RNA (lncRNA) such as *Embryonic ventral forebrain 2* (*Evf*2) are also involved in the regulation of the *Dlx* expression (Feng et al., 2006). Since *Evf2* plays an important regulatory role in *Dlx* expression, it has also been referred to as transcription-regulating ultraconserved ncRNAs (trucRNAs). It has been suggested that *Evf2* binds to the DLX2 protein and acts on I56i enhancer, regulating the activity of *Dlx5* and *Dlx6*. Furthermore, loss-of-function mutations in *Evf2* have shown to decrease the number of GABAergic interneurons in the developing mouse forebrain (Bond et al., 2009; Feng et al., 2006).

1.10 Evolution of *Dlx* genes and discovery of forebrain intergenic enhancers

Dlx genes are arranged as convergently transcribed bigene clusters. This genomic organization is thought to have occurred through a tandem duplication followed by cluster duplications during whole-genome duplication events (Zerucha & Ekker, 2000). The supporting

evidence for the tandem duplication hypothesis comes from the fact each mammalian *Dlx* bigene cluster harbors one member from each of the two major *Dlx* groups; namely *Dlx1/Dlx4/Dlx6*, and *Dlx2/Dlx3/Dlx5*, which are classified based on sequence similarities of their homeodomains region (Zerucha & Ekker, 2000). It is likely that the duplication events that led to multiple *Dlx* clusters occurred at the same time as the *Hox* clusters were undergoing genome duplications (Zerucha & Ekker, 2000). The exact timing of the duplication events leading to multiple copies of *Dlx* genes have been highly debated. Through genome sequencing, the earliest *Dll* orthologue was discovered in *C. elegans*, and it was shown that it was linked to a *HOM/Hox* cluster. Therefore, it has been suggested that the first *Dlx* duplication event occurred after the divergence of nematodes from eucoelomates; very early during the evolution of chordates (Stock et al., 1996; Zerucha & Ekker, 2000).

Dlx bigene clusters harbor conserved *cis*-regulatory elements within their intergenic region that spans ~2.5-10 Kb (Ghanem et al., 2003). These intergenic regulatory regions have been shown to act as potential binding sites for different transcription factors (Ghanem et al., 2003; Ghanem et al., 2008; Park et al., 2004; Poitras et al., 2007). The *Dlx1* and *Dlx2* cluster harbors I12a (550 bp) and I12b (400 bp) enhancers within the intergenic region (Ghanem et al., 2003; Park et al., 2004). A study by Park and colleagues (2004) demonstrated that the I12a enhancer is not active in the forebrain but is predominantly active in the branchial arches, apical ectodermal ridge and hyoid arches starting at E9.5 and until E16.5 of developing mouse embryos (Park et al., 2004); whereas the I12b enhancer, in addition to the apical ectodermal ridge, is mainly active in the developing mouse forebrain including telencephalon and diencephalon at E10.5 (Ghanem et al., 2007). Further functional investigation of the I12b enhancer using a *lacZ* reporter construct demonstrated that the I12b-*lacZ* positive cells are detected in tangentially migrating cells that are originated from the

medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE) in the developing forebrain (Ghanem et al., 2007). It was also suggested that since 93% of the adult cortical GABAergic interneurons exhibit I12b enhancer activity; thus it is suggested that the I12b enhancer could be involved in the regulation of *Dlx1* and *Dlx2* (Ghanem et al., 2007). Upstream regulatory element 2 (URE2) located upstream of the *Dlx1/Dlx2* bigene cluster has also been identified in the mouse and exhibits activity starting at E11.5 in the subpallial telencephalon, prethalamus, hypothalamus, somites and apical ectodermal ridge (Ghanem et al., 2007).

Similar to the *Dlx1/Dlx2* bigene cluster, the *Dlx5*/*Dlx6* cluster also comprises two highly conserved *cis*-regulatory elements within its intergenic region. These enhancer sequences known as I56i (400 bp) and I56ii (300 bp) have been shown to have 100% and 98% sequence identity between mouse and human, respectively (Ghanem et al., 2003; Ghanem et al., 2008). The I56i enhancer has been shown to be active in the subpallial telencephalon, prethalamus, hypothalamus, branchial arches as well as apical ectodermal ridge, beginning at E10.5 (Ghanem et al., 2008). Furthermore, spatiotemporal analysis of I56i enhancer activity via *lacZ* transgenesis demonstrated that the I56i *cis*-regulatory element (CRE) is mainly active in the tangentially migrating cells similar to that of I12b-*lacZ*+ cells (Ghanem et al., 2007). It was also shown that the I56i enhancer was active in most of the subventricular zone (SVZ) cells in the lateral ganglionic eminence (LGE), MGE and CGE. The I56i-*lacZ* activity was also detected in a large number of cells located in the mantle zone (MZ) of the developing forebrain (Ghanem et al., 2007). On the other hand, the I56ii enhancer shows activity only in the developing forebrain including subpallial telencephalon, prethalamus, hypothalamus, starting at E11.5 (Ghanem et al., 2008). More specifically, it has been shown that the I56ii enhancer is active in a group of post-mitotic projection neurons that are thought to be derived from the LGE and have tangentially migrated deep in the mantle zone of the

developing forebrain, also known as corridor cells (Ghanem et al., 2008; Lopez-Bendito et al., 2006; López-Bendito & Molnár, 2003). *C*haracterization of *Dlx* forebrain enhancers activity with *LacZ* reporter constructs showed that the activity patterns of the forebrain enhancers overlap significantly despite their divergence at the nucleotide level (Ghanem et al., 2008). This intriguing fact emphasizes the possibility that different intergenic enhancers may be responding to unique regulatory factors (Poitras et al., 2007).

1.11 Statement of Inquiry

Dlx bigene clusters are the mammalian homologs of the *Drosophila Distal-less* (*Dll*) gene, which plays an important role in the development of the fly's appendages (Zerucha & Ekker, 2000*). Dlx* homeobox genes are arranged in bigene clusters in a convergent configuration with enhancers located in the intergenic region, on distinct chromosomes (Lopez-Bendito et al., 2006). The expression patterns of these genes overlap significantly despite their distinct overall identity (Ghanem et al., 2007). The expression of the *Dlx*1/*Dlx*2 and *Dlx*5/*Dlx*6 bigene clusters in the forebrain and/or branchial arches could be attributed to intergenic enhancer sequences, namely I12a/I12b as well as I56i/I56ii, respectively (Ghanem et al., 2003; Ghanem et al., 2007; Ghanem et al., 2008; Park et al., 2004; Poitras et al., 2007). The intergenic location of these enhancers within each *Dlx* cluster suggests that these enhancers may act on either promoters of each *Dlx* cluster. This raises the provocative question of how do these intergenic enhancers regulate *Dlx* expression during development? This intriguing question of how is *Dlx* regulated at the chromatin level by means of enhancer-promoter interactions has not been investigated and provides the opportunity for my PhD research project to examine this possible phenomenon in great detail.

My PhD research project employs the Chromosome Conformation Capture (3C) technique in order to further investigate *Dlx* regulation at the chromatin level through enhancer-promoter interactions in each *Dlx* bigene cluster. The 3C method was initially developed to study the complete conformation of a chromosome in yeast (Dekker et al., 2002). It is now used as an emerging research tool to analyze the organization of complex genomic domains and investigate the relationship between genome architecture and gene expression (Dekker, 2003; Dekker, 2006; Miele & Dekker, 2008; Miele et al., 2001). There are still many questions regarding the mechanism of "DNA looping" and the factors underlying this process. Furthermore, this process has not been investigated for the *Dlx* bigene clusters but is particularly relevant considering the peculiar *Dlx* genomic configuration has been conserved throughout evolution and their important role during different developmental stages. Therefore, the first main objective underlying my PhD research project was to determine how *Dlx* expression is regulated at the chromatin level by means of enhancer-promoter interactions. In rectifying this intriguing question I have also addressed several questions including (1) do the *Dlx* enhancers form a loop with their respective promoters? Additionally, do these *Dlx* enhancers act on both promoters or act on one promoter specifically or preferentially in each *Dlx* cluster? (2) Do the enhancer-promoter interactions change with time during different developmental stages? (3) Is there any *trans* interaction between *Dlx1/Dlx2* and *Dlx5/Dlx6* bigene clusters?

Concomitant to studying *Dlx* regulation at the chromatin level, I was interested to study the functional importance of these intergenic enhancers since *Dlx* genes are involved in forebrain development and play an essential role in neurogenesis, especially in GABAergic interneuron proliferation, differentiation, migration and survival. Mis-regulation of *Dlx* genes have been associated with abnormal GABAergic interneuron development, which has been suggested to be

linked to neurological disorders including autism spectrum disorder (ASD), Rett syndrome, schizophrenia and epilepsy. To date, the exact regulatory cascade controlling *Dlx* expression is not fully understood, but it has been shown through *LacZ* reporter construct analysis that the exogenous activity pattern of the intergenic enhancers overlap with the endogenous activity of *Dlx* in the developing forebrain (Ghanem et al., 2008). For a number of years our laboratory has been studying the *cis*-regulatory sequence elements that control *Dlx* gene expression in the forebrain. Thus, multiple targeted deletion mouse-lines are generated where the I12b, I56i and I56ii intergenic enhancers are deleted. Since the role of the I56ii enhancer is relatively less well known during the development of telencephalon; my second main objective is to characterize the functional consequence of mice harboring one or two *Dlx* intergenic enhancer deletion(s). More specifically, I am interested in investigating the impact of I56ii enhancer deletion on the differentiation, and migration of GABAergic interneurons from the ventral telencephalon. In characterizing the functional importance of I56ii enhancer on development, migration and differentiation of GABAergic interneurons I addressed various questions including (1) what is the phenotypic consequence of enhancer deletion in the developing mouse? (2) Does enhancer deletion have an impact on *Dlx* function in developing mouse forebrain? (3) Does enhancer deletion have an impact on the activity of corridor cells and ultimately neuronal migration? (4) Is there a behavioural phenotype associated with the mice harboring I56ii enhancer deletion?

In addressing the above questions, I have successfully determined the DNA looping mechanism within and between the enhancer and promoter regions of *Dlx* bigene clusters, in which I12b and I56i enhancer acts on promoters of *Dlx1/Dlx2* and *Dlx5/Dlx6*, respectively and these interactions persist over different developmental stages. The I56ii enhancer however, only interacts with the promoter region of *Dlx6* at E13.5 and is undetectable at E15.5, suggesting that

this enhancer is only active within a very small window during development. Furthermore, a *trans* interaction may exist between the two *Dlx* loci in the developing forebrain at E13.5. I have also been able to successfully characterize the functional consequence of *Dlx* intergenic enhancer deletion(s) on forebrain development. Enhancer deletion(s) impair *Dlx* expression in the developing forebrain as well as other targets including the striatal markers *Islet1* and *Meis2*. I56ii enhancer deletion also results in severe decrease in the activity of the corridor cells, which may alter the thalamocortical axonal pathfinding in the developing forebrain. Overall my PhD thesis project highlights the functional significance of the *Dlx* intergenic enhancers in regulating *Dlx* expression through formation of loops with promoter region both in *cis* and *trans*. Additionally, my research demonstrates the importance of intergenic enhancer function for proper development and migration of GABAergic interneurons in the developing mouse forebrain.

Chapter 2: Capturing interactions: three-dimensional structural conformation of *Dlx* bigene clusters in the developing mouse forebrain.

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2.0 Abstract

An important aim of current biology is to understand how non-coding regulatory elements exert their effect over large genomic distances in order to control switch genes on and off at the appropriate times during differentiation and development. *Dlx* homeobox transcription factors are the mammalian homologs of the *Drosophila Distal-less* (*Dll*) gene that play an important role during developmental processes including forebrain development. *Dlx1/2* and *Dlx5/6* are expressed in forebrain and are arranged in convergently transcribed bigene clusters, with I12a/I12b and I56i/I56ii enhancers located in the intergenic region of each cluster, respectively. To determine the presence of a looping mechanism between the intergenic enhancers and the *Dlx* promoters during development, we employed Chromosome Conformation Capture (3C) technique on E13.5 and E15.5 developing mouse forebrain. Here we report that the I12b enhancer interacts predominantly with both promoters of *Dlx1* and *Dlx2* in the developing forebrain and the interactions are maintained during developmental stages. In addition, the I56i enhancer sequence interacts with both *Dlx5* and *Dlx6* promoters at E13.5 and persists during development at E15.5; however, the I56ii enhancer from *Dlx5/6* cluster only interacts with the promoter region of *Dlx6* and is undetectable at E15.5. Lastly, *trans* interactions exist between *Dlx1/2* and *Dlx5/6* clusters, which could imply that intergenic enhancers play a role in bringing these two loci together to regulate and maintain transcription of *Dlx* as well as other genomic targets. Collectively these results demonstrate that the enhancer sharing mechanism occurs within and between *Dlx* clusters of the developing forebrain.

2.1 Introduction

Chromosomes contain many non-coding elements that determine both chromosome structure and the activity of the underlying DNA (Dekker, 2008). *Cis*-acting regulatory sequences may be located tens or even hundreds of kilobases from the genes they control (Kleinjan & van Heyningen, 2005), but how they act over such long distances and the mechanisms by which they influence gene expression remains elusive. The ability to store, retrieve and translate genomic instructions are the essential steps in maintaining life in any given cell (Cook, 2010). It requires a very precise process to control gene regulation within the nuclear space. Considering its enormous size, the human genome, needs to be tightly packaged and organized in order to fit within the small size of the nucleus (Crutchley et al., 2010; Fraser & Bickmore, 2007).

The three-dimensional organization of genomes plays an essential role in the regulation of chromosomal processes including gene regulation and genomic stability (Fraser & Bickmore, 2007). This precise transcriptional regulation is mediated by short DNA sequences called enhancers, which regulate gene expression by interacting with the promoter region of the controlled gene (Bulger & Groudine, 2010; Noonan & McCallion, 2010). This regulatory process is achieved through long-range chromatin interactions within the cell nucleus. Long-range intrachromosomal (*cis*) and interchromosomal (*trans*) interactions have been shown to regulate gene expression by bringing the regulatory elements within close physical proximity of the target gene (Carter et al., 2002). Enhancer-promoter interactions could aid in stable recruitment of components of the transcription machinery to the promoter. In addition, enhancer-bound enzymatic activities could be brought in contact with promoter complexes that are then modified; for instance phosphorylated or methylated, which leads to modulation of promoter activity. Alternatively, given the very transient nature of these associations, the two loci may acquire

distinct but stable marks that direct assembly of protein complexes at later time points when the loci no longer interact (Dekker et al., 2002; Vernimmen et al., 2007).

Long-range chromatin contacts were found to regulate genes from a wide variety of cellular pathways, indicating that such chromatin contacts are thought to be one mode of general transcriptional regulation (Dmitriev et al., 2009; Petrov et al., 2008; Sexton et al., 2009). Nonetheless, co-regulated genes located far from each other or on different chromosomes can also co-localize and form foci in the nuclear space (Schoenfelder et al., 2010). This type of genomic organization likely participates in coordinating the proper timing or relative expression of various genes (Schoenfelder et al., 2010). Well characterized examples of spatial association of genomic elements involve interactions between enhancers and target genes. One of the well-known examples concerns the interactions at the β-globin locus (Tolhuis et al., 2002). The locus contains several β-globin-like genes that are regulated by a single *cis*-acting element, the Locus Control Region (LCR), which is located about 10 to 60 kb upstream of the globin gene promoters (Tolhuis et al., 2002). The LCR was found to physically associate with the active globin gene (Tolhuis et al., 2002). Many more examples of long-range looping events have been identified; namely, the α-globin locus (Vernimmen et al., 2007), and the interleukin gene cluster (Spilianakis & Flavell, 2004). Highly specific associations between loci located on separate chromosomes have also been described. These *trans* interactions can be between enhancers and putative target genes, as in the case of olfactory receptor genes (Lomvardas et al., 2006). However, in other cases, they appear to play a role in a higher level of gene control to coordinate the regulation of multiple loci (Lomvardas et al., 2006).

These long-range chromatin interactions are particularly intriguing to investigate in *Dlx* bigene clusters due to their peculiar genomic organization. *Dlx* genes are mammalian homologs of

the *Drosophila Distal-less (Dll)* gene that plays an important role in the development of the fly's appendages (Zerucha & Ekker, 2000). Prior to divergence of vertebrates from their ancestor and eventual diversification, the ancestral *Dll* gene underwent a series of duplication events, which led to the presence of up to three gene clusters found in vertebrate species; namely the *Dlx1/Dlx2*, *Dlx5/Dlx6*, and *Dlx3/Dlx4* clusters (Zerucha et al., 2000). The clusters take a convergent configuration, with enhancers located in the intergenic region, on distinct chromosomes (Zerucha & Ekker, 2000). In addition to their role in appendage development, *Dlx* clusters are thought to be involved in the development of the forebrain (including the telencephalon and diencephalon), branchial arches, neural crest, jaw and craniofacial bones, and sensory organs (Ghanem et al., 2007; Panganiban & Rubenstein, 2002). It has been suggested that the expression levels of the *Dlx1/Dlx2* and *Dlx5/Dlx6* clusters in the developing forebrain can be attributed to enhancer sequences such as I12b and I56i/I56ii (Fig. 1.1) (Ghanem et al., 2003; Ghanem et al., 2007; Ghanem et al., 2008). Partial functional redundancy amongst *Dlx* genes has been suggested by their overlapping expression patterns and by the phenotypic analysis of mice with targeted *Dlx* mutations (Acampora et al., 1999; Anderson et al., 1997a; Depew et al., 1999; Mengsheng Qiu et al., 1997). Overlap in expression could possibly be attributed to an enhancer sharing model (Bateman et al., 2012; Yang et al., 1995). The genomic arrangement of the *Dlx* genes has been conserved throughout evolution and a direct link has been suggested between the genomic organization of *Dlx* genes and their expression patterns in different species (Zerucha et al., 2000).

We have employed the Chromosome Conformation Capture (3C) technique to address the enhancer sharing model, within and between *Dlx* clusters and to examine *Dlx* regulation at the chromatin level by the virtue of a looping mechanism between the enhancer and promoter regions in the developing mouse. The 3C method was initially developed to study the complete

conformation of a chromosome in yeast (Dekker et al., 2002). It is used to analyze the organization of complex genomic domains and investigate the relationship between genome architecture and gene expression (Miele & Dekker, 2008; Miele et al., 2001). The 3C technique utilizes chemical cross-linking of proteins to other proteins and to DNA. The overall result is the cross-linking of physically touching segments throughout the genome via contacts between their DNA-bound proteins. This step provides a "snap shot" of the chromatin structure at a given cell stage. Following this step, cross-linked DNA is digested with restriction enzymes and then subjected to ligation at very low DNA concentration. Under such conditions, ligation of cross-linked fragments, which is intramolecular, is strongly favoured over ligation of random fragments, which is intermolecular. Cross-linking is then reversed and individual ligation products are detected and quantified by polymerase chain reaction (PCR) using locus-specific primers (Crutchley et al., 2010; Dekker, 2008; Dekker et al., 2002). Here we report that *Dlx* intergenic enhancers interact in *cis* with their respective promoters both specifically and preferentially. Additionally, we demonstrate that *trans* interactions exist between *Dlx1/2* and *Dlx5/6* clusters in the developing mouse forebrain.

2.2 Materials and Methods

2.2.1 Tissue preparation:

Chromosome Conformation Capture (3C) was performed following standard procedures as previously described (Miele & Dekker, 2008; Miele et al., 2001). The ventral telencephalon of the developing mouse forebrain was dissected at E13.5 and E15.5 developmental time-points. Tissues were washed 3X with cold Hank's Balanced Salt Solution (HBSS) (Invitrogen Life Technologies). Once tissues were equilibrated, they were minced into small pieces with sterile scissors and incubated in 200 U/mL of collagenase in HBSS supplemented with 3 mM $CaCl_2$. Tissues were incubated at 37°C for 3 hrs; following incubation, the cell suspension was washed 3X with 1 mL of DMEM medium supplemented with 10% calf serum and centrifuged at 300 ×g for 1 min. After each wash, the cell pellet was resuspended in 1 mL of DMEM medium supplemented with 10% calf serum. A single cell population was achieved by filtering the cell suspension through a 70 µm sterile nylon cell strainer (BD Falcon^{TM}). Cells were counted using a 0.1 mm deep Bright-Line hemocytometer. The cells were divided into 1×10^7 aliquots and were cross-linked immediately by adding to each aliquot a mixture of 8.1 mL DMEM medium supplemented with 225 µL of 37% formaldehyde. A mixture was obtained by inverting the tube 4-6X and incubated at room temperature (RT) for 10 min, mixing every 2 min. Cross-linking was stopped by adding 446 µL of 2.5 M glycine. The mixture was left at RT for 5 min followed by 15 min incubation on ice. The cell suspensions were centrifuged at 400 ×g for 10 min at 4°C. After centrifugation and removal of the supernatant, cell pellets were instantly frozen in liquid nitrogen and stored at -80°C until used.

2.2.2 Constructing 3C libraries:

2.2.2.1 Digestion of chromatin:

The cross-linked cells were thawed on ice for 30 min and lysed by resuspending the cells in a cocktail of 200 µL lysis buffer supplemented with 20 µL protease inhibitor (Sigma Aldrich). Following incubation on ice for 15 min, cells were disrupted by 2 × 20 strokes of a pestle B tissue homogenizer, on ice. Cell lysate was transferred to a 1.5 mL tube and centrifuged at 2000 ×g for 5 min at RT. The supernatant was removed and cells were washed twice using 100 µL of 1X restriction buffer and centrifuged at 2000 ×g for 5 min at RT. Following the wash steps, cells were resuspended in 100 µL of 1X restriction buffer and divided into two 50 µL aliquots. To each aliquot, 337 µL of 1X restriction buffer and 38 µL 1% SDS were added, mixed by pipetting and incubated at 65°C for 10 min. Following incubation, 44 µL 10% Triton X-100 were added to each tube and the solution was mixed gently to avoid bubbles. Lastly, 400U of the appropriate restriction enzyme was added to each tube, which was mixed and incubated in a 37°C water-bath overnight. In order to generate the *Dlx1/2* and *Dlx5/6* libraries (including both the negative [liver] and the experimental [forebrain] libraries), the *Bgl*II and *Bam*HI restriction enzymes were used to digest the DNA, respectively. A restriction enzyme is chosen such that it would cut frequently enough between the enhancers and the promoters in order to allow measuring the multiple interaction frequencies between them (Crutchley et al., 2010). The mouse liver tissue served as a negative control since *Dlx1/2* and *Dlx5/6* are not expressed in the liver during these developmental stages and was used to account for the random collisions that happen naturally in the cell.

2.2.2.2 Ligation of chromatin:

Following the overnight incubation, 86 µL 10% SDS were added to each tube followed by incubation at 65°C for 30 min to inactivate the restriction enzyme. The ligation cocktail was prepared by adding 5960 µL ddH_2O, 750 µL 10X ligation buffer (12.5 mL 1M Tris-HCl pH=7.5, 2.5 mL 1M $MgCl_2$ and 7.5 mL ddH_2O), 750 µL 10% Triton X-100, 80 µL 10 mg/mL BSA, 80 µL 100 mM ATP. The cocktail was mixed by inverting 4X before adding 10 µL 300 U/µL T4 DNA ligase. The ligation cocktail was mixed again by inverting the tube 4X. A 575 µL volume of cell lysate was added to the ligation cocktail and was mixed by inverting the tube 8X gently to avoid bubbles. The ligation mixture was incubated at 16°C for 2 hrs.

2.2.2.3 Protein removal and purification of the DNA:

To each ligation tube, 50 µL 10 mg/mL proteinase K were added followed by incubation at 65°C overnight. Following the overnight incubation, 50 µL 10 mg/mL proteinase K was added to each tube followed by incubation for 2 hours at 65°C. Following incubation, the ligation mixture was transferred to 50 mL conical tubes and the DNA was purified by adding 8 mL phenol to each tube and vortexing for 2 min. Each tube was centrifuged at 1500 ×g for 10 min at RT. The top phase was transferred to a new tube and was further cleaned by adding 8 mL phenol-chloroform to each tube. Tubes were vortexed for 2 min and centrifuged at 1500 ×g for 10 min at RT. The top phase was transferred to a SS34 centrifuge tube and the DNA precipitated by adding 0.1X volumes (800 µL) 3M sodium acetate (NaOAc, pH=5.2) and 2.5X volumes (22 mL) cold 100% ethanol. The DNA mixture was mixed by inverting the tube 4-6X and was incubated at -80°C for at least 1 hr or overnight. Following overnight incubation, tubes were centrifuged at 17,000 ×g for 25 min at 4°C. DNA pellets were washed with 10 mL 70% ethanol and centrifuged at 17,000 ×g for 10

min at 4°C. After removing all of the ethanol, the DNA pellet was resuspended in 400 µL of 1X TE pH=8.0 (DNA pellet was pooled by carrying over the TE buffer). The DNA suspension was cleaned using one volume (400 µL) of phenol-chloroform, vortexed for 1 min and centrifuged at 17,000 ×g for 5 min. The DNA was precipitated by adding 0.1X volumes (40 µL) 3M NaOAc pH=5.2 and 2.5X volumes (1100 µL) cold 100% ethanol. After mixing by inverting the tubes, a fluffy white precipitate was immediately formed in the tube, indicating the presence of a DNA pellet. The mixture was incubated at -80°C for at least 1 hr or overnight. After incubation, the tubes were centrifuged at 17,000 ×g for 25 min at 4°C. The DNA pellet was desalted by washing the pellet with 1 mL 70% ethanol (8-10X) or until the pellet no longer collapsed and remained the same size. After removing all of the ethanol, the DNA pellet was resuspended in a total of 100 µL 1X TE buffer pH=8.0. One µL of 10 mg/mL RNase A was added to each tube and incubated at 37°C for 15 min. Following the incubation, the 3C library was aliquoted and stored at -20°C until use.

2.2.3 Generating a BAC positive library:

Equal amounts of three BACs corresponding to the *Dlx1/2* locus (510G1 BAC), the *Dlx5/6* locus (564M8 BAC) and the *USP22* gene desert (RP23-305I5 BAC) were used to generate each positive library. The equal copy numbers of each BAC were determined by performing qRT-PCR on a common region in the backbone of the BAC templates using pBAC108.For (5´-ACAGATTTGAGGGTGGTTCG-3´) and pBAC108.Rev (5´-TAACCCCCGATATCAGGTCA-3´) primer-pair. The ΔCt values from this preparation was used to normalize the BAC volumes used in constructing the positive library. Therefore, 6.8 µL *USP22* BAC, 12.91 µL *Dlx1/2* BAC and 37.15 µL *Dlx5/6* BAC were mixed and used to generate the BAC positive libraries. In order

to generate *Dlx1/2* and *Dlx5/6* positive libraries, the *Bgl*II and *Bam*HI restriction enzymes were used to digest the DNA respectively.

To generate a positive library from BACs, the aforementioned BAC volumes were mixed together and the final volume was adjusted to 150 µL with ddH_2O. A 2 µL aliquot was taken for future gel analysis on a 0.8% agarose gel. To the 148 µL DNA, 150 µL of 10X NEB 3.1 and 131 µL *Bgl*II or *Bam*HI were added (New England Biolabs). The total volume was adjusted to 1500 µL using ddH_2O and the tube was incubated at 37°C in a water-bath overnight. Following incubation, the sample was split into 4×375 µL aliquots. The DNA was cleaned using an equal volume of phenol-chloroform. Each sample was vortexed for 1 min and centrifuged at 17,000 ×g for 5 min at RT. The top phase was transferred to a new tube and DNA was precipitated be adding 0.1X volume (37.5 µL) 3M NaOAc pH=5.2 and 2.5X volume (937.5 µL) cold 100% ethanol to each tube. The tubes were mixed gently by inverting 4-5X and incubated at -80°C for 1 hr. Following incubation, the tubes were centrifuged at 17,000 ×g for 20 min at 4°C. DNA pellets were washed using 1 mL 70% ethanol and centrifuged at 17,000 ×g for 10 min at 4°C. Each pellet was dissolved in 143 µL of ddH_2O followed by incubation at 37°C for 15 min. A 2 µL aliquot was removed and analyzed for successful complete digestion by running on a 0.8% agarose gel.

Upon validation of successful digestion of BAC DNAs, a ligation mix consisting of 141 µL digested BAC DNA, 40 µL 5X ligation buffer and 19 µL T4 DNA ligase was prepared and incubated at 16°C overnight. Following incubation, the enzyme was deactivated by heating the samples at 65°C for 15 min. The DNA was extracted twice by adding equal volumes of phenol-chloroform (200 µL) and once by adding equal volumes of chloroform (200 µL). After each step, tubes were vortexed for 1 min and centrifuged at 17,000 ×g for 5 min at RT. The top phase was

transferred to a new tube and the DNA was precipitated by adding 0.1X volume (20 µL) 3M NaOAc pH=5.2 and 2.5X volume (500 µL) cold 100% ethanol to each tube. The tubes were incubated at -20°C for 1 hr and centrifuged at 17,000 ×g for 20 min at 4°C. The DNA pellet was desalted by washing 3-4X with 1 mL 70% ethanol and centrifuging at 17,000 ×g for 20 min at 4°C. The DNA pellet was resuspended in 150 µL of 1X TE buffer pH=8.0. The resuspended DNA was incubated at 37°C for 15 min and ran on a 0.8% agarose gel alongside the undigested and digested BAC DNA to verify a successful library construction.

2.2.4 Library titration and PCR amplification:

The suitable concentrations for PCR reaction of each of the 3C libraries were verified by titrating the library on a 0.8% agarose gel using DNA high-mass ladder (Invitrogen Life Technologies). The adequate amount of DNA was deduced from titration (approximately 50 ng) and used for PCR amplification of the fragments. A 29 µL of master mix and 1 µL of 3C DNA template (experimental [forebrain] and negative [liver]) were used for each PCR reaction. The master mix for every PCR reaction contained 3.0 µL of 10X PCR buffer (100 mM Tris-HCl pH=8.5, 500 mM KCl), 2 µL 50 mM $MgCl_2$, 1.5 µL of 10 mM dNTPs, 0.25 µL (1U) Taq polymerase, 0.5 µL 8 µM each primer (Table 2.1) and 21.25 µL ddH_2O (RNase-free water). The initial denaturation step was 95°C for 5 min. The subsequent denaturation step was 95°C for 1 min, followed by an annealing step at 66°C for 1 min and an extension step at 72°C for 1 min. These 3 steps were repeated for 39 cycles and a final extension step at 72°C for 8 min completed the reaction. The BAC positive library was amplified by using 0.5 µL (1:5 dilutions) of BAC positive library and 29.5 µL of master mix for each PCR reaction. The master mix for the BAC positive library is similar to the previously described master mix; however, the volume of ddH2O was adjusted to accommodate for change in the amount of template used in each PCR reaction.

The same PCR program was used to amplify the products with the exception of having steps 2-4 cycled 34 times. PCR amplicons were fractionated on a 1.5% agarose gel containing 0.1 μg/mL red safe at 120V for 45 min and viewed on an UV transilluminator (Alpha Imager® EC, Alpha Innotech.).

2.2.5 Data analysis

After constructing libraries, successive PCR amplification and gel electrophoresis, PCR bands were quantified using Quantity One 1-D analysis software (Bio-Rad). The signal corresponding to each band was corrected for the background intensity by subtracting the background value from the intensity of each band. Upon this correction, the impact of the primer-pair efficiency was accounted for by dividing each normalized value with the corresponding signal from the BAC PCR. The corresponding ratios for both negative (liver) and experimental (forebrain) PCR reactions were plotted using excel.

Table 1: Complete list of primers used for 3C analysis. (P1= promoter *Dlx1*; F1=Forward primer1; R1=Reverse primer 1).

Target	Primer Name	Primer sequence (5´-3´)
	P1	GAGATAAGGTCCTAGTTGGGCTCATCTGG
	F1	CTTCAGGACCAATGGGAGAGTCTAAGTTCC
	F2	CAGTCTGTCTCTAGATTTAGGTTGGAGCTGGG
Dlx1	F3	CCAGGTCGCTTTAAAGTAAGACACAAGCAGCG
	F4	CGCAGGCTTTTTGCATCTCTGAGCTTGC
	F5	CAGGCGTTGTTACATCATTGCCTCATTAGGGG
	F6	GAGGCCTCAAGAGGGAGTTGAGATGAATTTGG
	P2	CCATAGCCAGAGAGTAGGTAGTCAAACTAGCC
	R1	GAATGGGTGGATCAAGCTACACTCTGAGG
	R2	CACTGAAGTCTGTTGTTGGAAGCACAGC
Dlx2	R3	GAGCCTACGCAGAATTAGCCTGAATTGCATGG
	R4	CACAACAGAACGGAAAGAGGCTAAGCTTGTGC
	R5	CGTTTATGGAAGACCTCATGCAGCACAATGC
	R6	CTCAAGGAAGCAAGTCTTGGGAGAAACTGAGC
	P6	GATTTCTCCCACCCAGTCTCTATCATCTGG
	F1	GAGAAGAGTGAGGAAGAGAACCACCTCTGC
	F2	GGTATTCACACTGGTATTCCTCACCACACC
	F3	CCAAGCCCTGTTCTGACTATACTAAAGGGC
	F4	GCTCCTTCTACAACTCGCAGAAACCTGG
Dlx6	F5	ACTTCCCAACCACCTCTCTGAGCTACTGC
	F6	GTTGTCCTTAGGCGGGGAGATACTGATCTGG
	F7	GAGAGAGAGATAGAGATAGATCAAGGGCAGCG
	F8	CGCTCACCCAGAATTCCAACTAACTTTCC
	F9	CTACAAGTCTGGTCAGCACTTTTGGAGC
	F10	CAGTAACCTGGGCGAGTTTAAGTCATCG
	P5	CCTAGAACTTGCTTGCCGTTTGACTAGG
	R1	CAGCTACCCAAAGAAATTACAGGCTCCC
	R2	GACTGCAAACAGGACAGCACCTCACACC
	R3	CTTTGTTTGAGACTTGGGTTGGGCTAGG
	R4	CACACACACACACACACACTGAAGAACC
Dlx5	R5	CCCTCTGCTTCTTGCTTCCTATTGTTCC
	R6	CTTTGGTTTCAAGGTTTGGGCTCTGACC
	R7	GAAGCCAAGGAACCAGTGTAGTCTATGTACCG
	R8	GAGAGAGAGAGAGAGAGAGAGAGATCACAGGG
	R9	CATCAAAACTTTGGGGGCTGGAGAAAGC
	R10	CATTAAGTTTGGAGCAGCTCAGTGCTACGG

2.3 Results

2.3.1 DNA looping mechanism explains the enhancer-promoter interactions within the *Dlx* clusters.

There are still many questions regarding "DNA looping" model and the factors underlying this process (Chambeyron & Bickmore, 2004; Cook, 2010; Fraser, 2006; Marsman & Horsfield, 2012; Naumova et al., 2013; Naumova et al., 2012). Studying the DNA looping mechanism is particularly relevant in the *Dlx* bigene clusters due to their genomic arrangement, where some of their enhancers are located in the intergenic region of each locus. In addition, the *Dlx* genomic configuration has been conserved throughout evolution and, given their important role during development including forebrain development, this makes the *Dlx* clusters an ideal study model for DNA looping mechanisms. In examining the possibility of enhancer-promoter interactions in each *Dlx* cluster, we generated chromosome conformation capture (3C) libraries using forebrain tissues, more specifically from the ventral telencephalon at various developmental time points. During these experiments, mouse liver was used as a negative control since *Dlx* genes are not expressed in liver tissue.

We investigated the possibility of an enhancer-promoter interaction between the promoters of each *Dlx* gene in the cluster with the enhancers located in the intergenic region of each given locus. At first, we explored the possible interactions that may occur between the promoter region of *Dlx1* with the intergenic region of *Dlx1/2* cluster. In parallel, we also examined the potential interactions between the *Dlx2* promoter region with the intergenic region of the *Dlx1/2* locus. As depicted in Fig. 2.1A, our 3C analysis demonstrated that the *Dlx1* promoter region showed interactions with I12b and/or I12a enhancers located in the intergenic region of the *Dlx1/2* cluster in the developing forebrain at E13.5 (Fig. 2.1A). In contrast to the interactions that were detected

between the *Dlx1* promoter and the intergenic region, we did not observe any major peaks between the *Dlx2* promoter region and the intergenic enhancers of *Dlx1/2* cluster (Fig. 2.1B). Therefore, this experiment did not provide any evidence for an enhancer-promoter interaction for *Dlx2*. Our data from the *Dlx1/2* cluster suggest that I12b and/or I12a intergenic enhancers interact preferentially with the promoter regions of *Dlx1* in the developing forebrain at E13.5. This may also suggest that other regulatory elements such as upstream regulatory element 2 (URE2) could be acting with the *Dlx2* promoter in order to maintain the expression levels of *Dlx2* in the early stages of the forebrain development at E13.5.

In parallel to this line of work, we also examined the interactions that may take place between the promoter regions of *Dlx5* and *Dlx6* with the intergenic region of the *Dlx5/6* cluster. Our 3C data suggested that the *Dlx6* promoter region interacts with the I56i and I56ii enhancers at E13.5 (Fig. 2.2A). In contrast, the promoter region of *Dlx5* seems to interact specifically with the I56i intergenic enhancer at E13.5 (Fig. 2.2B). Based on these data, the I56i enhancer may be playing an important regulatory role in activating and maintaining the transcription of both *Dlx5* and *Dlx6* in the developing forebrain. Additionally, the data obtain from the forebrain library at E13.5 suggests that the I56ii enhancer only interacts with *Dlx6* which could imply that I56ii enhancer is a regulator of *Dlx6* but not of *Dlx5* during early stages of the developing forebrain.

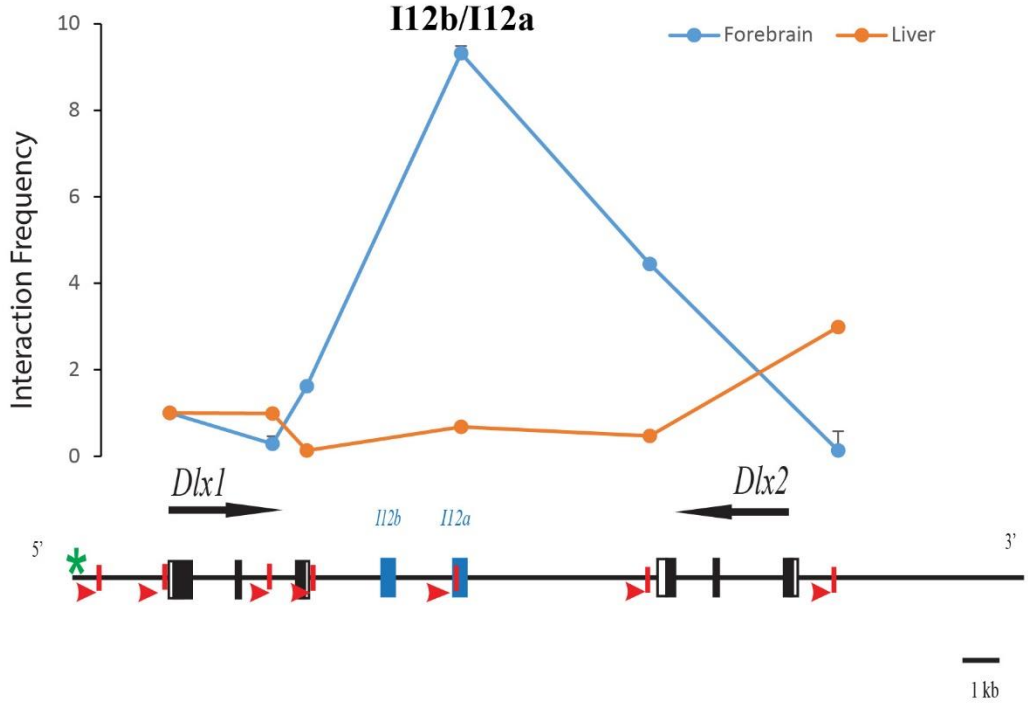
It is important to note that in all cases, we did not capture any interactions in the liver library. The lack of interactions in liver (negative library) suggests that the interactions that we did observe at E13.5 in the forebrain are results of intergenic enhancers and the promoter regions being brought to a close physical proximity to regulate *Dlx* expression. Additionally, in order to further validate the interactions that we observed within each *Dlx* cluster at E13.5 in the developing

forebrain, we are currently using the enhancer regions, where “peaks” were observed, as the fixed point and examining the presence of interactions with either promoter region of each *Dlx* cluster.

Collectively, our 3C analysis of E13.5 forebrain library indicates that the intergenic enhancers seem to interact with at least one of the promoters of the flanking *Dlx* genes, for both the *Dlx1/Dlx2* and *Dlx5/Dlx6* loci.

Figure 2.1: *Dlx1/2* intergenic region interacts with the promoter region of *Dlx1* at E13.5. Enhancer-promoter interactions between the *Dlx1/2* intergenic enhancers with the promoter regions of (A) *Dlx1* and (B) *Dlx2* at E13.5 in the developing mouse forebrain. Green asterix represents the fixed point at the promoter region of either *Dlx* genes. Error bars represent the standard error of the mean for three experimental replicates. The blue line corresponds to the 3C forebrain library (experimental library), while the orange line corresponds to the 3C liver library (negative library).

(A) E13.5


I12b/I12a
Forebrain
Liver
Interaction Frequency
10
8
6
4
2
0
Dlx1
Dlx2
Il2b
Il2a
5'
3'
1 kb

(B) E13.5

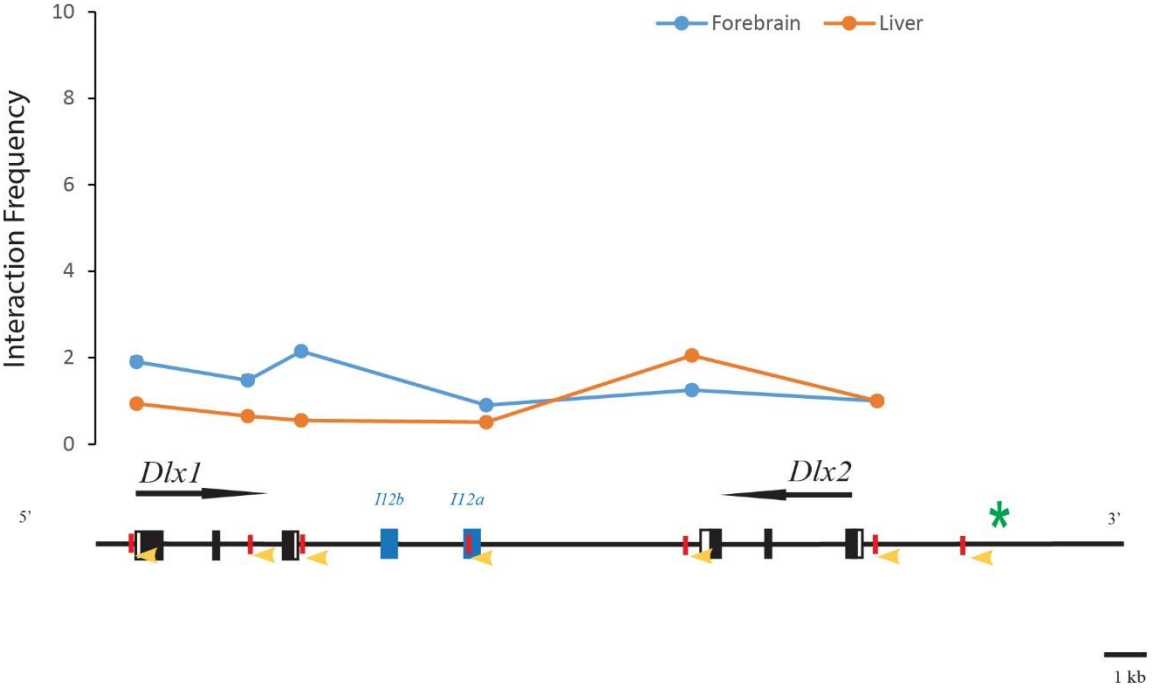
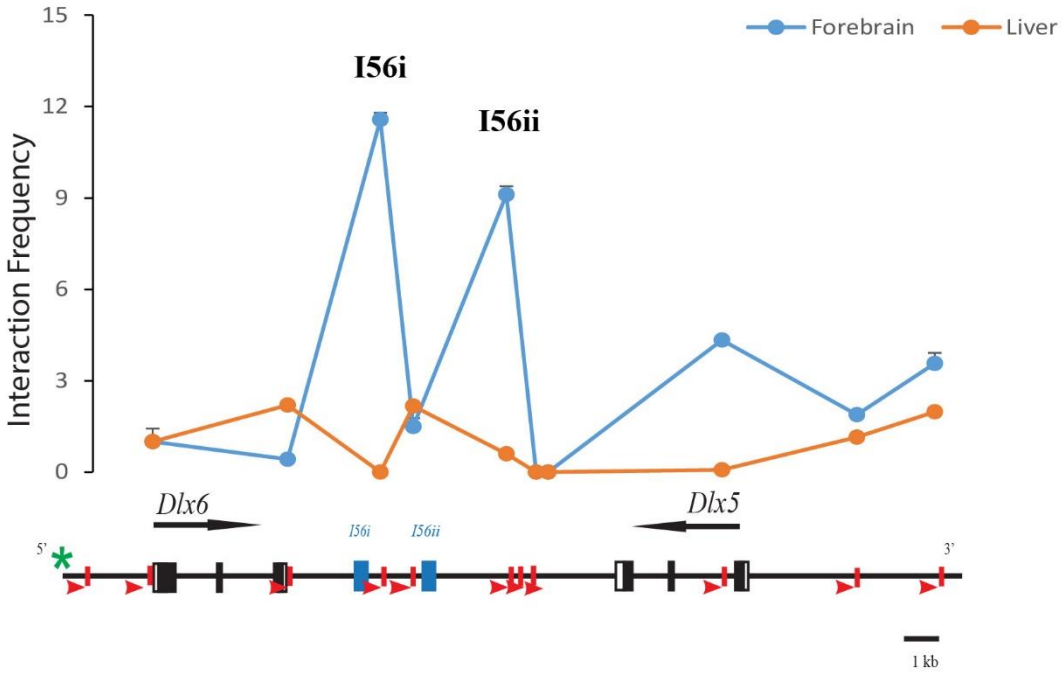
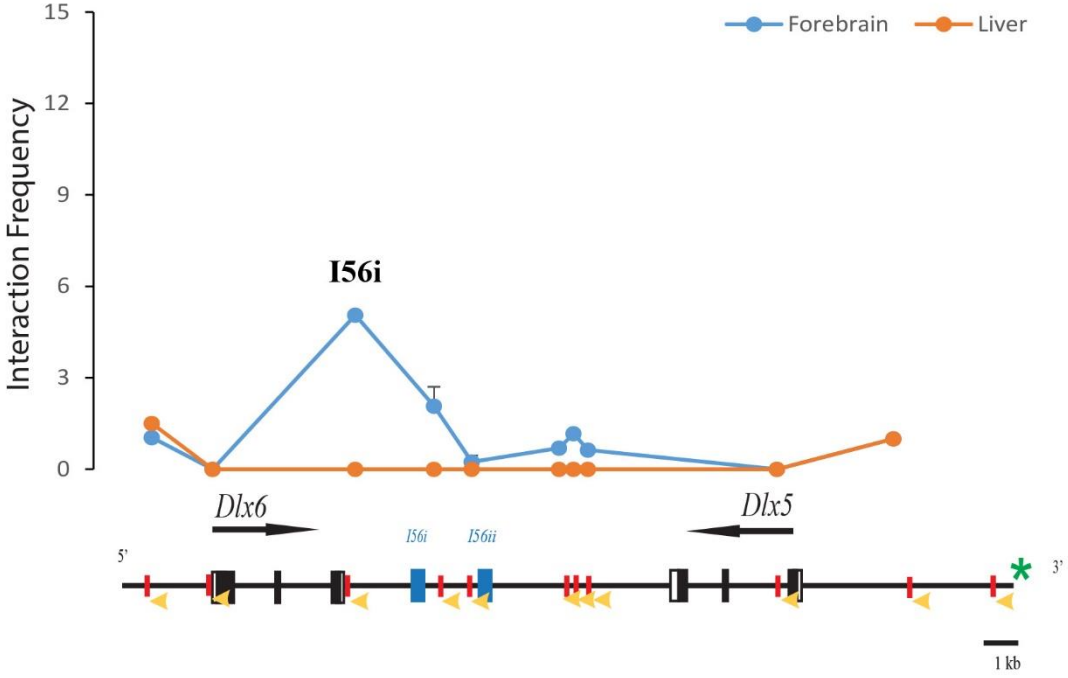

Forebrain
Liver
Interaction Frequency
10
8
6
4
2
0
Dlx1
Dlx2
Il2b
Il2a
5'
3'
1 kb

Figure 2.2: I56i and I56ii enhancers interact with the promoter regions of *Dlx5/6* cluster at E13.5. Enhancer-promoter interactions between the *Dlx5/6* intergenic enhancers with the promoter regions of (A) *Dlx6* and (B) *Dlx5* at E13.5 in the developing mouse forebrain. Green asterix represents the fixed point at the promoter region of either *Dlx* gene. Error bars represent the standard error of the mean for three experimental replicates. The blue line corresponds to the 3C forebrain library (experimental library), while the orange line corresponds to the 3C liver library (negative library).

(A) E13.5


Forebrain
Liver
I56i
I56ii
Interaction Frequency
15
12
9
6
3
0
Dlx6
Dlx5
I56i
I56ii
5'
3'
1 kb

(B) E13.5


Forebrain
Liver
I56i
Interaction Frequency
15
12
9
6
3
0
Dlx6
Dlx5
I56i
I56ii
5'
3'
1 kb

2.3.2 The effect of development on DNA looping mechanism within each *Dlx* cluster.

In our quest to understand the role of *Dlx* intergenic enhancers in regulating and/or maintaining the chromatin structure by the means of DNA looping mechanism within each *Dlx* locus, we were interested to determine whether promoter-enhancer interactions would change at different developmental stages. Therefore, in addition to the E13.5 forebrain libraries, we generated libraries using cells from the ventral telencephalon of E15.5 embryos. The E15.5 developmental time-point was chosen in order to minimize the number of cells that do not express *Dlx* genes in the developing forebrain. Similar to the previous set of experiments, we used liver tissue as a negative control.

First, we addressed the interactions between the *Dlx1* promoter region with the intergenic region of the *Dlx1/2* locus. Based on the results shown in Fig. 2.3, the *Dlx1* promoter region still interacts with I12b and/or I12a intergenic enhancers at E15.5 (Fig. 2.3A). This interaction is very similar to the interactions that we previously observed at E13.5; however, the size of the peak at E15.5 is reduced compared with the one detected at E13.5. The decrease in the size of the peak could suggest that the *Dlx1* expression is required more during the early stages of the development. Since we did, however, capture this interaction at E15.5 it can be deduced that the interactions between the *Dlx1* promoter region and the intergenic region of *Dlx1/2* locus are maintained at different developmental time-points.

We also examined the potential interactions between the *Dlx2* promoter region with the intergenic enhancers of the *Dlx1/2* cluster. Our data indicated that the *Dlx2* promoter region interacts with the I12b enhancer at E15.5 (Fig. 2.3B). It is noteworthy that no interaction between the *Dlx2* promoter and I12b was detected at E13.5. The change in the DNA looping mechanism with regards to the interaction between the I12b enhancer and the *Dlx2* promoter may suggest that

the I12b enhancer is not the only enhancer interacting with the promoter of the *Dlx2* in order to initiate and/or maintain the expression of *Dlx2* during development. However, the change in the DNA looping could suggest that the activity of the I12b enhancer is required to control and/or maintain the expression of *Dlx2* during development.

In addition to these set of experiments, we also examined the impact of development on the enhancer-promoter interactions taking place at the *Dlx5/6* locus. Our 3C investigation suggested that the *Dlx6* promoter region interacts with the I56i intergenic enhancer at E15.5 (Fig. 2.4A). It is important to note that the interaction between the I56ii enhancer and the promoter region of *Dlx6* is no longer detectable at E15.5 (Fig. 2.4A) when compared to the interactions that were captured at E13.5 (Fig. 2.2A). It is noteworthy that the size of the peak corresponding to the I56i enhancer is much bigger at E15.5 than the one observed at E13.5. It can be hypothesized that, due to the lack-there-of the I56ii interaction with the *Dlx6* promoter, the increase in the enhancer-promoter interaction between the I56i enhancer and the *Dlx6* promoter is required to maintain the transcript levels of *Dlx6* during development.

In parallel, we also examined the interactions that occur between the promoter region of *Dlx5* in the developing forebrain at E15.5. As shown in Fig. 2.4B, our results indicate that the I56i intergenic enhancer interacts with the promoter region of *Dlx5* in the developing forebrain at E15.5 (Fig. 2.4B). The interactions between I56i enhancer and the promoter region of *Dlx5* that are captured at E15.5 are similar to those seen at E13.5 (Fig. 2.2B); however, the size of the peak, which corresponds to the I56i enhancer, is a lot smaller at E15.5 (Fig. 2.4B) than the one detected at E13.5 (Fig. 2.2B). This phenomenon is intriguing since the decrease in the I56i interaction frequency with the promoter of *Dlx5* is accompanied by a decrease in the interactions between I56i enhancer and the promoter of *Dlx6* at E15.5 (Fig. 2.4). Our data from investigating the interactions

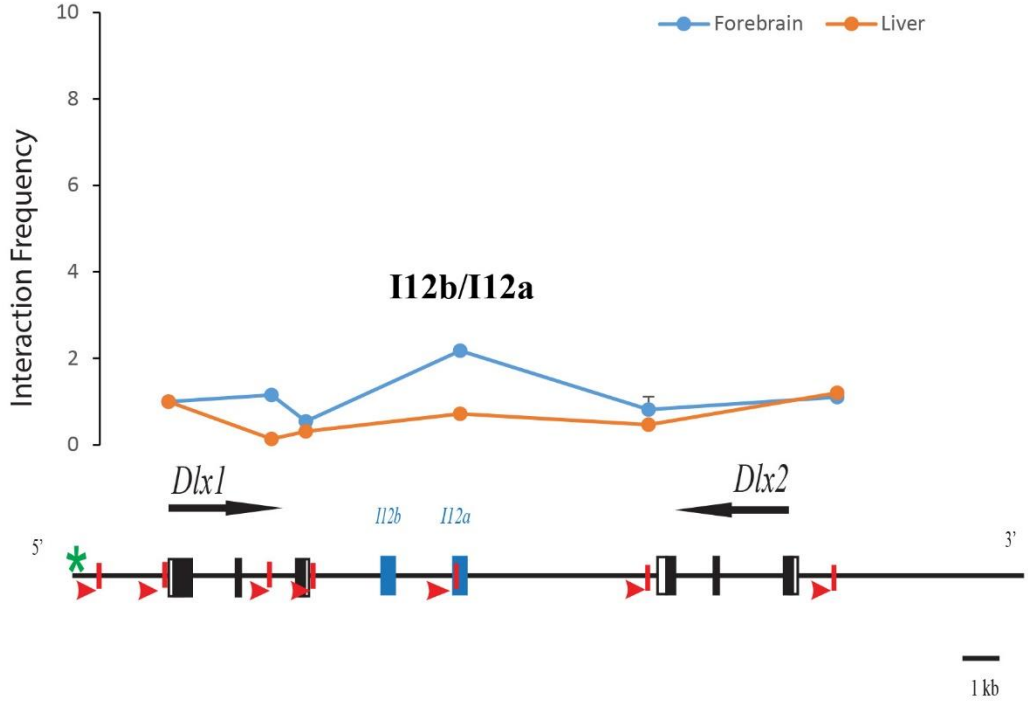
within the *Dlx5/6* cluster suggests that the activity of the I56i enhancer is maintained at different developmental stages and interacts with both promoter regions of *Dlx5/Dlx6* on either sides of the locus.

Similar to the previous set of experiments at E13.5, it is noteworthy that we did not observe any enhancer-promoter interactions within *Dlx* clusters in the liver library. The absence of enhancer-promoter interactions in liver may suggest that DNA looping mechanism that is shown to take place to activate and/or maintain *Dlx* expression does not occur in liver since *Dlx* genes are not expressed in liver. Moreover, we are currently validating the enhancer-promoter interactions that are observed at E15.5 within both *Dlx* clusters by using the intergenic enhancers, where "peaks" were observed, as our reference point and examining the DNA looping with either promoter within each cluster.

The data presented in this set of experiments demonstrated that the promoter-enhancer interactions did in fact change during development; where, I12b showed interactions with *Dlx2* at E15.5 and the interactions between I56ii enhancer and the promoter regions of *Dlx6* is no longer detectable.

Figure 2.3: *Dlx1/2* intergenic enhancers interact with the promoters of *Dlx1/2* cluster at E15.5. Enhancer-promoter interactions between the *Dlx1/2* intergenic enhancers with the promoter regions of (A) *Dlx1* and (B) *Dlx2* at E15.5 in the developing mouse forebrain. Green asterix represents the fixed point at the promoter region of either *Dlx* gene. Error bars represent the standard error of the mean for three experimental replicates. The blue line corresponds to the 3C forebrain library (experimental library), while the orange line corresponds to the 3C liver library (negative library).

(A) E15.5


Forebrain
Liver
Interaction Frequency
10
8
6
4
2
0
I12b/I12a
Dlx1
Dlx2
I12b
I12a
5'
3'
1 kb

(B) E15.5

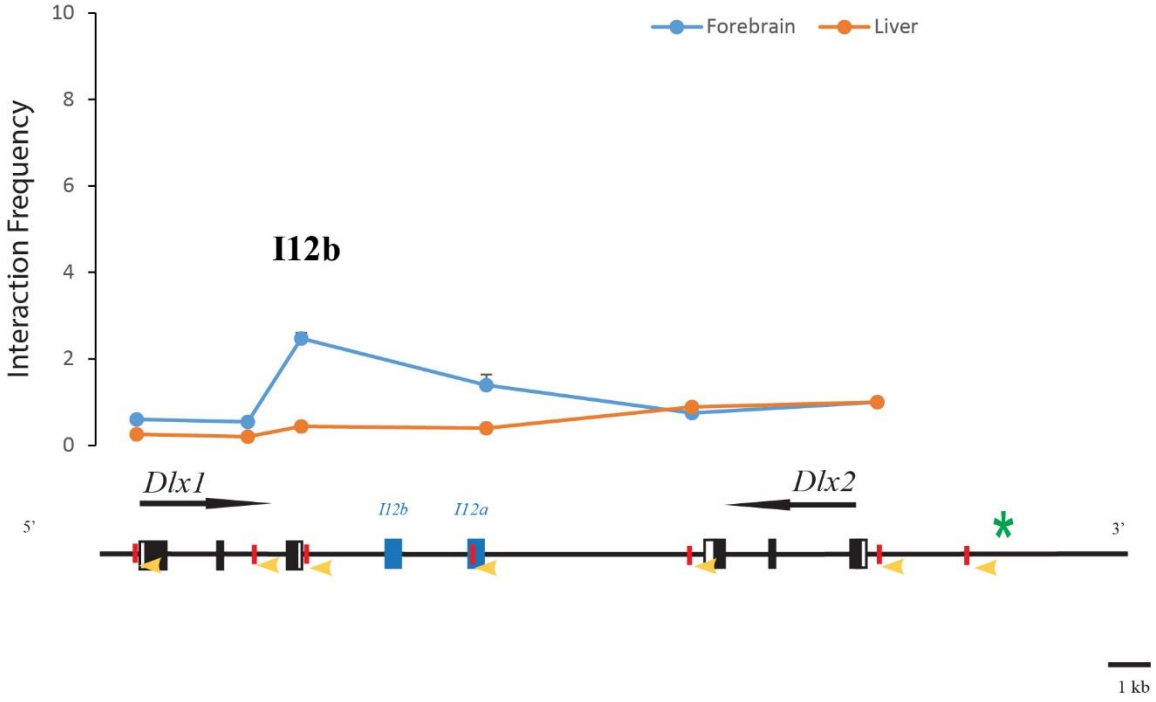
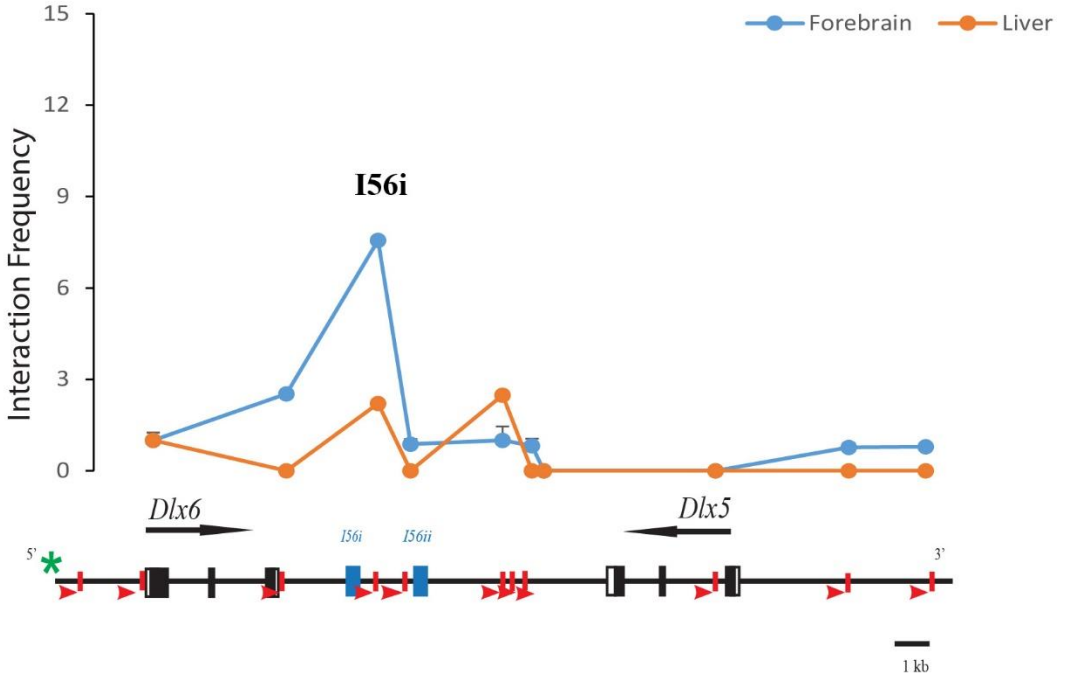
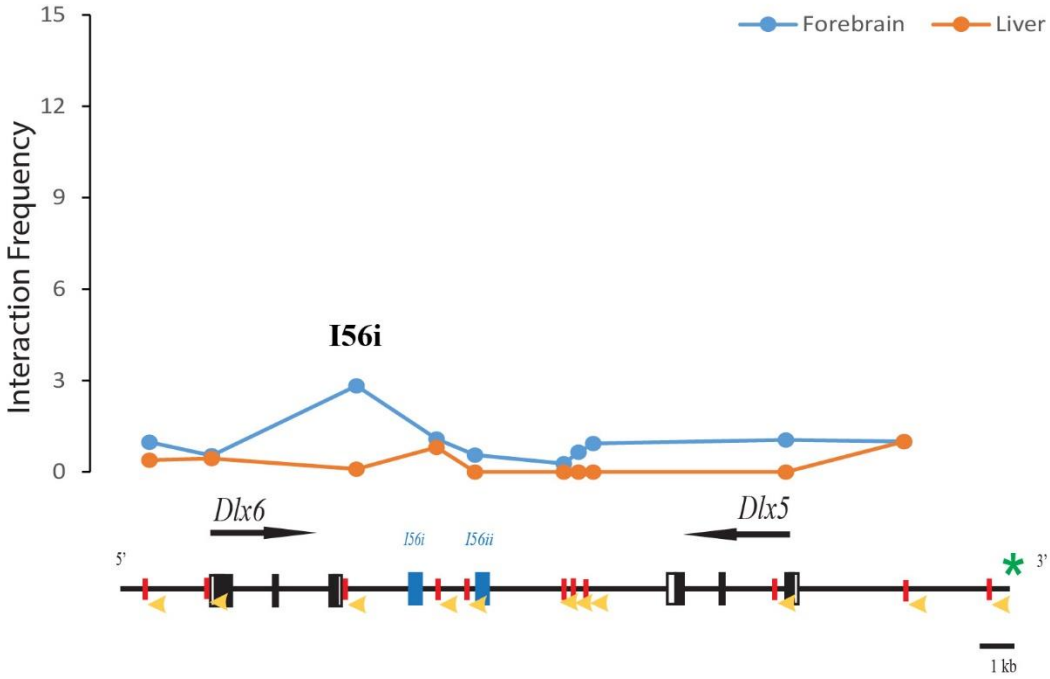

Forebrain
Liver
Interaction Frequency
10
8
6
4
2
0
I12b
Dlx1
Dlx2
I12b
I12a
5'
3'
1 kb

Figure 2.4: I56i enhancer interacts with the promoters of *Dlx5* and *Dlx6* at E15.5. Enhancer-promoter interactions between the *Dlx5/6* intergenic enhancers with the promoter regions of (A) *Dlx6* and (B) *Dlx5* at E15.5 of developing mouse forebrain. Green asterix represents the fixed point at the promoter region of either *Dlx* gene. Error bars represent the standard error of the mean for three experimental replicates. The blue line corresponds to the 3C forebrain library (experimental library), while the orange line corresponds to the 3C liver library (negative library).

(A) E15.5


Forebrain
Liver
Interaction Frequency
15
12
9
6
3
0
I56i
Dlx6
Dlx5
I56i
I56ii
5'
3'
1 kb

(B) E15.5


Forebrain
Liver
Interaction Frequency
15
12
9
6
3
0
I56i
Dlx6
Dlx5
I56i
I56ii
5'
3'
1 kb

2.3.3 *Trans* interactions exist between *Dlx1/2* and *Dlx5/6* bigene clusters in the developing forebrain at E13.5.

After investigating and understanding enhancer-promoter interactions within each *Dlx* locus, we were also interested to examine whether there could be physical interactions between the *Dlx1/2* and *Dlx5/6* bigene clusters in the developing forebrain at E13.5. In order to address this intriguing question, we generated a *trans* 3C forebrain library using the ventral telencephalon of developing forebrain at E13.5. In order to generate the *trans* library we used the same enzymes that were used to generate our *cis* libraries (namely, *Bam*HI-*Bgl*II double-digest) to generate compatible restriction sites. The ligation principle was similar to that of the *cis* libraries. A close physical proximity in the nuclear space resulting from physical interactions between the two loci would enable the ligation of the compatible restriction ends from one chromosome containing the *Dlx1/2* locus with the compatible ends located on the other chromosome containing the *Dlx5/6* locus. Similar to our previous experiments, mouse liver tissue was used as a negative control. We chose E13.5 for studying *trans* interactions since previous work using *lacZ* transgenesis has demonstrated that all forebrain enhancers are active at this developmental time-point.

First, we examined the possibility of the promoter regions of *Dlx1* and *Dlx2* genes interacting with the *Dlx5/6* cluster. The 3C analysis of the potential interactions between *Dlx1* promoter regions with *Dlx5/6* cluster showed that the *Dlx1* promoter region interacts with the I56i and/or I56ii intergenic region of the *Dlx5/6* locus (Fig. 2.5A). In parallel, we were also able to capture an interaction between the promoter region of *Dlx2* with an intergenic region that encompasses I56i although PCR signals appeared to be weaker than for the *Dlx1* interaction (Fig. 2.5B).

We then investigated the possibility of a *trans* interaction between the promoter regions of *Dlx5* and *Dlx6* with the *Dlx1/2* cluster, again at E13.5. As shown in Fig. 2.6A, our data showed that the promoter region of *Dlx6* interacts with the enhancers located in the intergenic region of *Dlx1/2* cluster. As for the *Dlx5* promoter, the signal that was obtained corresponded to the intergenic region of *Dlx1/*2 locus where I12b andI12a enhancers are located (Fig. 2.6B). The *trans* data were confirmed using two independent E13.5 forebrain libraries (data not shown). It is important to note that the location of the peaks between the two sets of libraries were the same; however, the intensity or height of the peaks varied between the two sets of libraries.

The lack of any interactions in the liver *trans* library strengthens the possibility of a *trans* interaction existing between *Dlx* loci in the developing forebrain at E13.5. Furthermore, we are currently examining the validity of the interactions that we observed in *trans* between the two *Dlx* loci by using the intergenic region, that corresponded to the signal, as our new reference point and investigating the *trans* interactions between the intergenic region of each cluster with the promoter regions of the other cluster. In addition to the validation PCRs, the interactions that are observed here are currently being validated using chromosome fluorescent *in situ* hybridization (FISH).

The presence of a *trans* interaction could suggest a positive cross-regulation or a cross-talk (Gould et al., 1997) between *Dlx1/2* and *Dlx5/6* loci in the developing forebrain at E13.5. Furthermore, the existence of a *trans* interaction between *Dlx* bigene clusters strengthens the possibility of an enhancer sharing mechanism (Eun et al., 2013) between the two *Dlx* loci in the developing forebrain. The presence of these *trans* interactions between the two *Dlx* clusters may suggest that the intergenic enhancers in each locus play an important role in bringing the two *Dlx* clusters together. This mode of cross-regulation could further suggest that *Dlx* genes are in a "poised state", which could represent an important regulatory role for *Dlx* genes during forebrain

development at E13.5. The cross-regulation by means of enhancer sharing between the two *Dlx* clusters may suggest a novel regulatory mechanism that would control the expression levels of *Dlx* genes during forebrain development.

Figure 2.5: *Trans* interactions may exist between *Dlx1/2* and *Dlx5/6* loci at E13.5. *Trans*-interactions between *Dlx1/2* intergenic enhancers with the promoter regions of (A) *Dlx6* and (B) *Dlx5* at E13.5 of developing mouse forebrain. Green star represents the fixed point at the promoter region of either *Dlx* gene. Error bars represent the standard error of the mean for two experimental replicates. A' and B' are the schematic representation of the interactions detected by 3C gel analysis represented in A and B respectively. The blue line corresponds to the 3C forebrain library (experimental library), while the red line corresponds to the 3C liver library (negative library).

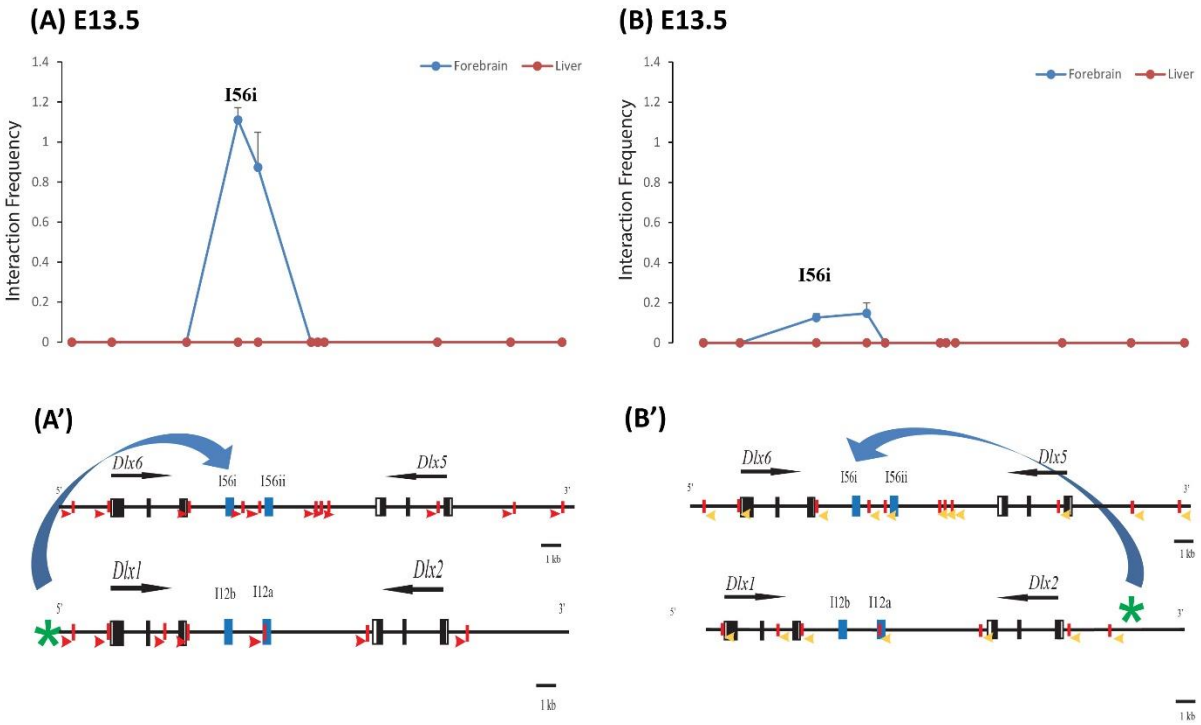
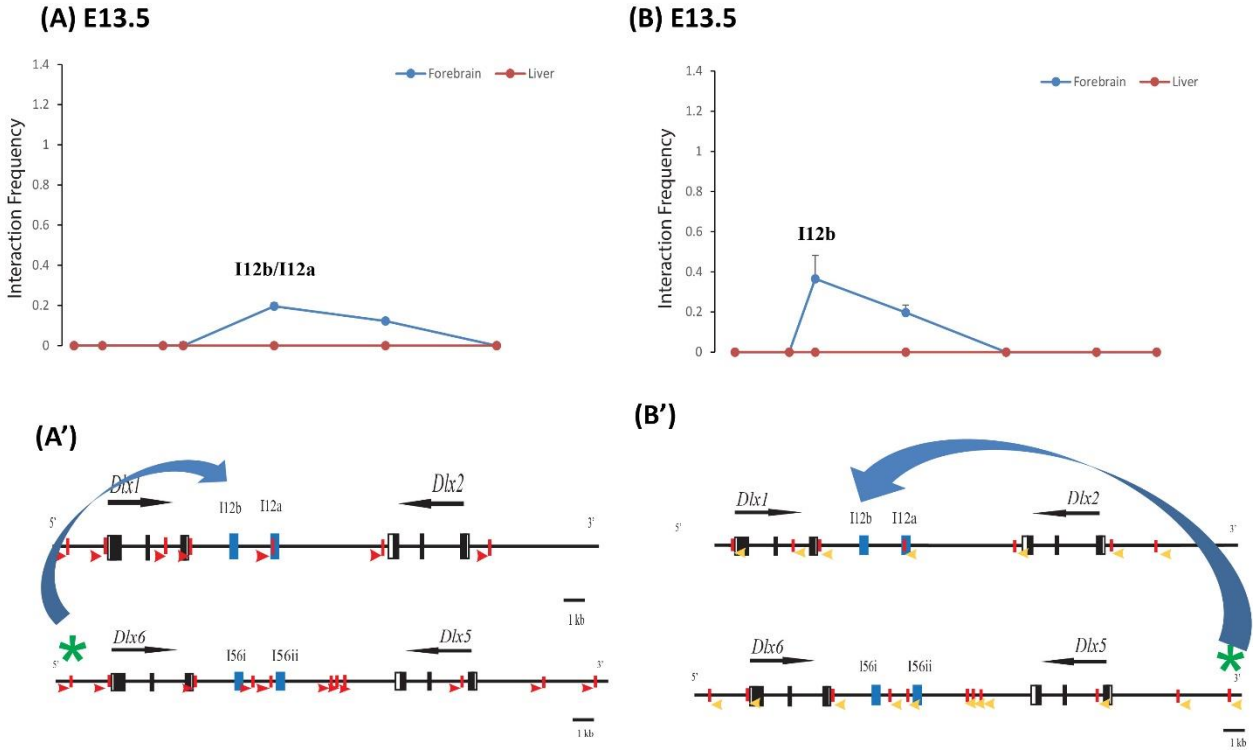

(A) E13.5
Forebrain
Liver
I56i
Interaction Frequency
1.4
1.2
1
0.8
0.6
0.4
0.2
0
(B) E13.5
Forebrain
Liver
I56i
Interaction Frequency
(A')
Dlx6
I56i
I56ii
Dlx5
5'
3'
1 kb
Dlx1
I12b
I12a
Dlx2
(B')
Dlx6
I56i
I56ii
Dlx5
Dlx1
I12b
I12a
Dlx2

Figure 2.6: *Trans* interactions may exist between *Dlx5/6* and *Dlx1/2* loci at E13.5. *Trans*-interactions between *Dlx5/6* intergenic enhancers with the promoter regions of (A) *Dlx1* and (B) *Dlx2* at E13.5 of developing mouse forebrain. Green star represents the fixed point at the promoter region of either *Dlx* gene. Error bars represent the standard error of the mean for two experimental replicates. A' and B' are the schematic representation of the interactions detected by 3C gel analysis represented in A and B respectively. The blue line corresponds to the 3C forebrain library (experimental library), while the red line corresponds to the liver library (negative library).


(A) E13.5
Forebrain
Liver
Interaction Frequency
1.4
1.2
1
0.8
0.6
0.4
0.2
0
I12b/I12a
(B) E13.5
Forebrain
Liver
Interaction Frequency
1.4
1.2
1
0.8
0.6
0.4
0.2
0
I12b
(A')
Dlx1
Dlx2
I12b
I12a
5'
3'
1 kb
Dlx6
Dlx5
I56i
I56ii
5'
3'
1 kb
(B')
Dlx1
Dlx2
I12b
I12a
5'
3'
Dlx6
Dlx5
I56i
I56ii
5'
3'
1 kb

2.4 Discussion

2.4.1 *Dlx1/2 cis*-regulatory elements play an essential role in controlling the *Dlx1/Dlx2* expression in the developing forebrain.

Studies aimed at discovering the *Dlx* intergenic enhancers concentrated on the expression patterns of the *lacZ* reporter gene in the developing transgenic mice (Ghanem et al., 2003; Ghanem et al., 2007; Ghanem et al., 2008). It has been demonstrated that the I12b-*lacZ* positive cells are detected in tangentially migrating cells that originate from the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE) between E11.5 and E13.5 in the developing forebrain (Ghanem et al., 2007). It was also suggested that since 93% of the adult cortical GABAergic interneurons exhibit I12b enhancer activity (Ghanem et al., 2007). Therefore, it is interesting that the I12b enhancer does not show any interactions with the promoter regions of *Dlx2* in the developing forebrain at E13.5 (Fig. 2.1B). Through *lacZ* transgenesis, it has been shown that URE2-*lacZ* expression patterns overlaps markedly with the expression patterns of I12b-*lacZ* in the developing forebrain (Ghanem et al., 2007). As a result, the lack of interactions between the I12b enhancer and the *Dlx2* promoter region at E13.5 can potentially be attributed to the activity of the upstream regulatory element 2 (URE2) located upstream of *Dlx1*. Interestingly, we were able to capture interactions between the I12b enhancer and the promoter region of *Dlx2* at E15.5 (Fig. 2.3B). This change in the DNA looping may suggest that the I12b enhancer plays an important role in maintaining the expression levels of *Dlx2* during development.

It is noteworthy that according to the 3C data obtained so far, the I12b and/or I12a enhancer regions interact with the promoter region of *Dlx1* in the developing forebrain at E13.5 and E15.5 (Fig. 2.1A, 2.4A). However, through analysis of the *lacZ* reporter construct, Park and colleagues (2004) demonstrated that the I12a enhancer located in the intergenic region of *Dlx1/2* cluster is

active in the branchial arches and the developing limb buds; however, I12a activity is undetectable in the developing forebrain (Park et al., 2004). The branchial arches were not investigated in the context of 3C due to the very low number of I12a-*lacZ*+ cells in the branchial arches, which would have decreased the chance or possibility of detecting a true interaction. Therefore, it can be concluded that the interactions that are captured between the *Dlx1/2* intergenic region and the promoter region of *Dlx1* can be attributed to the activity of I12b enhancer at E13.5 and E15.5.

By comparing the interaction patterns between the intergenic region of *Dlx1/2* cluster with the promoters of *Dlx1* and *Dlx2* at E13.5 and E15.5, it can be proposed that the I12b enhancer activity is essential in maintaining the transcript levels of *Dlx1* during development. In addition, it is important to note that the regulatory elements that may exert their effect on the *Dlx2* promoter to initiate the expression of *Dlx2* during development have not been identified, but it can be proposed that the I12b enhancer may be required to maintain the expression levels of *Dlx2* at E15.5. The above theory may suggest that there could be a positive feedback mechanism between the activity of the I12b enhancer and the maintenance of the expression levels of *Dlx1/Dlx2* in the developing forebrain. Additionally, there may be other unknown regulatory elements that may also contribute to the I12b regulatory pathway that are not understood and/or discovered yet.

2.4.2 The I56i and I56ii intergenic enhancers interact with promoters of *Dlx5/6* bigene cluster in the developing forebrain.

In addition to investigating the I12b activity patterns using *lacZ* transgenic mice, the activity patterns of the I56i-*lacZ* reporter gene were also examined. Spatiotemporal analysis of the activity of the I56i-*lacZ*+ cells demonstrated that the I56i *cis*-regulatory element (CRE) is mainly active in the tangentially migrating cells similar to that of I12b-*lacZ*+ cells (Ghanem et al., 2007).

It was also shown that the I56i enhancer was active in most of the subventricular zone (SVZ) cells in the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) as well as the caudal ganglionic eminence (CGE). Expression of I56i-*lacZ* was also detected in a large number of cells located in the mantle zone (MZ) of the developing forebrain (Ghanem et al., 2007). These former observations are consistent with the interaction patterns that are observed within the *Dlx5/6* cluster. According to Fig. 2.2, according to the 3C data obtained thus far, the I56i enhancer interacts with both promoters of *Dlx5* and *Dlx6* at E13.5. It is noteworthy that the interactions between I56i enhancer and the promoter regions of *Dlx5* and *Dlx6* are maintained during development at E15.5 (Fig. 2.4). This could suggest that the I56i enhancer plays an important regulatory role in controlling the expression levels of *Dlx5* and *Dlx6* during forebrain development.

Furthermore, the spatiotemporal *lacZ* analyses demonstrated that unlike the I12b and I56i enhancers, the I56ii enhancer is not active in GABA-expressing cells nor is it active in tangentially migrating GABAergic neurons (Ghanem et al., 2008). The I56ii enhancer has been shown to be active in a small subpopulation of post-mitotic projection neurons that are thought to be derived from LGE progenitors and have tangentially migrated deep to the mantle of the LGE and MGE between E11.5 and E13.5, a region also known as corridor cells (Ghanem et al., 2008; Lopez-Bendito et al., 2006; Maroof & Anderson, 2006; Wichterle et al., 2003). Since I56ii-*lacZ* expression patterns overlap markedly with the expression patterns of *Dlx6*, it was suggested that *Dlx6* expression could potentially be under control of the I56ii enhancer (please refer to Chapter 4 for more detailed analysis of the regulatory role of I56ii enhancer in the developing forebrain).

This hypothesis was addressed using 3C analysis of the developing mouse forebrain at E13.5. As depicted in Fig. 2.2A, our 3C data obtained thus far suggest the I56ii enhancer only interacts with the promoter region of *Dlx6* at E13.5 (Fig. 2.2A). The previously published work by

Ghanem et al (2008) demonstrated that the *lacZ* reporter staining was no longer detectable in the forebrain of the developing transgenic mice beyond E13.5 (Ghanem et al., 2008). This is also consistent with our 3C data at E15.5, where we did not observe any interactions between the I56ii enhancer and the promoter region of *Dlx6*. This unique interaction between I56ii enhancer and the promoter region of *Dlx6*, could suggest that I56ii enhancer is a regulator of *Dlx6* expression in the developing forebrain at E13.5. This potential regulatory role between I56ii enhancer and *Dlx6* may suggest that *Dlx6* could be involved regulating the forebrain development by coordinating the proper function and activity of the corridor cells (please refer to Chapter 4 for more in-depth analysis of the regulatory role of I56ii enhancer).

The changes that occurred in the interactions between the intergenic enhancers of the *Dlx5/6* cluster and the promoter of *Dlx6* gene is particularly interesting since previous study through *lacZ* transgenesis, reported that the I56i and I56ii intergenic enhancers are not active in the same cell population in the developing forebrain (Ghanem et al., 2008). Therefore, the interactions that are captured at E13.5, where both I56i and I56ii seemed to interact with the promoter of *Dlx6*, could be due to the limitation of 3C technique. This limitation is due to the inability of this technique to capture interactions in a single cell. Thus, it is important to note that the overall interactions that are captured do not necessarily mean that these interactions occur in the same cell at the same time. As a result, since the interactions that are presented here are derived from a heterogeneous cell population, our 3C data present an overall view of the interactions that occur in the developing forebrain. Therefore, the interactions between the I56i and I56ii enhancers with the *Dlx6* promoter suggest that *Dlx6* may be playing different regulatory roles in the different cell populations of the developing forebrain. More specifically, since it has been shown that the I56ii enhancer is active in the corridor cells at E13.5 (Ghanem et al., 2008), the interactions

between the I56ii enhancer and the promoter of *Dlx6* may contribute to the maintenance of the activity of the corridor cells. Whereas, in a different cell population where the I56i enhancer is interacting with the promoter region of *Dlx6*, the expression of *Dlx6* may serve different regulatory functions, for instance, being involved in the proper development and differentiation of the GABAergic interneurons.

The importance of *Dlx6* expression in the developing forebrain is further highlighted by the changes that are captured in the enhancer-promoter interactions at E15.5. According to our 3C data from the E15.5 library, an increase in the interaction frequency between the I56i enhancer with the promoter of *Dlx6* is accompanied by a decrease in the frequency of interactions between the I56i enhancer and the promoter region of *Dlx5*. The changes in the interaction frequencies at E15.5 imply that the interactions between the I56i enhancer and the *Dlx6* promoter were captured in a higher number of cells at E15.5 than the interactions between the I56i enhancer and the promoter of *Dlx5* (please refer to Chapter 4 for a more detailed mechanistic model highlighting the potential role of *Dlx6* in the developing forebrain).

2.4.3 *Trans* interactions may suggest a very sophisticated regulatory mechanism for the I12b and I56i enhancers during forebrain development.

The functional importance of such interchromosomal (*trans*) genomic interactions are not very well understood and are highly debated. It has been suggested that interchromosomal (*trans*) interactions could be a result of co-regulated genes sharing common nuclear space in order to respond to similar regulatory factors (Sexton et al., 2009). Previous cases of *trans* interactions were reported in a study conducted by Spilianakis and colleagues (2005) who demonstrated that the Locus Control Region (LCR) upstream of the globin promoter regulates the TH2 cytokine

genes as well as *Ifng* gene (a TH1 cell-expressed gene) in immature T-cells (Spilianakis et al., 2005). It was demonstrated that as the cells mature and become polarized, these interchromosomal interactions (*trans* interactions) were diminished and changed into intrachromosomal interactions (Spilianakis et al., 2005). Therefore, it was suggested that co-association of two different gene loci could represent a "poised state" for either gene involved. The "poised state" could allow for a rapid activation of either gene locus under appropriate conditions during specific developmental stages (Spilianakis et al., 2005).

Transgenic analysis of *Dlx* intergenic enhancer activities has demonstrated that the I56i and I12b CREs located in the intergenic regions of *Dlx5/6* and *Dlx1/2* clusters, respectively have overlapping activities in the majority of the cells in the SVZ and MZ, suggesting that these CREs are mainly active in the same cell populations and could potentially respond to similar regulatory factors (Ghanem et al., 2007). Therefore, it is possible that the I12b and I56i enhancer sequences co-regulate the expression levels of the *Dlx* clusters in order to properly coordinate the timing and relative expression of the *Dlx* genes in the developing forebrain. This level of co-regulation can potentially be due to the fact that *Dlx* genes may share a common nuclear space, which can further be explained by the long-range chromatin interactions observed in *trans* between *Dlx1/2* and *Dlx5/6* clusters (Fig. 2.5 and 2.6). As it is depicted in Fig. 2.5 and 2.6, the I12b and I56i CREs are communicating with each other via *trans* interactions between the two clusters. The *trans* interactions that are observed between *Dlx1/2* and *Dlx5/6* clusters may be a result of the intergenic regulatory elements interacting with *Dlx* genes on the other cluster, which might serve as an internal regulatory role in controlling the expression levels of *Dlx* genes in the developing forebrain. This intricate level of genomic organization could indicate a new mode of cross-

regulatory mechanism for the transcriptional regulation of *Dlx* genes in the developing forebrain (Dmitriev et al., 2009; Petrov et al., 2008; Sexton et al., 2009).

2.4.4 *Trans* and *cis* interactions between and within *Dlx* loci may reveal a novel but provocative role for the I56i enhancer.

As discussed extensively, our 3C data did not capture any interactions between the I12b enhancer and the promoter region of *Dlx2* at E13.5, which led to the hypothesis that the expression of *Dlx2* at E13.5 can be attributed to the activity of the URE2 enhancer upstream of *Dlx1*. In addition to the above hypothesis, the data from *trans* library at E13.5 showed that the I56i enhancer interacts in *trans* with the promoter regions of *Dlx1/Dlx2*. Therefore, it can be hypothesized that in addition to URE2, the I56i enhancer could possibly be responsible for regulating the expression levels of *Dlx2* in the developing forebrain at E13.5. This hypothesis may suggest a novel function for the I56i enhancer, in which, in addition to regulating the expression levels of the *Dlx5* and *Dlx6* by means of interacting with their promoter regions at E13.5, the I56i enhancer also regulates the expression of *Dlx1/Dlx2* in *trans* through interacting with the promoter regions of *Dlx1/2* cluster at E13.5. It is important to note that a study conducted by Ghanem and colleagues (2008) through *lacZ* transgenesis demonstrated that the expression patterns of I56i-*lacZ,* I12b-*lacZ,* and URE2-*lacZ* overlapped markedly in the developing forebrain at E13.5 (Ghanem et al., 2008). Such pronounced overlap in the activity patterns of I56i, I12b and URE2 at E13.5 may support the above hypothesis, in which I56i and/or URE2 may be regulating the expression levels of *Dlx2* at E13.5.

If this hypothesis holds true, one would expect that the promoter regions of *Dlx1/Dlx2* locus would be brought to a close physical proximity with the *Dlx5/Dlx6* promoters in the nuclear space. Thus we should have been able to detect *trans* interactions between the promoter regions of *Dlx1/Dlx2* locus with that of the *Dlx5/Dlx6* cluster. However, such interactions were not captured

in our 3C *trans* data. The absence of the interactions between the promoter regions of the two loci could possibly be explained by several factors. First, since we generated our 3C libraries from a heterogeneous cell population, the interactions that are captured represent an overall picture of the interactions that occur in the developing forebrain at E13.5. It is paramount to note that the 3C data that are shown here do not imply that these interactions are happening in the same cell. Therefore, it is possible that the interactions that are captured either in *cis* or in *trans* at E13.5 are in fact occurring in different cell populations. Secondly, the absence of a signal pertaining to interactions between the promoter regions could also be due to experimental challenges including cross-linking efficiency, lack of complete digestion and successive ligation. Therefore, it is possible that some of these interactions are not captured using a PCR-based approach. Therefore, the absence of the interactions between the promoter regions does not necessarily imply that these interactions are not happening.

In conclusion, the data presented in this chapter reflect a complex and dynamic regulation of *Dlx* genes through enhancer-promoter interactions in *cis* and *trans* during the early stages of embryonic development through the activity of various regulatory elements. Furthermore, the enhancer-promoter interactions are shown to be maintained over different developmental stages at E13.5 and E15.5. These data demonstrated an intricate cross-regulatory mechanism, which highlights the important regulatory role of *Dlx* intergenic enhancers (more specifically I56i and I12b) in maintaining the *Dlx* expression.

Chapter 3: Developmental and behavioral abnormalities in mice with targeted mutations in *Dlx* enhancer sequences.

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Preface:

The work presented in this chapter is a collective effort by several members of Dr. Ekker's laboratory and provides an overview of the developmental and behavioural analyses of the various *Dlx* mutant lines where either forebrain intergenic enhancers were deleted or a SNP knock-in (vI56i) was introduced in the I56i intergenic enhancer. This chapter is a compilation of the results obtained by Man Yu (2011), Cindy Lesage-Pelletier (2011), Crystal Esau (2013) and myself. I was assigned to compile all the data and prepare a manuscript for submission. The original manuscript was expanded in this chapter to make sure that its style was more suitable for my thesis. Man Yu (2011) characterized the phenotype associated with the ΔI12b mutant line; Cindy Lesage-Pelletier (2011) was involved with characterizing the vI56i mutant line. Crystal Esau (2013) worked on the ΔI56i mutant line and I was mainly involved with the ΔI56ii mutant line, but was also involved in assisting Crystal Esau with her experiments. The next chapter (chapter 4), provides a more detailed functional analysis of the I56ii and I56ii-I12b enhancer deletions on the developing mouse forebrain.

3.0 Abstract

The *Distal-less* homeobox (*Dlx*) genes encode homeodomain transcription factors that are involved in early vertebrate development of limbs, sensory organs, branchial arches and forebrain (telencephalon and diencephalon). The mouse and human genomes each have six *Dlx* genes organized into three convergently transcribed bigene clusters (*Dlx1/2*, *Dlx3/4* and *Dlx5/6*) with *cis*-regulatory elements (CREs) located in the intergenic region of each cluster. The *Dlx1/2* intergenic region harbors the I12b/I12a CREs, while *Dlx5/6* includes I56i/I56ii. In order to determine the functional importance of these CREs on *Dlx* expression and forebrain development, we have generated several mutant mouse-lines where *Dlx* forebrain intergenic enhancers are deleted (ΔI56i, ΔI56ii, ΔI12b) or mutated (vI56i). Upon characterizing the phenotypic changes that occur in mice harboring the aforementioned mutation(s), mouse-lines are viable, fertile and do not show obvious developmental defects. Here we demonstrate that as result of mutation in *Dlx* intergenic enhancers, the expression levels of *Dlx*, *Evf2* and some of their potential downstream targets, including *Gad2* are significantly impaired when compared to wildtype littermates. Mutations in *Dlx5/6* intergenic enhancers (ΔI56i, vI56i and ΔI56ii) result in decreased levels of GABA in the developing forebrain. Furthermore, a fear conditioning test demonstrated that the ΔI56i mutant mice have learning deficits. Characterizing mice with mutated *Dlx* intergenic enhancers will help us to further enhance our understanding of the role of these CREs in controlling *Dlx* genes during forebrain development.

3.1 Introduction

The precise control of gene transcription is crucial for the development of an organism. The genome contains the necessary information to ensure that genes are expressed at a suitable rate and in appropriate place (Uchikawa et al., 2003). Interpretation of genomic information involves integration of cellular history and extracellular environment, which ultimately happens at the level of chromatin and is mediated by *cis*-regulatory elements (CREs) including enhancers, promoters, silencers and insulators (Bulger & Groudine, 2010; Bushey, Dorman, & Corces, 2008; Riethoven, 2010). Amongst these CREs, enhancers play a particularly prominent role in spatiotemporal gene regulation during embryogenesis (Noonan & McCallion, 2010). The defining characteristic of enhancers is their ability to drive gene expression at a distance to the transcription start site (Ong & Corces, 2011; Williamson, Hill, & Bickmore, 2011). A central feature of enhancers is their ability to function as a binding platforms for transcription factors (TFs) and other regulatory molecules that are needed to activate and sustain transcription (Ong & Corces, 2011). Enhancers are usually located upstream (5'), downstream (3') or within the intronic region of the gene that they regulate (Kleinjan & van Heyningen, 2005). The genomic location of functionally homologous enhancers is often, but not always, conserved between species (Kalay & Wittkopp, 2010).

Gene families such as *Dlx* provide intriguing models for studying gene regulation and functional divergence between *cis*-regulatory elements. The *Dlx* bigene clusters are arranged in a convergent configuration with some of the enhancers located within the intergenic region (Fig. 1.1). Sharing of *cis*-regulatory elements between members of a *Dlx* bigene cluster may contribute to the overlap in gene expression and to their partial functional redundancy. Two highly conserved enhancer elements, I12b and I12a were discovered in the intergenic region of *Dlx1* and *Dlx2*

(Ghanem et al., 2003; Park et al., 2004; Poitras et al., 2007). Similarly, the I56i and I56ii enhancers were identified in the intergenic region of the *Dlx5/Dlx6* genes of zebrafish, mouse, and human and were able to target expression of reporter transgenes to the forebrain of both mouse and zebrafish in patterns that mimic the endogenous gene expression (Zerucha et al., 2000). The two genes from the *Dlx1/Dlx2* cluster are expressed in the developing forebrain with patterns that overlap partially with those of *Dlx5* and *Dlx6* (Ghanem et al., 2008). The arrangement of *Dlx* genes is conserved throughout evolution and a direct association has been observed between the genomic organization of the *Dlx* genes and their expression pattern in different species (Zerucha et al., 2000). Partial functional redundancy between *Dlx* paralogs is suggested by the overlapping gene expression patterns and phenotypes of mice with targeted *Dlx* mutations (Acampora et al., 1999; Anderson et al., 1997a; Depew et al., 1999; Mengsheng Qiu et al., 1997; Robledo et al., 2002).

To address the functional importance of the *Dlx* intergenic enhancers in the *Dlx1/Dlx2* and *Dlx5/Dlx6* intergenic regions, we have generated mutant mouse-lines where *Dlx* forebrain enhancers (namely I56i, I56ii and I12b) have been deleted from the intergenic region of each respective *Dlx* bigene cluster. A SNP associated with cases of autism has also been introduced in the ultraconserved region of the I56i enhancer (vI56i). Here we report that the enhancer deletions impair *Dlx* and *Evf2* expression levels with a profound effect on the downstream targets of *Dlx* including *Gad2*. Furthermore, mice harboring I56i deletion, demonstrate an increased learning deficit when compared to wildtype littermates.

3.2 Materials and Methods

3.2.1 Generation of mutant mice lines:

The ΔI56i, vI56i, ΔI56ii and ΔI12b mutant mice were generated at the Transgenic Mouse Core Facility at the McGill Cancer Center. Through homologous recombination, a LoxP-flanked PGK-neomycin-resistant cassette from pL452 replaced the enhancer sequence of I56i (6869406-6869829; Chromosome 6; *Eco*RI-*Bam*HI), I56ii (6871522-6871962; Chromosome 6; *Eco*RI-*Not*I) and I12b (71535989-71536569; Chromosome 2; *Eco*RI-*Bam*HI) in Bacterial Artificial Chromosomes (564M8 BAC and 510G1 BAC) harboring 200 Kb of the *Dlx5/Dlx6* and *Dlx1/Dlx2* loci, respectively. In addition, the vI56i mutant line was generated by replacing 6869406-6869829 (Chromosome 6) with 6869419-6869819 containing A6869601G SNP, which was followed by an adjacent neomycin cassette. The BAC clones were screened and sequenced to ensure a successful recombination. Following screening and sequencing, desired vectors were electroporated into 129SV mouse embryonic stem (ES) cells. Cells were selected with gentamicin to detect positive cells harboring the mutant BAC. Additionally, 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. Chimeric mice were mated with C57BL/6 wildtype mice and genotyped to ensure germ-line transmission of the mutant allele and to generate heterozygote progeny that were positive for the enhancer mutations in their germ line. The mutant mice were mated with SoxCre mice to remove the neomycin cassette from the intergenic region of *Dlx* clusters. This step was important to prevent the potential impact of the neomycin cassette during expression analysis.

3.2.2 Genomic DNA extraction:

Tissue samples from the tail end of weaned mice (approximately 5 mm), or extra-embryonic tissue of embryos, were digested in 300 – 500 µL of digestion cocktail, containing 10 µL of 10 mg/mL proteinase K per 100 µL of digestion buffer (50 mM Tris-HCl pH=8.0, 100 mM EDTA, 100 mM NaCl and 1% SDS). Samples were incubated in a heat bath at 55°C for 3 hours while vortexing the tubes every 30 min until samples were digested. Genomic DNA was extracted using a salt and ethanol precipitation protocol, where 0.4 volumes of 5M NaCl was added for every 500 µL of digestion buffer/proteinase K solution used in the previous step. Following centrifugation at maximum speed at 13 000 ×rpm (Microlite, Thermo Electron Corporation) for 5 min at room temperature, genomic DNA was precipitated by adding 0.8 volumes of isopropanol to the supernatant. Upon addition of isopropanol, a compact DNA pellet was formed. Subsequently samples were centrifuged at maximum speed at room temperature for 3 min and the resulting pellet was washed with 500 µL of cold 70% ethanol. After an additional centrifugation for 3 min at maximum speed at room temperature, the pellet was resuspended in 300 – 500 µL (the same volume as of the starting digestion cocktail) of room temperature TE buffer (100 mM Tris-HCl pH=8.0 and 10 mM EDTA).

3.2.3 Genotyping using Polymerase Chain Reaction:

Mice harboring the enhancer mutations on one or both alleles were screened using the polymerase chain reaction using a set of primers flanking the enhancer region (Table 3.1). A second PCR was used to detect the presence of the wildtype allele to help confirm the results of the first PCR and to further confirm the genotype of the screened mice. A 24 µL master mix and 1 µL genomic DNA template (equivalent of approximately 100 ng of genomic DNA) were used for each PCR reaction. The master mix for every PCR reaction contained 2.5 µL of 10X PCR buffer

(100 mM Tris-HCl pH=8.5, 500 mM KCl, 12 mM $MgCl_2$), 1.25 µL of 2 mM dNTPs, 1 U Taq polymerase, 0.5 µM of each forward and reverse primer and 18.55 µL of ddH2O (RNase-free water). The initial denaturation step was 95°C for 5 min. The subsequent denaturation step was 95°C for 30 sec, followed by an annealing step at 58°C for 45 sec and an extension step at 72°C for 45 sec. These 3 steps were cycled 39 times and a final extension step at 72°C for 4 min completed the reaction. PCR reaction products were fractionated on a 1% agarose gel containing 0.1 µg/mL red safe at 120V for 45 min and viewed on an UV transilluminator (Alpha Imager® EC, Alpha Innotech.). During the genotyping process, fetal haemoglobin was used as a positive control by utilizing FH.for (5´-GATCATGACCGCCGTAGG-3´) and FH.rev (5´-CATGAACTTGTCCCAGGCTT-3´) primers to ensure that there was adequate amount of DNA present in each PCR reaction for a successive amplification.

Table 3.1: Complete list of primers used for genotyping mutant mice using a Polymerase Chain Reaction.

Primer Name	Primer Sequence (5´-3´)
ΔI56iΔNeo.for	TGCTCCTGCCGAGAAAGTAT
ΔI56iΔNeo.rev	CAACAGATGGCTGGCAACTA
vI56i.for	CCCCAATGTCTGCTTCAAAT
vI56i.rev	GGAAGCCCCATACTGTGAGA
ΔI56iiΔNeo.for	GAGGGAAGAAAGACGGGAGT
ΔI56iiΔNeo.rev	GTCAGAGCCCAAACCTTGAA
ΔI12bΔNeo.for	TGAGTCTGTAATGGCAAAATGC
ΔI12bΔNeo.rev	CAGGTGCAGATTCCCTGAAG

3.2.4 Gene expression analysis:

3.2.4.1 RNA extraction from fresh tissue:

Total RNA was extracted from wild type, ΔI56ii and ΔI56ii/ΔI12b mutant mice forebrain at E13.5 embryonic stages. The ventral telencephalon was carefully dissected, instantly frozen in liquid nitrogen, and stored at -80°C until use. A maximum of 30 mg of tissue was used to extract total RNA using QIAGEN RNeasy Plus® Kit following the manufacturer's protocol. The total RNA concentration and purity were determined using nanodrop spectrophotometry as well as 1% agarose gel detection. Agarose gel detection was used to validate the absence of RNA degradation and genomic DNA contamination in the extracted RNA samples. A cDNA synthesis followed immediately after total RNA quantification to best maintain total RNA integrity (or in other words, to avoid any potential RNA degradation prior to cDNA synthesis). The remaining RNA was frozen in liquid nitrogen and stored at -80°C for future use.

3.2.4.2 cDNA synthesis from the extracted RNA:

Two μg of total RNA were used as template to synthesize first strand cDNA. A 20 μL solution of total RNA and 0.05 μg/μL of random primers (random hexamers; New England BioLabs) was heated for 10 min at 70°C and immediately chilled on ice. Once the solution was chilled, 8 μL of 5X First-Strand buffer, 4 μL of 0.1 M DTT, 1 μL of RNasin and 2 μL of 10 mM dNTP were added to the solution and mixed thoroughly. The solution was incubated for 10 min at 25°C for random primers and then equilibrated for 2 min at 42°C. Subsequent to the incubation, 1 μL of SuperScript Reverse Transcriptase II (Invitrogen Life Technologies) was added to the solution and mixed by pipetting. The final volume was adjusted to 40 μL with RNase-free water and samples were incubated for 50 min at 42°C followed by a heat inactivation step at 70°C for

15 min. A 10-fold dilution of cDNA template was used for subsequent experimentations. cDNA samples, both diluted and undiluted, were stored at -20°C until use.

3.2.4.3 Quantitative Real-Time PCR (qRT-PCR):

Quantitative Real-Time PCR (qRT-PCR) was done using SYBR Green, which is a fluorescent dye that binds to the double-stranded DNA during PCR amplification. In addition to the gene-specific primers for (*Dlx1, Dlx2, Dlx5, Dlx6, Evf2* as well as striatal markers *Islet1* and *Meis2* [Table 3.2]), qRT-PCR experiments were carried out in parallel with elongation factor 1 alpha (ef1α) housekeeping gene (Table 3.2). Primer design and experimental set up was carried out in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE Guidelines) (Bustin et al., 2010; Bustin et al., 2009). Specificity of the primers was confirmed by performing reverse transcriptase PCR and direct sequencing of the PCR products. The qRT-PCR reaction was performed using the illumina Eco™ Real-Time PCR System (Illumina Inc.). A 15 µL total reaction volume was used for each qRT-PCR reaction, consisting of 7.5 µL SYBR Green master mix, 1.5 µL each of forward and reverse primers at 10 µM concentration, 1 µL cDNA template (1:10 dilution of synthesized cDNA) and 3.5 µL ddH_2O. The initial denaturation step was 95°C for 8 min, followed by a subsequent denaturation step of 95°C for 15 sec, an annealing step at 58.5°C for 30 sec and a 30 sec extension step at 72°C. The reaction was performed for 39 cycles. The PCR plate was read after each cycle to measure the signal intensity emitted from the reaction. After the final cycle, a melting curve was monitored by gradually heating the plate from 55°C to 95°C and measuring expression at 0.5°C intervals. A single peak was determinant of a single PCR product in the test tube. In case of DNA contamination in the PCR tube, the peak corresponding to the contaminant would appear upstream in the melting curve analysis. The results of the qRT-PCR analysis were collected and analyzed

using the Eco™ illumina software (Illumina Inc.). For all replicates, the threshold value was manually set at 0.010. In order to determine the relative expression of each gene, the efficiency of designed primers in binding and amplifying the target gene was determined by generating a standard curve from undiluted and 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of cDNA template. Trials were run in triplicates and the average concentration threshold (Ct) value was plotted against the log(dilution). The slope of the best-fit line was used as a parameter to determine the efficiency (E) value ($E = 10^{\frac{-1}{slope}}$; thus a lower slope, implies greater primer efficiency in binding to and amplifying the target). The actual E value for each primer pair did not vary significantly and was very close to 2. The data were analyzed using the Pfaffl method of analyzing qRT-PCR data (Pfaffl, 2001). The mean normalized expression (MNE) levels in ventral telencephalon were also calculated (in 5 biological replicate runs, in which there were triplicate experimental runs for each biological run), since it is statistically more robust than normalized expression; MNE = $\frac{(E_{\mathrm{Re}f})^{\overline{Ct_{ref}}}}{(E_{T\mathrm{arg}et})^{\overline{Ct_{t\mathrm{arg}et}}}}$. Subsequently the standard error of MNE (SEMNE) was calculated as an estimate of standard deviation, computed from the data samples being analyzed.

SEMNE $= (\frac{(E_{\mathrm{Re}f})^{\overline{\Delta Ct_{ref}}}}{(E_{T\mathrm{arg}et})^{\overline{\Delta Ct_{t\mathrm{arg}et}}}}) \times (\sqrt{((\ln(E_{ref}) \times (SE\overline{\Delta Ct_{ref}})^2 + (\ln(E_{t\mathrm{arg}et}) \times (SE\overline{\Delta Ct_{t\mathrm{arg}et}})^2)})$; where SE $= \sqrt{(\frac{\sum_{i=1}^{n}(x_i - x)^2}{n \times (n-1)})}$ and $\Delta Ct = Ct_{HKG} - Ct_{target}$. The wildtype tissue was chosen as the control or calibrator tissue. The normalized relative expression of target genes in homozygotes and heterozygotes compared to wild type were calculated by using $2^{-\Delta\Delta Ct}{}_{target}$, where $\Delta\Delta Ct_{target} = \Delta Ct_{wildtype} - \Delta Ct_{target}$. A two-tailed t-test was used to investigate the statistical significance of the changes that were observed between the mutant mice and the wildtype littermate.

Table 3.2: Complete list of primers used for qRT-PCR experiments.

Primer Name	Primer Sequence (5´-3´)
Ef1a.for	AAGCTCTTCCTGGGGACAAT
Ef1a.Rev	ATGCTATGTGGGCTGTGTGA
Dlx1.For	CAGTTGCAGGCTTTGAACC
Dlx1.Rev	ACTTGGAGCGTTTGTTCTGG
Dlx2.For	GCCTCACCCAAACTCAGG
Dlx2.Rev	GCCGCTTTTCCACATCTTC
Dlx5.For	CGACTTCCAAGCTCCGTTC
Dlx5.Rev	TTCTTTCTCTGGCTGGCTG
Dlx6.For	CGGACCATTTATTCCAGCC
Dlx6.Rev	CGCTTATTCTGAAACCATATC
Islet1.For	GCCTCAGTCCCAGAGTCATC
Islet1.Rev	GGCTGGTAACTTTGCACCTC
Meis2.For	CCAGAGTGGAGACAACAGCA
Meis2.Rev	TCTGAAGGGTACGGGTGTGT
Evf2.For	CAATGCGAATGCTAGAAAATGA
Evf2.rev	ACAGCAGTGGGAAAGCAATC
Gad2.For	TCATTGCCCGCTATAAGATG
Gad2.Rev	GCAGCTCCCTTCTTGAGAGA1
Gsh2.For	CTTCCAATATGTACCTGTCCC
Gsh2.Rev	GCTTGTGTGATTGTTCCTCG
Mash1.For	TCTCCTGGGAATGGACTTTG
Mash1.Rev	AGGTTGGCTGTCTGGTTTG
Nkx2.1.For	AGCACACGACTCCGTTCTCA
Nkx2.1.Rev	CCCTCCATGCCCACTTTCTT

3.2.5 Embedding and cryosectioning of fresh samples:

Forebrain samples were collected from wild type, heterozygous and homozygous mouse embryos at E11.5 and E13.5. Following the dissection, the samples were immediately fixed for 2 hours at room temperature or overnight at 4°C in 4% paraformaldehyde (PFA) in 1X PBS. Following fixation, the samples were washed twice in 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH=7.4) for 5 min and immersed in 30% sucrose overnight at 4°C. Once tissues were calibrated in sucrose they were embedded using OCT cryomatrix embedding medium (Thermo Shandon; Fisher Scientific). A thin layer of cryomatrix was applied to the embedding mold and then the sample was positioned onto the mold and pressed lightly in order to eliminate any entrapped air. Additional cryomatrix was added to provide a supporting coat for the tissue section. Lastly, the mold was floated on liquid nitrogen and the matrix was allowed to harden. Molds were stored at -80°C until ready for sectioning. Prior to sectioning, the embedded forebrains were allowed to acclimatize to the cryostat chamber temperature (-18°C) for 45 min in order to further enhance the quality of the sections. The chamber temperature was set at -18°C in order to optimize the quality of sections. Twenty µm sections were obtained from the embedded specimen, utilizing a LEICA CM 1850 cryostat-microtome and collected on Fisherbrand SuperFrost® Plus microscope slides (Fisher Scientific). Slides were stored at -80°C until further use.

3.2.6 *In situ* hybridization on mouse forebrain sections:

3.2.6.1 Plasmid linearization:

A 200 µL solution containing 10 µg of cloned cDNA, 20 µL of restriction enzyme buffer, and 10 U of the appropriate enzyme were used to linearize the plasmid in order to generate

antisense probes. Five U of enzyme were added initially to the solution, after incubation for 1 hour at the appropriate temperature (depending on the enzyme), another 5 U of enzyme were added, mixed and then incubated overnight at the appropriate temperature as indicated in Table 3.3. Following incubation, digested and undigested plasmids were fractionated using 0.8% agarose gel electrophoresis to ensure a successive digestion.

Table 3.3: cDNA clones used to synthesize DIG-labelled RNA probes for *in situ* hybridization on frozen sections.

Clone ID	Vector	Insert Size (Kb)	Linearizing Enzyme	Promoter
mDlx1	pBS-SK	0.24	BamHI	T3
mDlx2	pBS-SK	1.7	HindIII	T3
mDlx5	pBS-SK	1.6	SmaI	T3
mDlx6	pCR2.1 TOPO	0.37	HindIII	T7
Islet1	pDrive	1.01	XhoI	T7
Meis2	pDrive	1.18	XhoI	T7
Evf2 (Bq)	pDrive	0.540	XhoI	T7

3.2.6.2 Phenol-Chloroform extraction of linearized plasmid:

Phenol-Chloroform extraction of linearized DNA was done by adding 0.1X volume (20 µL) 3M NaOAc and 1X volume (200 µL) of phenol-chloroform followed by vortexing the reaction for 2 min. The reaction was then centrifuged at maximum speed (13,000 ×rpm) for 10 min. The top phase was transferred to a new tube and 1X volume (200 µL) of chloroform was then added to the solution. The solution was vortexed for 2 min following a centrifugation for 10 min at maximum speed. The top phase was transferred to a new tube and the linearized plasmid was precipitated by adding 0.1X volume (20 µL) 3M NaOAc, 2.5X volume (500 µL) of cold 100% ethanol and incubating at -20°C overnight or at -80°C for at least an hour. The reaction was then centrifuged at maximum speed for 25 min at 4°C and washed with 1 mL 70% ethanol. Subsequent to centrifugation at 4°C for 10 min, the linearized plasmid was dissolved in 30 µL Nuclease-Free water. The concentration of linearized plasmid was quantified by nanodrop spectrophotometry (Thermo Scientific, Nanodrop 2000).

3.2.6.3 Antisense RNA probe synthesis:

One µg of linearized plasmid was used as a template to synthesize digoxygenin-11-UTP-labelled ribonucleotide probes. A 20 µL solution containing 1µg of linearized plasmid, 2 µL of 10X transcription buffer, 2 µL of DIG-dNTP, 0.5 µL of RNase inhibitor, and 2 µL of RNA polymerase (1 µL was added and incubated at 37˚C for an hour then added the second 1 µL and incubated for another hour at 37˚C) was incubated at 37°C for 2 hours. After incubation, 2 µL of DNAse I recombinant was added to the solution and was incubated for 15 min at 37°C. The synthesized probe was purified by adding 4 µL of 4M LiCl and 3X volume of cold 100% ethanol. The solution was subsequently centrifuged at maximum speed for 20 min at 4°C. The sample was

washed with 1 mL of 70% ethanol and centrifuged at max speed for 5 min at 4°C. The pellet was air dried on ice for a maximum of 4 min and resuspended in 20 µL of Nuclease-free water. The final solution was incubated at -20°C for 10 min. Upon synthesizing the probes, 1 µL of the synthesized RNA probes, 1 µL of RNA loading dye, and 3 µL of water were combined in one tube and 3 µL of RNA ladder, 1 µL of loading dye, and 1 µL of water were combined in a second tube. Both tubes were heated for 10 min at 70°C and subsequently chilled for 5 min on ice. The quality of the probes was then assessed using 1% agarose gel electrophoresis for 15 min at 120 V. If the quality of the probe was good a single clean band was observed on the gel. Lastly, the concentrations of the RNA probes were quantified using a nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000 Spectrophotometer). Probes were then stored in 2 µL aliquots at -80°C until use.

3.2.6.4 Hybridization and staining:

The *in situ* experiment was conducted using three biological replicates, and for each biological replicate, three sections corresponding to rostral, medial and caudal regions of each of the developing brains were examined. The following in situ hybridization protocol was adapted from Strahle and colleagues (Strähle et al., 1994). Frozen slides were thawed for 2 hours at room temperature. The edges of each slide were sealed using a pap-pen to keep liquid on the slide during the hybridization process. Probes were diluted in 1 mL hybridization buffer consisting of 100 µL of 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, pH=7.5), 500 µL of deionized/ultrapure formamide, 200 µL of 50% dextran sulphate, 100 µL of 10 mg/mL yeast tRNA, 20 µL of 50X Denhardts solution and 80 µL of ddH2O, at a dilution of 1:200. Prior to hybridization, the probe mixture was denatured for 10 min at 70°C. Slides were placed in a sealed Tupperware box lined with paper towel and Whatman paper soaked

with solution A (1X SSC, 50% formamide, 0.1% Tween 20) to make a humidification chamber. A 350 µL volume of the probe mix was added to each slide that was then covered with a coverslip and incubated at 70°C overnight. Following the incubation, slides were transferred to a Coplin jar and washed 2 × 30 min at 70°C with solution A. This step was followed by 2× 30 min washes in 1X TBST (140 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl pH=7.5, 0.1% Tween 20) at room temperature. Slides were then blocked in 10% fetal bovine serum (FBS) in 1X TBST (blocking solution) for at least 1 hour at RT. Subsequent to the blocking step, slides were placed back in a sealed Tupperware box with water-soaked paper towels at the bottom. A 350 µL volume of 1:1000 dilution of anti-Digoxygenin AP Fab fragment antibody (Roche) in blocking solution was added to each slide, which were then covered with a coverslip, and incubated overnight at 4°C. The next day, slides were placed in Coplin jars and washed 4-5X for 20 min in 1X TBST at RT. Slides were then equilibrated for 2 × 10 min in 1X NTMT staining buffer (100 mM NaCl, 100 mM Tris-HCl pH=9.5, 50 mM $MgCl_2$, and 0.1% Tween 20). Following the washes, each slide was covered with 400 µL of 1X NTMT containing 1.4 µL of 50 mg/mL BCIP and 2.7 µL of 50 mg/mL NBT and stained in the dark for 2 hours. Washing the slides twice in distilled water stopped the staining reaction. Upon deactivating the staining process, slides were fixed by adding 400 µL of 4% PFA in 1X PBS for 20 min at RT. Following the fixation, slides were rinsed with ddH_2O and mounted using aquamount mounding media. Lastly, slides were visualized using an Olympus BX-60 microscope.

3.2.7 Immunohistochemistry on mouse forebrain sections:

Slides were retrieved from the -80°C freezer and were allowed to thaw at RT for 2 hours. Antigen retrieval was performed by adding 300 µL of 1X PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.1% Tween 20 pH=7.4) to each slide and incubated for 10 min

in a humidified chamber. Slides were then equilibrated by adding 300 µL 1X TE buffer and incubated at RT for 10 min in the humidified chamber. Following incubation, the slides were blocked using 500 µL blocking solution (1X PBST supplemented with 10% heat-inactivated calf serum) at RT for 2 hrs in a humidified chamber. Slides were washed with 1X PBS for 10 min at room temperature. The primary antibody was diluted as recommended by the supplier in the blocking solution. Approximately 300 µL of diluted antibody were added to the slides which were incubated overnight at 4°C. After overnight incubation, the slides were washed three times in 1X PBS for 10 min each at room temperature. The secondary antibody was diluted 1:1000 fold in blocking solution (a complete list of all primary and secondary antibodies used in this project are available in Table 3.4). Approximately 300 µL of antibody were placed on each slide and incubated in the dark for 2 hrs at RT. Next, the slides were washed 3X with 1X PBS for 10 min each at RT. The slides were then cover slipped with mounting media and left in the dark at RT for 1 hr.

Alternatively, diaminobenzidine (DAB) staining was used to enhance the visualization of the signal on the sections. About 300 µL of 1X PBST was added to each slide and incubated for 10 minutes in a humidified chamber. 400 µL of 3% H_2O_2 in 1X PBST was added to each slide and slides were incubated for 15 min at RT. Slides were then washed 3×5 minutes in 1X PBS at room temperature. About 400 µL of blocking solution was added to each slide and incubated for 2 hours in a humidified chamber. Slides were washed with 1X PBS for 10 minutes at room temperature. The primary antibody was diluted as recommended by the supplier in blocking solution. Approximately 300 µL of diluted antibody were added to the slides which were incubated overnight at 4°C. After overnight incubation, the slides were washed three times in 1X PBS for 10 min at RT. The secondary antibody was diluted 1:500 fold in the blocking solution. Approximately 300 µL of antibody were placed on each slide and incubated in the dark for two hours at room

temperature. Next, the slides were washed three times with 1X PBS for ten minutes each at room temperature. 300 μL of DAB solution was added to each slide and slides were incubated in the dark for one hour. Slides were checked every 5 min to monitor the staining. Slides were then washed 3 times for 5 min each in 1X PBS at RT. Slides were fixed in 300 μL of 4% PFA in 1X PBS for 20 min at RT. Next, slides were washed in ddH_2O and mounted using aquamount mounting media.

Table 3.4: Complete list of antibodies and dilutions used for immunohistochemistry on coronal sections of the developing mouse forebrain at E13.5.

Primary antibody	Source	Dilution	Secondary antibody	Dilution
Anti-Islet1	Rabbit	1:250	Goat anti-Rabbit	1:1000
Anti-Ctip2	Rat	1:500	Goat anti-Rat	1:1000
Anti-Semaphorin 3A	Rabbit	1:200	Goat anti-Rabbit	1:1000
Anti-Slit1	Rabbit	1:200	Goat anti-Rabbit	1:1000
Anti-Ephrin A5	Rabbit	1:250	Goat anti-Rabbit	1:1000
Anti-GABA	Rabbit	1:500	Goat anti-rabbit	1:1000

3.2.8 Behavioural Analysis of mutant mice:

The behavioral analysis were performed at the Mouse Behavioral Core Facility at the University of Ottawa. All protocols that were used here were adapted from Crawley (2007) and were approved by Animal Care and Veterinary Services (ACVS) at the University of Ottawa (Crawley, 2007). We performed our behavioral testing on mice that were about 8-10 weeks old. For all mutant lines, a minimum of 10 wildtype and 10 mutant mice were examined for each gender. All the behavioral tests were performed with the help of an observer who was blinded to the genotype of the animals. We performed a variety of behavioral tests including beam break, pre-pulse inhibition, elevated plus maze, open field, adult and juvenile social interactions, and fear conditioning to test for the behaviors associated with possible deficiencies in forebrain development. More specifically, these tests were used to monitor any potential differences between motor and sensory coordination, fear and anxiety, learning and memory as well as the social behaviors of the mutant mice and their wildtype littermates. In all cases, the mice were allowed to habituate to the testing environment an hour before performing any of the tests with a background white-noise that was set to 70 dB. Between different tests, mice were allowed to rest for 2-3 days. Before and after examining the behavior of each mouse, the surface was cleaned with an antiseptic solution to avoid any potential interference with the behavior of the proceeding animal due to the odors left by the previous test mouse. Lastly we used Noldus Ethovision tracking software to detect the movement of each mouse in tests where this was appropriate. Multivariate ANOVA (IBM SPSS statistics 2.0 software) was used to investigate whether there is a statistical significance between the behaviour of the mutant mice compared to their wildtype littermates.

3.2.8.1 Beam Break:

The beam break test was designed to assess the animal's motor activity. Each individual is place in a separate cage surrounded by infrared emitters and receptors. This specific orientation of the laser beams provided linear infrared beams that were broken as results of the movement of the test animal. The number of times that the beams were crossed as a result of the movement of the test animal in the test cage was recorded every 5 min for a total of 15 hours. These data were used as an estimate to assess the locomotor activity of the test mouse.

3.2.8.2 Pre-pulse inhibition:

The pre-pulse inhibition test was designed to examine the sensory (auditory) coordination of the test animal. Each individual was placed inside a chamber that was just big enough to fit the test animal without having any freedom of movement. Each chamber was located inside a sealed box that was equipped with mechanical sensors measuring the reaction ("flinching") of the test animal to the stimulus. Each mouse was allowed to habituate to the chamber with no stimuli for 2 min. During the first day of the test, each mouse received 120 db pulses and the level of their reaction was scored by a computer as "pulse alone". During the second day of the test, animals were placed in the same chamber and a series of 4 db, 8 db and 16 db stimulus followed by 120 db pulses for 100 msec were given to each test mouse and the extent of their reaction to the stimulus was compared between different intensities (db) and the prepulse data. The percent prepulse inhibition was used to compare the mutant mice with their wildtype littermates.

3.2.8.3 Elevated plus maze:

The elevated plus maze test was used to measure anxiety levels of the mutant mice compared to wildtype littermates. The elevated plus maze consists of two arms perpendicular to

each other. One has open platforms, while the other arm is enclosed with walls surrounding it. The maze was raised 1 m above the floor. The testing room was well lit to increase the anxiety levels of the test mice. Each mouse was placed in the center, where both arms meet and was allowed to explore the maze freely for 10 min. The amount of time that each mouse spent in either open or closed arms and the number of entries in either arm were used to estimate the anxiety levels of the test mouse.

3.2.8.4 Open field:

The open field test was performed to further investigate the anxiety levels of the mutant mice compared to the wildtype littermates. The open field test consisted of a square box that is 45×45×45 cm in dimension. Each mouse is placed gently in the box and is allowed to explore the area for 10 min. The amount of time each mouse spends in the corners of the box compared to the center of the box is used as an estimate of the anxiety levels of the test animal.

3.2.8.5 Adult social interaction:

Adult social interaction was used to evaluate the social affiliation of mice harboring mutation in the I56i enhancer based on their ability to recognize members of their own group. The adult social interaction test consisted of two trials that were performed under red light and white noise. Each trial was performed for 5 min. In the first trial, the mouse was placed in the box that includes a wire mesh cage in the corner and was allowed to explore the area and then was removed from the box. During the second stage of this test, an unfamiliar mouse of the same gender, age and strain was placed in the wire mesh cage (target mouse). And then the mouse from the first trial (test mouse) was placed back in the box. The amount of time that the test mouse spent interacting

with the target mouse in the interaction zone around the wire mesh was used as an estimate of their sociability. The interaction zone was defined as a 15 cm × 30 cm area surrounding the wire mesh.

3.2.8.6 Juvenile social interactions:

The juvenile social interaction test was also employed to address the impact of mutations in the I56i enhancer on social behavior of the mutant mice. A test animal and a juvenile of the same gender were used to measure their sociability. All trials were performed under red light with white noise. The juvenile social interaction test consisted of two trials that were three days apart. Test mice were allowed to habituate to the testing environment for 15 min prior to the test. The test mouse and the juvenile mouse were placed in a novel cage simultaneously and were allowed to explore for 2 min. The length of time that the test mouse spent interacting with the juvenile mouse was recorded. Trial 2 was performed three days later, in which the same two mice were placed back in a new cage for 2 min. The amount of time that the test mouse spent interacting with the juvenile mouse was recorded. The difference between the interaction time from the first trial and the second trial were used as an estimate of their social behavior.

3.2.8.7 Fear Conditioning:

The fear conditioning test was utilized to investigate if the ΔI56i, ΔI56ii and ΔI12b enhancer deletions had any impact on learning and memory of the mutant mice compared to wildtype littermates. We examined learning through the ability of the mouse to be able to associate a neutral stimulus (cue) with a more severe consequence (shock). Once learning takes place and the animal associates the cue with the shock, the cue alone should be adequate to generate a fear response. The fear conditioning test spans over three days. During day one (training), the mice were trained to associate the cue with the shock. Each mouse was placed in a box and was allowed

to explore and habituate for 2 min. After 2 min, a tone (cue) was played for 30 sec, which was followed by a 2 sec shock at 0.5 mA. The mouse was allowed to calm down for 1 min and another similar round of cue and shock were applied. Overall during the 6 min for each trial the freezing behavior of the animal was recorded. The second day was dedicated to the contextual fear conditioning, where the mice were placed in the same environment as day one. The freezing behavior of each mouse was recorded for a total of 6 min in response to hearing the cue every 2 min for 30 sec at a time. The freezing behaviors in day 2 were used as an estimate to measure the ability of the mouse to remember the environment in which an aversive shock was delivered. Lastly, on the third day of the fear conditioning test, the mouse was placed in a novel environment. The freezing behavior of each mouse was recorded for 3 min to validate that the mouse did not associate the novel environment with that of day 1 and 2. Following this step, a cue similar to that of day 1 and 2 was played for 30 sec and the freezing behavior of the animal was recorded. The percent freezing from day 1, day2 and day3 were used to measure the learning and memory of the animal in associating the cue with the shock.

3.3 Results

3.3.1 *Dlx* mutant mice do not exhibit any morphological abnormalities:

Upon obtaining the ΔI56i, ΔI56ii, vI56i and ΔI12b mutant lines, we examined the potential impact of these mutations on the viability and fertility of the mutant mice. To address the question, we mated heterozygous male from each of the mutant lines with a heterozygous female of the same line and screened for homozygous offspring. Homozygous mice from all mutant lines did not exhibit any obvious morphological abnormalities when compared to wildtype littermates (data not shown). Furthermore, we examined the potential impact of the mutations on the fertility of the homozygous mice. The fertility of the ΔI56i, ΔI56ii, vI56i and ΔI12b homozygous mice did not seem to be affected as a result of the enhancer mutations.

3.3.2 The mutation in the intergenic enhancers significantly affects the mRNA levels of *Dlx* and other genes in the developing forebrain.

First we addressed the impact of the enhancer mutations on gene expression levels for each mutant line in the ventral telencephalon at E13.5. Our qRT-PCR data showed that the expression levels of *Dlx1* and *Dlx2* expression levels were not affected in the ΔI56i mutant line at E14.5 (Fig. 3.1A) (Esau, 2013). In parallel, the expression levels of *Dlx5* and *Dlx6* were significantly decreased (~80%, $p<0.01$, Fig. 3.1A). Furthermore, the I56i enhancer deletion significantly reduced the expression of *Gad2* (~50%, $p<0.05$) and *Evf2* (~95%, $p<0.01$) in the ΔI56i mutant mice (Fig. 3.1A) (Esau, 2013). These data suggest an important regulatory role for the activity of the I56i enhancer in regulating and maintaining the expression of *Dlx* genes as well as *Gad2* and *Evf2*.

We also investigated the impact of a knock-in mutation in the I56i enhancer (vI56i) on the expression levels of *Dlx* genes, *Gad2* and *Evf2* at E13.5 (Lesage-Pelletier, 2011). As it is depicted in Fig. 3.1B, the SNP knock-in mutation did not affect the expression levels of *Dlx1* and *Dlx2* in

the developing forebrain at E13.5 (Fig. 3.1B). The SNP mutation in the I56i enhancer reduced the transcript levels of *Dlx5* and *Dlx6* by approximately 25% and 30% at E13.5 respectively ($p<0.01$) (Lesage-Pelletier, 2011). It is interesting to note that the decreases in mRNA levels of *Dlx5/Dlx6* did not affect the expression levels of *Gad2*. This is interesting since *Gad2* expression levels are thought to be regulated and/or maintained by the DLX proteins. Lastly, the SNP mutation significantly decreased the expression of *Evf2* non-coding RNA (~70%, $p<0.01$) (Lesage-Pelletier, 2011). Collectively, the SNP mutation in the I56i enhancer seems to have altered the activity of the I56i enhancer in the developing forebrain at E13.5, which in turn, may have affected the expression levels of *Dlx5/6* and *Evf2*.

Similar to the previous two mutant lines, we also examined the expression profiles of the ΔI56ii mutant mice in the ventral telencephalon at E13.5. Our data show that the I56ii enhancer deletion significantly reduced the expression levels of *Dlx5* (~25%, $p<0.01$), *Dlx6* (70%, $p<0.001$), and *Evf2* (70%, $p<0.001$) at E13.5 (Fig. 3.1C). The decreases in the expression of *Dlx5/6* were paralleled with a significant decrease in the transcript levels of *Gad2* (~70%, $p<0.001$). It is noteworthy that *Dlx1* expression was significantly increased (~55%, $p<0.01$) in the ΔI56ii mutants, while the expression levels of *Dlx2* remain unchanged (Fig. 3.1C). Lastly, the I56ii enhancer deletion significantly reduced the expression of *Islet1* and *Meis2,* two striatal markers (~50%, $p<0.001$) at E13.5. These changes collectively suggest a regulatory role for I56ii enhancer in the developing forebrain at E13.5 (please refer to chapter 4 for more detailed analysis of the I56ii mutant mice).

Lastly, we were interested in determining the changes in the expression levels of *Dlx* genes, *Gad2* as well as other transcription factors including *Mash1, Nkx2.1, Gsh* in the ΔI12b mutant mice at E13.5 (Yu, 2011). Our qRT-PCR data from the ΔI12b mutant mice showed that the

expression levels of *Dlx1* and *Dlx2* were decreased by approximately 25% ($p<0.01$) and 20% ($p<0.05$) respectively in the ventral telencephalon of the ΔI12b mice at E13.5 (Fig. 3.1D) (Yu, 2011). It is important to note that the I12b enhancer deletion did not have any significant impact on the expression of *Dlx5, Dlx6, Mash1, Nkx2.1, Gsh* and *Gad2* (Fig. 3.1D). The changes in the ΔI12b mutant mice are a lot less pronounced when compared to the other mutant lines discussed here. This could imply that other regulatory factors may regulate the activity of the *Dlx1* and *Dlx2* in the developing forebrain at E13.5 (Yu, 2011).

In addition to the qRT-PCR analysis of mutant lines, we also examined the effect of mutations of the spatial expression of *Dlx* genes as well as *Evf2* in the developing forebrain. We did not notice any changes in the expression patterns of the aforementioned genes in the ventral telencephalon of the ΔI12b and vI56i mutant mice at E13.5 (data not shown) (Lesage-Pelletier, 2011; Yu, 2011). However, our *in situ* hybridization results from the ΔI56i and ΔI56ii mutant mice showed a global change in the expression patterns of *Dlx1, Dlx5, Dlx6* and *Evf2* at E13.5 (Fig. 3.2). The expression patterns remained similar between the ΔI56i (Fig. 3.2A-C') and ΔI56ii (Fig. 3.2D-F') mutants and wildtype mice at E13.5. The expression level of *Dlx5, Dlx6* and *Evf2* were greatly reduced in the subventricular (SVZ) and mantle zones (MZ) (Fig. 3.2). Furthermore, our *in situ* experiments demonstrated that *Dlx1* expression is increased in the VZ and SVZ of the ΔI56ii mutant mice at E13.5 (Fig. 3.2D-D'; please refer to chapter 4 for more detailed expression analysis of I56ii mutant mice). Overall, the expression studies suggest an important regulatory role for the intergenic enhancers in regulating *Dlx* expression levels in the developing forebrain.

Figure 3.1: Mutant mice show impaired *Dlx, Gad2* and *Evf2* expression levels in the developing forebrain. Quantitative Real-Time PCR (qRT-PCR) results corresponding to relative expression levels of *Dlx1*, *Dlx2*, *Dlx5*, *Dlx6*, *Gad2* and *Evf2* transcripts in the mouse ventral telencephalon of wildtype and homozygous littermate embryos from (A) ΔI56i at E14.5, (B) vI56i, (C) ΔI56ii and (D) ΔI12b at E13.5. In all cases, gene expression levels were analyzed using five biological replicates each of which was assayed three times. The expression level of wild-type mice was defined as 1.0. Data are expressed as mean + SEM. (**P* <0.05) (***P* < 0.01) (****P*<0.001).

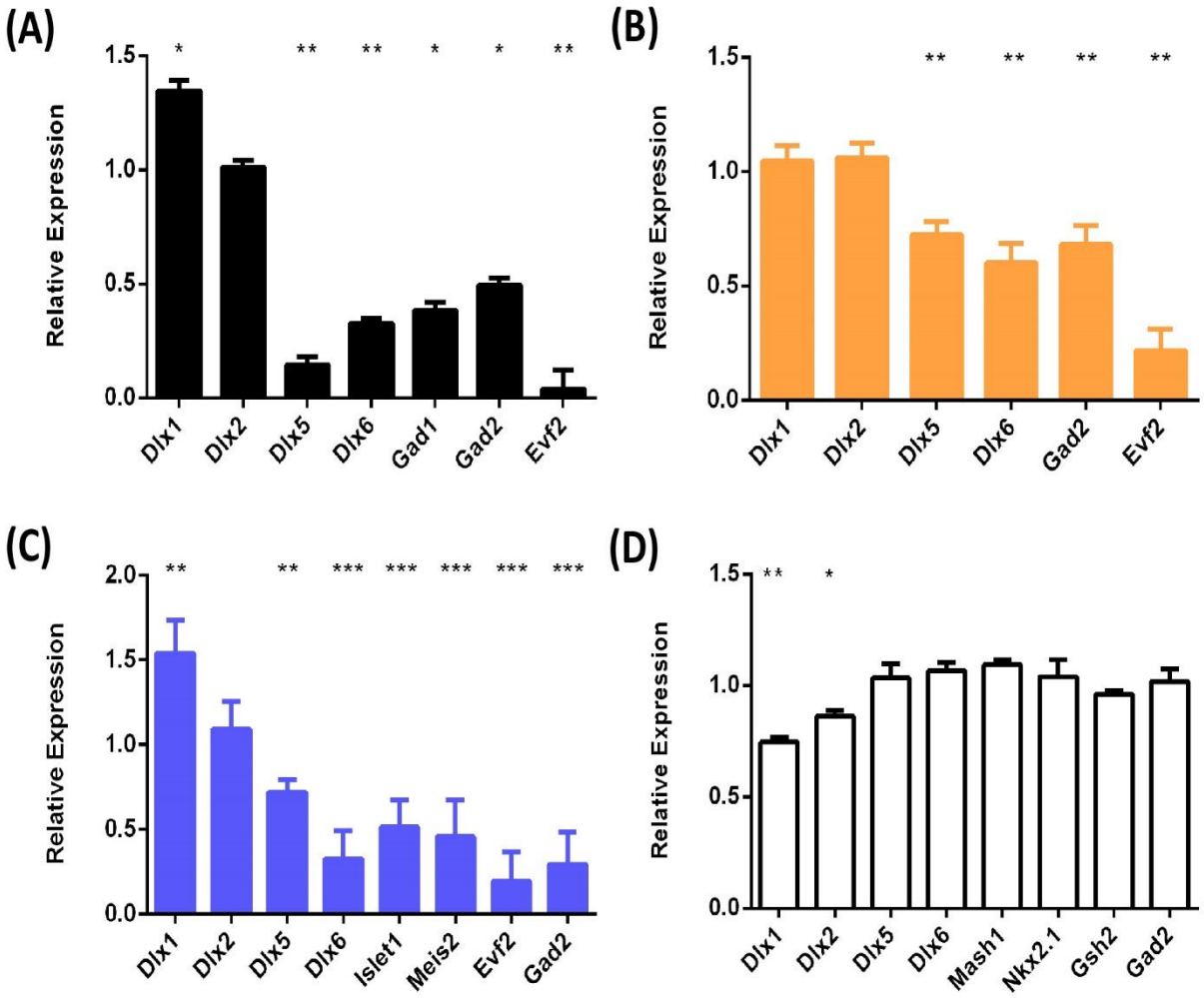
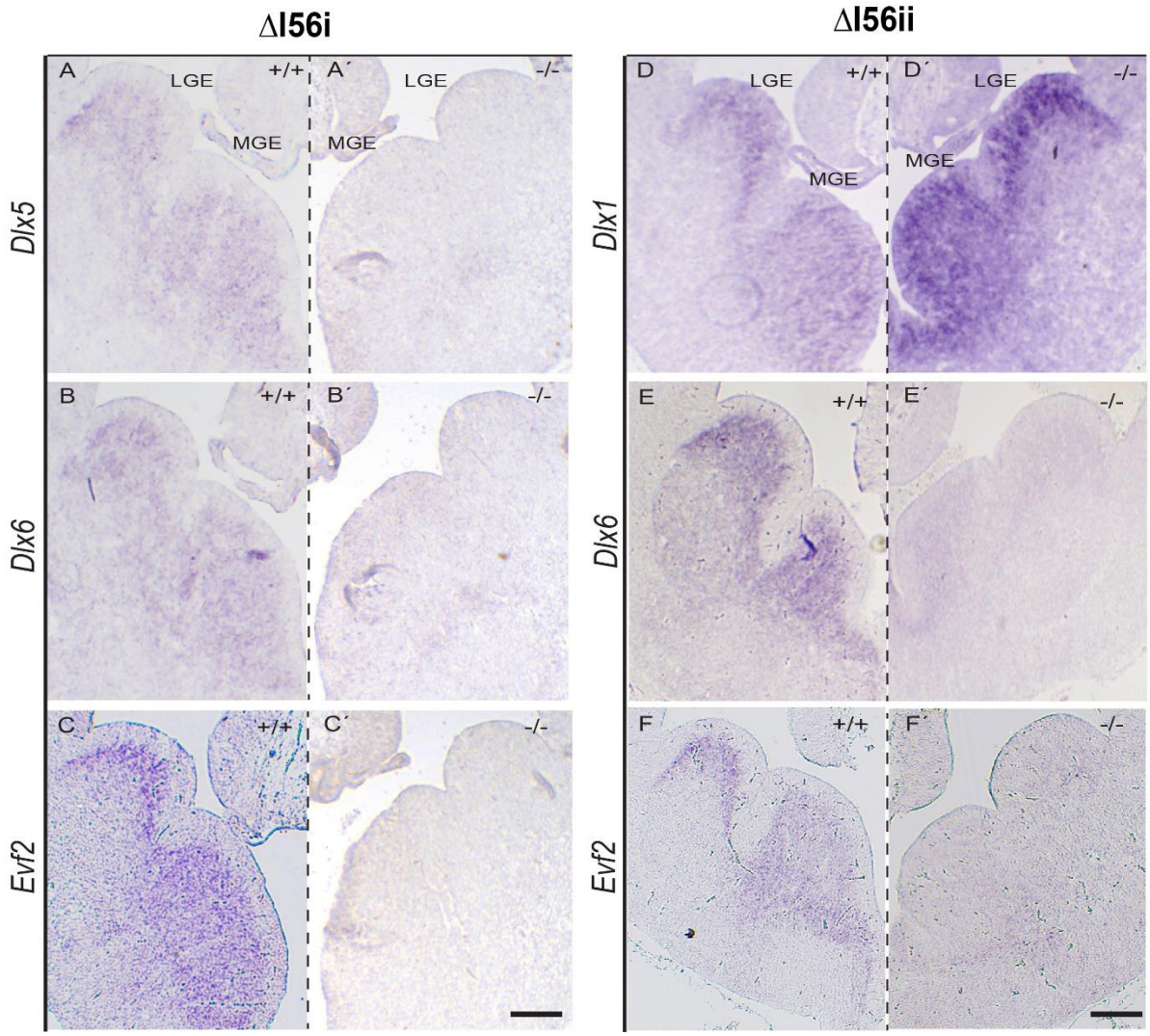

(A)
Relative Expression
1.5
1.0
0.5
0.0
* ** ** * * **
Dlx1
Dlx2
Dlx5
Dlx6
Gad1
Gad2
Evf2
(B)
Relative Expression
1.5
1.0
0.5
0.0
** ** ** **
Dlx1
Dlx2
Dlx5
Dlx6
Gad2
Evf2
(C)
Relative Expression
2.0
1.5
1.0
0.5
0.0
** ** *** *** *** *** ***
Dlx1
Dlx2
Dlx5
Dlx6
Islet1
Meis2
Evf2
Gad2
(D)
Relative Expression
1.5
1.0
0.5
0.0
** *
Dlx1
Dlx2
Dlx5
Dlx6
Mash1
Nkx2.1
Gsh2
Gad2

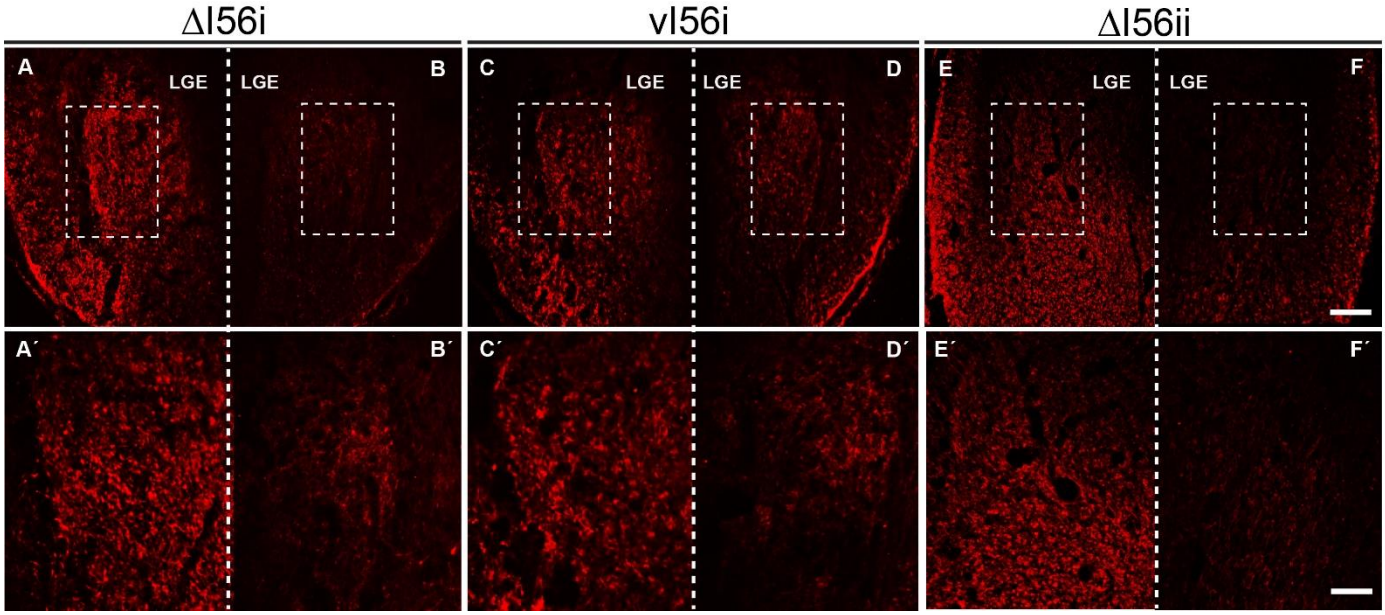
Figure 3.2: *In situ* hybridization on coronal sections of ventral telencephalon of E14.5 I56i wildtype (A-C), ΔI56i mutants (A'-C'), E13.5 I56ii wildtype (D-F) and ΔI56ii mutants (D'-F'). The ΔI56i mutant mice show a global decrease in *Dlx5* (A-A'), *Dlx6* (B-B') and *Evf*2 (C-C') transcript in the developing ventral telencephalon. The ΔI56ii mutant mice show a significant global increase of *Dlx1* (D-D'), and decrease of *Dlx6* (E-E') and *Evf*2 (F-F') transcript in the developing ventral telencephalon. Scale bar: 50μm.


ΔI56i
ΔI56ii
A
LGE
+/+
A´
LGE
-/-
MGE
MGE
D
LGE
+/+
D´
LGE
-/-
MGE
MGE
Dlx5
Dlx1
B
+/+
B´
-/-
E
+/+
E´
-/-
Dlx6
Dlx6
C
+/+
C´
-/-
F
+/+
F´
-/-
Evf2
Evf2

3.3.3 Mice harboring mutations in the intergenic region of *Dlx5/6* exhibit a decrease in the expression of GABA in the ventral telencephalon at E13.5.

Previous studies through gain-of-function experiments demonstrated that the expression patterns of *Dlx* genes overlap with the expression patterns of *Gad* in the developing forebrain, which suggested that *Dlx* genes are involved in controlling *Gad* expression (Stühmer et al., 2002). *Gad* codes for the enzyme GAD that is responsible for synthesizing GABA in the GABAergic interneurons. In order to further characterize the impact of changes in the expression levels of *Dlx* genes on the levels of GABA in the mutant mice, we performed immunohistochemistry against GABA on the ventral telencephalon of all mutant lines at E13.5. Our analysis of GABA expression in the developing forebrain of ΔI12b mutant mice did not show any changes between homozygous mice and wildtype littermates at E13.5 (data not shown) (Yu, 2011). In contrast, ΔI56i, vI56i and ΔI56ii homozygous mice showed a severe decrease in the expression of GABA in the LGE and the striatum of the developing forebrain when compared to the wildtype littermates at E13.5 (Fig. 3.3). Collectively, our data suggest that *Dlx5* and *Dlx6* may play an important role in regulating expression of GABA in the GABAergic interneurons.

Figure 3.3: GABA expression in the developing forebrain of the mutant mice at E13.5. Characterization of GABA distribution in the ventral telencephalon at E13.5, in wildtype (A, C, E) and (B) ΔI56i, (D) vI56i, (F) ΔI56ii mutants. Panels A', B', C', D', E', F' are high-magnification pictures of boxes shown in panels A, B, C, D, E, F, lateral ganglionic eminences. Scale bars: (A–F), 100 μm; (A'-F'), 50 μm.


ΔI56i
vI56i
ΔI56ii
A
LGE
LGE
B
C
LGE
LGE
D
E
LGE
LGE
F
A´
B´
C´
D´
E´
F´

3.3.4 Behavioral consequences of mutations in the *Dlx* enhancer sequence.

It has been well established that *Dlx* genes play an important role during developmental processes including forebrain development. As a result, we performed a series of behavioral tests to examine the impact of the enhancer mutations on the motor activity, learning, memory and anxiety of the mutant mice. We performed several behavioral tests including beam break, elevated plus maze, open field, pre-pulse inhibition and fear conditioning on ΔI56i, ΔI56ii and ΔI12b mutant lines. Since the vI56i mutant mice may constitute an autism model, in addition to the beam break and elevated plus maze tests, we performed adult and juvenile social interactions to examine if vI56i mutant mice demonstrate any Autism Spectrum Disorder (ASD)-like behaviors. Behavioral testing was performed at the Core Behavioral Facility of the University of Ottawa. We performed our behavior tests on homozygous mutants and their wildtype littermates. We used previously published results to estimate our group sizes for these behavioral tests.

Since all the behavioral tests that we performed on the mutant lines relied on measurements that were dependent on their locomotor activity, we first performed beam break test on all mutant lines (ΔI56i, vI56i, ΔI56ii, and ΔI12b). This was important in order to eliminate the possibility of any gross locomotor differences between the mutants and their wildtype littermates. We did not observe any significant differences between the locomotor activity of the ΔI56i, vI56i, ΔI56ii and ΔI12b mutant mice and their wildtype littermates (data not shown). Furthermore, we also examined the ability of the mutant mice to response to sensory stimulation using a pre-pulse inhibition test. This was particularly important since some of the later behavioral tests, including fear conditioning rely on the ability of the animal to hear the cue. Our data demonstrated that there were no significant difference between the auditory capabilities of the mutant mice and their wildtype

littermates (data not shown). Collectively, these data confirmed that the enhancer mutations did not result in any locomotor and/or sensory defects in the mutant mice.

After eliminating the possibility of any locomotor differences between the mutants and their wildtype littermates, we examined whether the enhancer mutations had any behavioral impact on the levels of fear and anxiety of the mutant mice. We investigated this using an elevated plus maze test. The elevated plus maze is based on the preference for dark and enclosed places, compared to bright and exposed places. It has been shown that the mice with higher levels of anxiety will spend more time in the dark and enclosed arms. Our data showed that there were no significant difference between the fear and anxiety levels of the ΔI56i, vI56i, ΔI56ii and ΔI12b mutant mice and their wildtype littermates (data not shown).

Since anxiety disorders have been associated with defects in *Dlx* genes, in addition to elevated plus maze, we also examined if the enhancer mutation had any effect on the anxiety levels of the ΔI56i, ΔI56ii and ΔI12b mutant mice. We used an open field to assess the difference in the anxiety levels of the ΔI56i, ΔI56ii and ΔI12b mutant mice and their wildtype littermates. Open field measures an individuals' anxiousness based on their willingness to explore a novel environment. The total distance that was traveled inside the novel environment in order to explore was used as an estimate to measure the levels of anxiety of mutant mice compared to their wildtype littermates. According to our data, there were no significant differences between the anxiety levels of the ΔI56ii and ΔI12b mutant mice and their wildtype littermates (data not shown). This data coincides with the data gathered from the elevated plus maze in which there was no significant difference between the anxiety levels of mutants and wildtype littermates. However, ΔI56i mutant mice showed significantly higher levels of anxiety compared to the wildtype littermates ($T(26.4) = -2.2$, $p = 0.035$; Fig. 3.4A) (Esau, 2013). This may imply that the mice harboring the I56i enhancer

deletion, may have defects in the neural circuitry involving the amygdala and hippocampus (Rosen & Schulkin, 1998). It is important to note, however, that the ΔI56i mutant mice did not show any increased levels of anxiety in the elevated plus maze test, which raises the provocative question whether the two tests target a different specific region of the amygdala and hippocampus.

In addition to studying the anxiety levels in the mutant mice, we used a fear conditioning test to examine the effect of the enhancer mutations on learning and memory in ΔI56i, ΔI56ii and ΔI12b mutant mice. The fear conditioning test was performed over a three days period. During the first day of training (referred to as baseline training), both mutant and wildtype mice were trained to associate a neutral stimulus (such as a sound) with a much more unpleasant stimulus (shock). The freezing behavior was used to measure the ability of the animal to learn and associate the two stimuli together. The baseline training was successful, since ΔI56i, ΔI56ii and ΔI12b mutant mice and their wildtype littermates showed very limited freezing behavior, indicating that they did not associate the cue with the shock. The second day of the fear conditioning test is the contextual training. The mice are placed back in the previous box and their ability to remember the baseline training and associating the environment with the aversive shock was measured through their freezing behavior. Our data demonstrated that the ΔI56i, ΔI56ii and ΔI12b mutant mice had a very similar freezing behavior to their wildtype littermates, indicating that the ΔI56i, ΔI56ii and ΔI12b mutant mice did not have any defects in their hippocampal function and their short-term memory (data not shown). The last day of the fear conditioning test examined the ability of the mice to remember the association between the cue and the shock. We placed the mice in a novel environment and allowed them to habituate for 3 min in silence (pretone). We then measured their freezing behavior to make sure they did not recognize their environment and did not associate it to the shock. The minimal freezing behavior was indicative that the mice were not able to

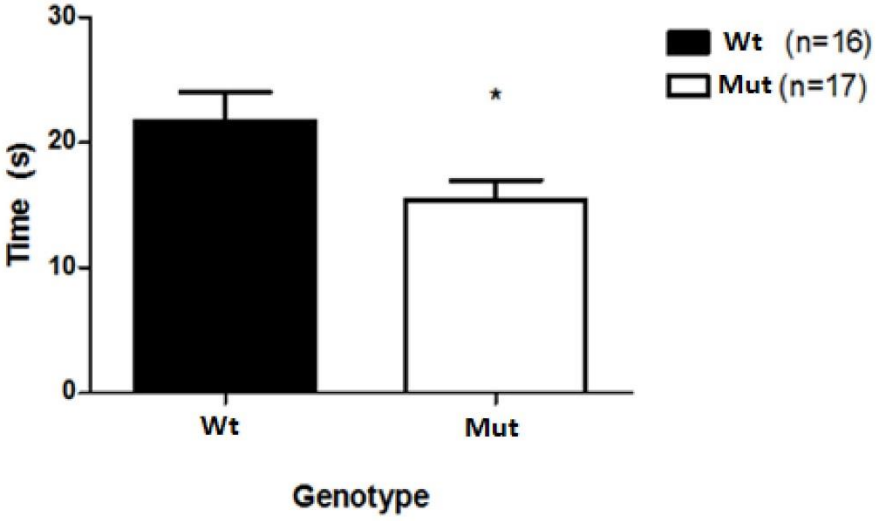
remember and associate their environment with the shock. Following the pretone, we gave the mice the same cue as in day 1 followed by a shock. Once the tone was initiated we did not observe any significant difference between the freezing time of the ΔI56ii and ΔI12b mutant mice and their wildtype littermates, indicating that I56ii and I12b enhancer removal did not have an impact on the learning ability of the ΔI56ii and ΔI12b mutant mice (data not shown). However, the ΔI56i mutant mice showed a significant reduction in their freezing behavior when compared to the wildtype counterparts (Fig. 3.4B) (Esau, 2013). Our data suggest that the ΔI56i mutant mice seemed to have learning deficits, which may be associated with defects within their amygdala (Esau, 2013).

Lastly, since lack of social interactions is considered one of the hallmarks of the autism spectrum disorders, we investigated the impact of vI56i mutation on the social behavior of animals. More specifically, we addressed the social behavior of the vI56i mutant mice by using both adult and juvenile social interaction and compared to their wildtype counterparts. The social interaction tests allowed us to evaluate the social behaviour of the mice based on their ability to recognize members of their own group. Our analysis of the social behavior of the vI56i mutant mice showed that there were no significant difference in the interaction time between the vI56i and the wildtype littermates (data not shown) (Lesage-Pelletier, 2011). It is important to note that congruent to the principles of the test, in which the animal should not spend much time socializing with the other individual on day 3 of the test, the vI56i mutant mice seemed to be more sociable than the wildtype littermates. However, this trend was not statistically significant (data not shown). This trend, even though not significant, led us to examine the social behavior of the ΔI56i mutant mice, since both cases are associated with a mutation in the I56i enhancer. Both adult and juvenile social interaction tests were performed on the ΔI56i mutant mice and were compared to the wildtype littermates.

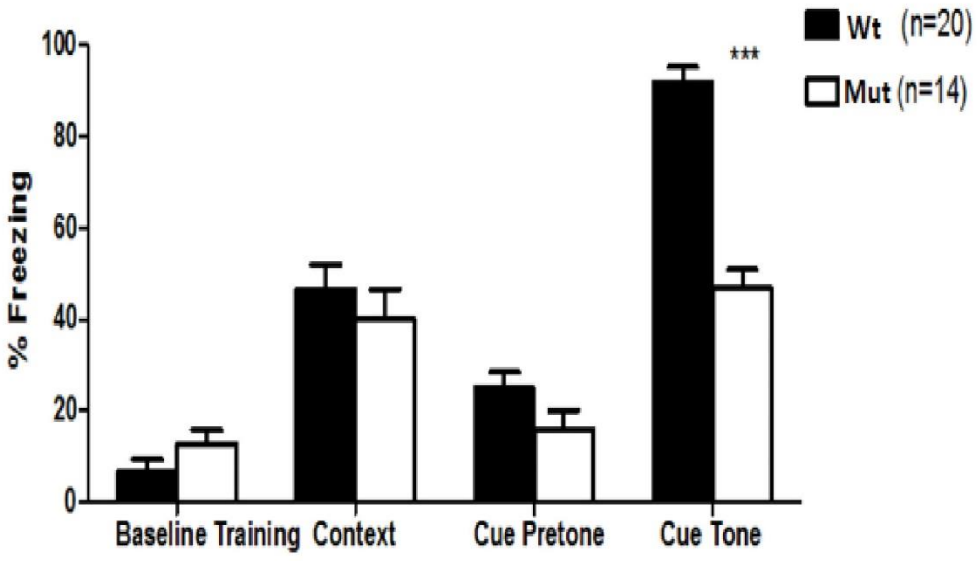
Interestingly, our results were very similar to the ones we collected from the vI56i mutant mice, in which mutant mice seemed to spend more time with the other individual (data not shown). The enhanced sociability behavior in the mutant mice is similar to that observed in Williams-Beuren Syndrome. Williams-Beuren Syndrome is a rare neurodevelopmental disorder characterized by an unusually cheerful demeanor and ease with strangers (Martens et al., 2008).

Figure 3.4: Open field and fear conditioning behavioral tests demonstrate increased levels of anxiety and learning deficit in mice harboring I56i enhancer deletion. (A) Open field. The amount of time spent in each corner of the apparatus was used to determine the levels of anxiety of the ΔI56i mutant mice. T-test shows a significant increase in the anxiety levels of the ΔI56i mutant mice ($T(26.4) = -2.2$, $p = 0.035$). (B) Fear conditioning test was performed on mice harboring I56i deletion. Repeated ANOVA showed no significant difference in percent freezing time between the ΔI56i mutant mice and the wildtype littermate during baseline training or context. However, the ΔI56i mutant mice exhibit a significant learning deficit when compared to the wildtype littermate ($F(3,96)=105.6$, $p<0.001$) (Esau, 2013).

(A)


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

(B)


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

3.4 Discussion

3.4.1 Mutations in the *Dlx* forebrain intergenic enhancers contradict the theory of functional redundancy between *Dlx* intergenic enhancers.

The gene regulatory apparatus, including *cis*-regulatory elements, that directs development serves a binding platform for various transcription factors to regulate gene expression during different developmental stages (Arnone & Davidson, 1997). 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). In determining the importance of *Dlx cis*-regulatory elements in regulating and/or maintaining *Dlx* expression levels during development, we characterized the phenotypic consequence of mutations in *Dlx* enhancer sequences by generating ΔI56i, vI56i, ΔI56ii and ΔI12b mutant lines. The mice harboring these mutations seem normal and do not have any morphological abnormalities. It is noteworthy that the normal appearance of the mice harboring enhancer mutations may suggest a functional redundancy between the *Dlx* intergenic enhancers in the developing forebrain. This hypothesis is also consistent with previous studies in which *lacZ* transgenesis revealed overlaps in the activity patterns of the *Dlx* intergenic enhancers in the developing forebrain, (Ghanem et al., 2007; Ghanem et al., 2008). The gene expression analyses of the ΔI56i, vI56i, ΔI56ii, and ΔI12b mutant mice lead us to refine the above hypothesis (Fig. 3.1 and 3.3). Our data shows that the *Dlx* expression levels are significantly impaired in some of the mice harboring a single enhancer mutation (Fig. 3.1). This may suggest that even though *Dlx* intergenic enhancers show overlapping activity patterns, they might be active in different cell populations or serve different functions within the same cell. In addition, the impaired levels of *Dlx* expression calls into question the proposed partial redundancy of the *Dlx*

intergenic CREs, since the single mutants do in fact demonstrate a statistically significant change in the transcript levels of *Dlx* genes that otherwise should not have been detected if a true partial functional redundancy existed between *Dlx* intergenic CREs. Collectively these data reinforce the certainty that the intergenic enhancers are essential in initiating and/or maintaining *Dlx* expression.

3.4.2 The *Dlx5/6* intergenic enhancers are involved in controlling GABA production in the developing forebrain.

The *Dlx* genes regulate the development of GABAergic interneurons, modulating the proper circuitry in the developing forebrain. GABA serves as an inhibitory neurotransmitter in interneurons late during developmental stages. As it is shown in Fig. 3.3, GABA expression has drastically decreased in the ΔI56i, vI56i, ΔI56ii mutant mice when compared to the wildtype littermates (Fig. 3.3). The decreased levels of GABA in the ΔI56i, vI56i, ΔI56ii mutant mice may suggest that intergenic enhancers are involved in regulating a genetic pathway that regulates *Gad* expression and ultimately GABA synthesis. As mentioned in Chapter 1, it has been hypothesized that the DLX1 and DLX2 proteins bind to the I56i enhancer to activate and/or maintain the expression of *Dlx5* and *Dlx6* in the developing forebrain. Subsequently, DLX5 and DLX6 proteins are thought to bind to the enhancer region of *Gad,* which codes for GAD enzyme responsible for GABA synthesis. Therefore, the decrease in the expression levels of *Dlx5* and *Dlx6* may disrupt this pathway by reducing the levels of DLX5 and DLX6 proteins. This could imply that the ΔI56i, vI56i, ΔI56ii mutant mice might have a delayed or disrupted GABAergic neuronal development. The mechanisms underlying *Dlx* gene regulation of the development of the GABAergic neuronal subtypes are not fully understood; however, these data could suggest an important functional relevance for the I56i and I56ii intergenic enhancers with regards to establishing and/or

maintaining *Dlx* expression, which will ultimately impact the proper differentiation and migration of GABAergic interneurons to the neocortex.

3.4.3 Mutations in the I56i intergenic enhancer have behavioral consequences.

Given the importance of the *Dlx* genes in regulating the migration and development of GABAergic interneurons in the developing forebrain, it has been 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 & Pearl, 2003). As has been demonstrated in this chapter, mice harboring enhancer mutations show a significant decrease in *Dlx* expression levels. Given the important role of *Dlx* genes during developmental stages including forebrain development, we examined the impact of these enhancer mutations on the behavior of the ΔI56i, vI56i, ΔI56ii and ΔI12b mutant mice. The vI56i, ΔI56ii and ΔI12b mutant mice did not exhibit any statistically significant behavioral abnormalities when compared to the wildtype littermates, implying that the mutations in the enhancer sequence did not have any impact on the development of the mutant mice.

Interestingly, the mice harboring I56i enhancer deletion show higher levels of anxiety, and impaired fear conditioning, the latter result suggesting that ΔI56i mice have learning deficiencies (Fig. 3.4). It is important to note that our data from the elevated plus maze test did not show any levels of anxiety in the ΔI56i mutant mice (data not shown). This contradicts with the behavior that we observed from the open field test, in which the ΔI56i mutant mice demonstrated an increase in hyperactivity and anxiety (Esau, 2013).

We further examined the learning and memory of the ΔI56i mutant mice. A fear conditioning tests allowed us to examine any changes in learning and memory of the ΔI56i mutant mice through associating a neutral stimulus with an aversive shock. Studies have suggested that in

mammals, learning and memory encompasses a highly complex system that is regulated in part by the amygdala and hippocampal complex (Phelps, 2004). During this process, the hippocampus is mainly involved with short-term memory that allows the individual to recollect certain information or events at the individual's will. In contrast, the amygdala is mainly involved more in long-term memory and allows the individual to associate emotional events (Phelps, 2004). Other studies have addressed the role of the amygdala and hippocampus in learning and memory by inducing lesions in both amygdala and hippocampal regions of the rat brain (Phillips & LeDoux, 1992). Phillips and LeDoux (1992) demonstrated that amygdala function is mainly involved in responses to simple stimulus and is mainly used to associate a series of events (Phillips & LeDoux, 1992). However, the hippocampus was shown to be mostly involved in memory of more complex events and relaying sensory information (Phillips & LeDoux, 1992). The ΔI56i mutant mice did not demonstrate any defects in hippocampal activity, suggesting that the I56i enhancer deletion did not impact the ability of the mutant mice to remember their surrounding environment (Fig. 3.4B). However, the ΔI56i mutant mice did show a clear defect in amygdala function. The ΔI56i mutant mice were not able to associate the cue (noise) with the aversive shock once they were placed in a novel environment. This deficiency in amygdala activity implies that the mice harboring I56i enhancer deletion had learning deficits when compared to wildtype littermates (Fig.3.5B).

In addition to these observed behavioral abnormalities, the mice harboring mutations in the I56i enhancer (both ΔI56i and vI56i) were tested for any potential changes in their social behavior. The adult and juvenile social interaction tests demonstrated that the ΔI56i and vI56i mutant mice tend to be more sociable than their wildtype littermates (data not shown). Even though this change in behavior was not statistically significant, there was a trend when comparing the two mutant lines with their wildtype littermates. These enhanced levels of social interactions with strangers is

very similar to the behavior observed in individuals with Williams-Beuren Syndrome. Previous work on the molecular impact of the knock-in mutation on the I56i enhancer, using electro-mobility shift assay (EMSA), co-transfection as well as affinity purification experiments, discovered general transcription factor 2i (*Gtf2i*) as a new regulator of *Dlx* genes (Poitras et al., 2010). *Gtf2i* is among a group of genes that are often deleted in Williams-Beuren Syndrome, a rare neurodevelopmental disorder (Martens et al., 2008). The molecular mechanism in which *Gtf2i* regulates DLX protein levels through interactions with the I56i enhancer is not understood. Two different modes of action have been hypothesized in which Gtf2i might regulate DLX protein levels. First, it has been suggested that Gtf2i could possibly bind to the putative binding site of the I56i enhancer, which is located between the two DLX binding sites (Poitras et al., 2010). The second mechanism suggests that Gtf2i could potentially interact with the DLX proteins both directly or through an intermediate factor. This indirect association is suggested to be independent of the ability of the Gtf2i to bind to the I56i enhancer sequence (Poitras et al., 2010). These data underlines the importance of repeating the adult and juvenile social interactions with a much bigger sample size and further investigate the possibility of this neurodevelopmental disorder in the I56i mutant mice.

In summary, the data presented in this chapter provide a detailed analysis of the functional importance of *Dlx* intergenic enhancers in the developing forebrain. Additionally, this project demonstrates that changes in gene expression resulting, directly and indirectly, from enhancer deletions may have consequences on the behavior of the mutant mice. Lastly, studying highly conserved regulatory elements involved in the *Dlx* regulatory network will shed light on the underlying mechanisms involved in neurological disorders associated with disrupted GABA circuitry.

Chapter 4: Functional consequence of *Dlx* enhancer deletion(s) in the developing mouse forebrain.

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4.0 Abstract

An important aim of current biology is to understand the role of *cis*-regulatory elements (CREs) in regulating the spatial and temporal expression patterns of their target genes. CREs that are involved in developmental processes are shown to have a high degrees of conservation amongst species. *Dlx* homeobox genes encode a group of transcription factors that play an essential role during developmental processes including forebrain development through regulating and maintaining the differentiation, proliferation and migration of GABAergic interneurons. The *Dlx1/2* and *Dlx5/6* genes are active in the forebrain and are arranged in convergently transcribed bigene clusters, with I12a/I12b and I56i/I56ii enhancers located in the intergenic region of each cluster respectively. To determine the regulatory role of the intergenic CREs on *Dlx* expression levels, we have characterized the phenotypic consequence of I56ii and I56ii-I12b enhancer deletions on forebrain development and neuronal migration through the activity of the corridor cells. Here we report that these enhancer deletion(s) impair *Dlx* expression and that of potential DLX targets, including *Gad2* as well as striatal markers *Islet1* and *Meis2*. In addition, I56ii enhancer deletion greatly reduces the expression of Islet1 and Ctip2 in corridor cells. Furthermore, enhancer deletion disrupts the expression patterns of Semaphorin 3A, Slit1 and Ephrin A5 guidance cues in the corridor cells of the developing forebrain at E13.5. These changes may disrupt the trajectory of the tangentially migrating thalamocortical axons to the cortex. These data suggest an important regulatory role for the I56ii enhancer and for *Dlx6* in the developing forebrain by means of a potential regulatory mechanism which may regulate the activity of the corridor cells.

4.1 Introduction

The majority of neurons that make up the cortex are of two types. The first type consists of excitatory projection neurons that release the neurotransmitter glutamate. The second type consists of inhibitory interneurons that release GABA as their primary neurotransmitter. These neurons are derived from progenitor cells in the ventricular zone of the telencephalon and migrate to their final destinations through two distinct mode of migration, namely radial or tangential migration (Poitras et al., 2010). One of the features of the developing central nervous system is the ability of the neuronal cells to migrate away from their origin to their final destination (Gleeson & Walsh, 2000; Lambert de Rouvroit & Goffinet, 2001). This process is essential for proper brain development and function (Wichterle et al., 2003).

Radial migration is the most studied form of neuronal movement in the developing cerebral cortex. The main feature of radial migration is that the neurons that were born in the proliferative zone move along the radially oriented glial fibers perpendicular to the surface of the brain (Hatten, 1999). Due to such intricate interactions between the glia fibers and migrating neurons, radial migration has also been referred to as "gliophilic migration". In contrast, cortical interneurons have been shown to migrate from the ventral telencephalon to the developing neocortex via tangential migration (Parnavelas, 2000). During tangential migration, also known as "neurophilic migration", neurons move parallel to the surface of the brain along the trajectory of axons and often disobey the regional boundaries within the developing brain (Hatten, 1999). It has been widely debated as to how neurons know where to go within the developing brain and how they recognize their final destination. Lineage studies have reconciled the divergent migratory patterns of the cortical interneurons, demonstrating that the migration of these interneurons have distinct patterns (Mione et al., 1997). It has been shown that the interneurons that originate from the ventral telencephalon,

in addition to intrinsic specification of production, differentiation and final destination, depend largely on a wide spectrum of guidance cues to navigate from the ventral telencephalon to the developing cortex (Zhu et al., 1999). For instance, SLIT1 is a secretory protein that is produced in both the lateral and medial ganglionic eminences (LGE and MGE) and has been shown to act as a chemorepellent for the tangentially migrating GABAergic interneurons (Yuan et al., 1999). Furthermore, it has been suggested that hepatocyte growth factor (HGF) could provide localized cues to interneurons and thus facilitate their migration towards the dorsal pallium (Powell et al., 2001).

Two different mechanisms have been proposed to play an essential role in establishing neuronal pathways to deliver information to the cortex. The first proposed mechanism for thalamocorticol axonal projections depended on specific attracting and/or repelling signals from different cortical regions. This idea led to the "handshake hypothesis", where thalamocortical and cortico-thalamic fibers reciprocally guide each other's navigation pattern (Molnár & Blakemore, 1995). According to the "handshake hypothesis", the axons from thalamocortical and the corticothalamic projections combine to form a scaffold. The thalamocortical and corticothalamic projections use this scaffold as they grow past one another towards their respective destinations within the cortex and the dorsal thalamus (Molnar et al., 1998; Molnár & Blakemore, 1995). Other knockout studies have supported this hypothesis, in which deletion of the genes that are strictly expressed in the cortex such as *Tbr1* or dorsal thalamus including *Gbx2*, disturbs the migration pattern of the thalamocortical and corticothalamic axons (Hevner et al., 2002; López-Bendito & Molnár, 2003). The second mechanism that is proposed for the proper establishment of neuronal pathways involve tangentially migrating cells within the ventral telencephalon, also known as "corridor cells", guiding thalamic projections towards the cortex (Lopez-Bendito et al., 2006).

These migrating cells (or corridor cells) are thought to create a permissive corridor between the boundaries of the diencephalon/telencephalon and pallial/subpallial regions of the developing forebrain (Lopez-Bendito et al., 2006). It has been proposed that the corridor cells not only function by providing positional cues to the thalamocortical projections, but also by forming a bridge between different regions of the telencephalon that otherwise would have been non-permissive for growth of the thalamocortical axons (Corbin et al., 2001). Interestingly, it has been demonstrated that, unlike the tangentially migrating thalamocortical cells, the corridor cells migrate in the opposite direction. The corridor cells originate from the lateral ganglionic eminence (LGE) and migrate downward deep into the mantle zone (MZ) of the medial ganglionic eminence (MGE) to provide a bridge for the incoming afferent thalamocortical axons (Corbin et al., 2001; Lopez-Bendito et al., 2006; López-Bendito & Molnár, 2003). They express a number of LGE-specific markers including *Islet1, Ebf1* and *Meis2* (Lopez-Bendito et al., 2006). Genetic regulatory mechanisms control this differentiation and migration of neurons, and allow for normal development of the brain.

The *Dlx* family is a group of genes that encode homeodomain transcription factors, which are involved in embryonic development of the telencephalon by regulating the differentiation and migration of GABAergic interneurons (Panganiban & Rubenstein, 2002). *Dlx* genes in mice are arranged in three bigene clusters; namely, *Dlx1/Dlx2*, *Dlx5/Dlx6*, *Dlx3/Dlx4* (Zerucha & Ekker, 2000). In these bigene clusters, *Dlx* genes are arranged in a convergent configuration with the *cis*-acting regulatory elements (CREs) located within the intergenic region of each cluster, namely I12a/I12b and I56i/I56ii located in the *Dlx1/Dlx2* and *Dlx5/Dlx6* clusters, respectively (Zerucha & Ekker, 2000; Zerucha et al., 2000). Functional analysis of *Dlx* intergenic enhancers using *lacZ* reporter transgenesis demonstrated a lot of overlap, yet some marked differences between the

activity patterns of I12b, I56i and I56ii CREs in the developing forebrain (Ghanem et al., 2008). Spatiotemporal analysis of *lacZ* transgenes demonstrated that the I56ii enhancer is neither active in GABAergic interneurons nor in the tangentially migrating cells to the cortex. However, transgene expression targeted a special subpopulation of post-mitotic cells deep in the mantle zone, also referred to as corridor cells (Ghanem et al., 2008). Additionally, it was demonstrated that I56ii-positive cells express *Islet1* and *Meis2* striatal markers. This was further investigated in co-transfection assays *in vitro* where these striatal markers were able to activate transcription via the activity of I56ii enhancer (Ghanem et al., 2008). Since the I56ii enhancer is seemingly active in the corridor cells, it is thought that *Dlx* genes may play a role in modulating the migration of the thalamocorticol projections through these permissive corridor cells.

The focus of this project was to elucidate the effect of the loss of *Dlx* function through enhancer deletion on forebrain development. Furthermore, we are interested in investigating the impact of the loss of *Dlx* function on the corridor cells, which are involved in the tangential migration of thalamocortical neurons to the developing cortex. In order to address these questions, we have generated a targeted deletion mouse line where the ΔI56ii intergenic enhancer was deleted. In addition to the ΔI56ii mutant line, we were interested to see the phenotypic impact of multiple enhancer deletions on neuronal migration and forebrain development. The ΔI56ii/ΔI12b double deletion line was generated by mating ΔI56ii and ΔI12b mutant mice. Here we report that enhancer deletion(s) impairs expression of *Dlx* genes. In addition, the I56ii enhancer deletion greatly reduces the expression of Islet1 and Ctip2 transcription factors in the corridor cells located in the striatum of the developing forebrain. Furthermore, the enhancer deletion disrupts the expression patterns of Slit1, Semaphorin 3A, and Ephrin A5 guidance cues in the corridor cells.

Changes in *Dlx* expression in the developing forebrain as a result of intergenic enhancer deletion(s) suggests an important regulatory role for the *Dlx6* gene in the developing forebrain.

4.2 Materials and Methods

4.2.1 Generation of I56ii targeted deletion mice:

The ΔI56ii targeted deletion mice were generated at the Transgenic Mouse Core Facility at the McGill Cancer Center. The strategy that was utilized to generate the ΔI56ii mutant mice is depicted in Appendix 1. Through homologous recombination, a LoxP-flanked PGK-neomycin-resistant cassette replaced the entire I56ii enhancer sequence on a Bacterial Artificial Chromosome (564M8 BAC) harboring 200 Kb of the *Dlx5/Dlx6* locus. The BAC clones were screened and sequenced to ensure a successful recombination. Following screening and sequencing, the positive vector was electroporated into 129SV mouse embryonic stem (ES) cells. Cells were selected with gentamicin to detect positive cells harboring the mutant BAC. Additionally, 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. Chimeric mice were mated with C57BL/6 wild-type mice and genotyped to ensure a germ-line transmission of the mutant allele and to generate heterozygote progeny that were positive for the I56ii deletion in their germ line. Once I56ii heterozygous mice were obtained, we mated two heterozygotes to produce homozygous ΔI56ii mutant mice. The ΔI56ii mutant mice were mated with SoxCre mice to remove the neomycin cassette from the *Dlx5/Dlx6* cluster to generate ΔI56iiΔNeo mutant mice (see Appendix 1). This step was important to prevent the potential impact of the neomycin cassette during expression analysis.

4.2.2 Genomic DNA extraction:

For detailed methodology on genomic DNA extraction please refer to Chapter 3, Section 2.2.

4.2.3 Genotyping using Polymerase Chain Reaction:

Mice harboring the I56ii enhancer deletion on one or both alleles were screened using the polymerase chain reaction and ΔI56iiΔNeo.for (5′-GAGGGAAGAAAGACGGGAGT-3′) and ΔI56iiΔNeo.rev (5′-GTCAGAGCCCAAACCTTGAA-3′) primers flanking the enhancer region. A second PCR was used to detect the presence of the wildtype I56ii allele to help confirm the results of the first PCR. Primers used for this second PCR reaction were I56ii forward (5′-GGATCCCTCAGCAACCCATTTGCAGT-3′) and I56ii reverse (5′-GGATCCCAGAGGCTCTGTCTCTATATT-3′). Additionally, a third PCR reaction was performed to screen for I12b enhancer deletion in the double mutant line using ΔI12bΔNeo.for (5′-TGAGTCTGTAATGGCAAAATGC-3′) and ΔI12bΔNeo.rev (5′-CAGGTGCAGATTCCCTGAAG-3′) primers flanking the enhancer region. Please refer to Chapter 3, Section 2.3 for detailed composition of each PCR master mix as well as the fractionation step. Presence of a 600 bp band was indicative of the I56ii deletion and a 900 bp corresponded to the I56ii wildtype allele. Similarly, the presence of an 800 bp band corresponded to the I12b wildtype allele, while a 400 bp band was indicative of I12b deletion.

4.2.4 Gene expression analysis:

The detailed methodology pertaining to the gene expression analysis of mutant mice is discussed in Chapter 3, Sections 2.4.1, 2.4.2 and 2.4.3.

4.2.5 Embedding and cryosectioning of fresh samples:

Please refer to Chapter 3, Section 2.5 for detailed description of the steps involved in the embedding and cryosectioning of the fresh tissue samples.

4.2.6 *In situ* hybridization on mouse forebrain sections:

Complete methodology pertaining to the *in situ* hybridization technique is discussed in detail in Chapter 3, Sections 2.6.1, 2.6.2, 2.6.3 and 2.6.4.

4.2.7 Immunohistochemistry on mouse forebrain sections:

Please refer to Chapter 3, Section 2.7 for the complete list of antibody dilutions and the methodology involved in the immunohistochemistry experiments.

4.3 Results

4.3.1 Morphological analysis of mutant mice with the I56ii and I56ii-I12b *Dlx* enhancer deletion(s):

In order to determine whether enhancer deletion impacts the viability and fertility of ΔI56ii mutant mice, I mated ΔI56ii heterozygous mice in order to generate ΔI56ii homozygous mice. The offspring from the heterozygous mice were screened for the absence of the I56ii enhancer. Homozygous mutants did not show any morphological abnormalities or size differences when compared to the wildtype littermates (data not shown). In addition, the I56ii enhancer deletion did not affect the fertility and the ability of the animals to mate. Throughout my studies, I have collected embryos at both E11.5 and E13.5, but did not observe any noticeable morphological abnormalities or size differences when compared to their wildtype siblings.

In order to examine the functional consequence of multiple *Dlx* intergenic enhancer deletions on the developing forebrain, I generated a second mutant line where both the I12b and I56ii enhancers were removed. This was achieved by mating the ΔI56iiΔNeo homozygous with the ΔI12bΔNeo homozygous mutants. After generating double-heterozygotes, a series of mating were carried out until ΔI56iiΔNeo/ΔI12bΔNeo double homozygous mutants were obtained. Double-mutants are viable and fertile; however, double homozygous litters were infrequently born and the litter sizes were smaller than the wildtype litters of the same age (data not shown).

4.3.2 I56ii intergenic enhancer deletion significantly impairs the expression levels of *Dlx5/6, Gad2, Evf2* as well as *Islet1* and *Meis2* striatal markers.

In order to characterize the functional consequences of enhancer deletion(s) on gene expression, the ventral telencephalon of the mutant and wildtype mice was dissected at E11.5 and

E13.5. After extracting RNA and synthesizing cDNA, qRT-PCR assays were performed to determine the impact of enhancer deletion on the expression of *Dlx* genes (*Dlx1, Dlx2, Dlx5,* and *Dlx6*) as well as other genes including *Gad2, Evf2* and striatal markers *Islet1* and *Meis2*. *Gad2* was an interesting candidate since it is thought that *Gad* expression is regulated by DLX5/DLX6 proteins (Ghanem et al., 2008; Poitras et al., 2007). *Evf2*, a long non-coding RNA was also examined due to its ability to bind and form a stable complex with DLX2 protein. It has been suggested that this stable complex contributes to the transcriptional regulation of *Dlx5/Dlx6* (Feng et al., 2006). In all cases, expression studies were carried out by comparing the mutants with the wildtype littermates.

Previous studies through *lacZ* transgenesis demonstrated that I56ii activity starts at E11.5 and is undetectable after E13.5 (Ghanem et al., 2008). Therefore, E11.5 and E13.5 were chosen as the two time-points to specifically determine the consequence of enhancer deletion on the expression levels of *Dlx* as well as other genes including *Islet1, Meis2, Evf2* and *Gad2*. As depicted in Fig. 4.1A, I56ii enhancer deletion moderately impairs the expression levels of *Dlx5, Dlx6, Islet1,* and *Meis2* at E11.5 (Fig. 4.1A). However, these changes are statistically significant when compared to the wildtype littermates. *Dlx5* and *Dlx6* expression levels are reduced 25% and 35% respectively at E11.5 ($p<0.01$). Furthermore, the expression levels of striatal markers *Islet1* and *Meis2* are decreased by approximately 35% and 30%, respectively at E11.5 ($p<0.01$; Fig. 4.1A). Lastly, the expression levels of *Dlx1* and *Dlx2* are not impacted as a result of I56ii enhancer deletion at E11.5 (Fig. 4.1A).

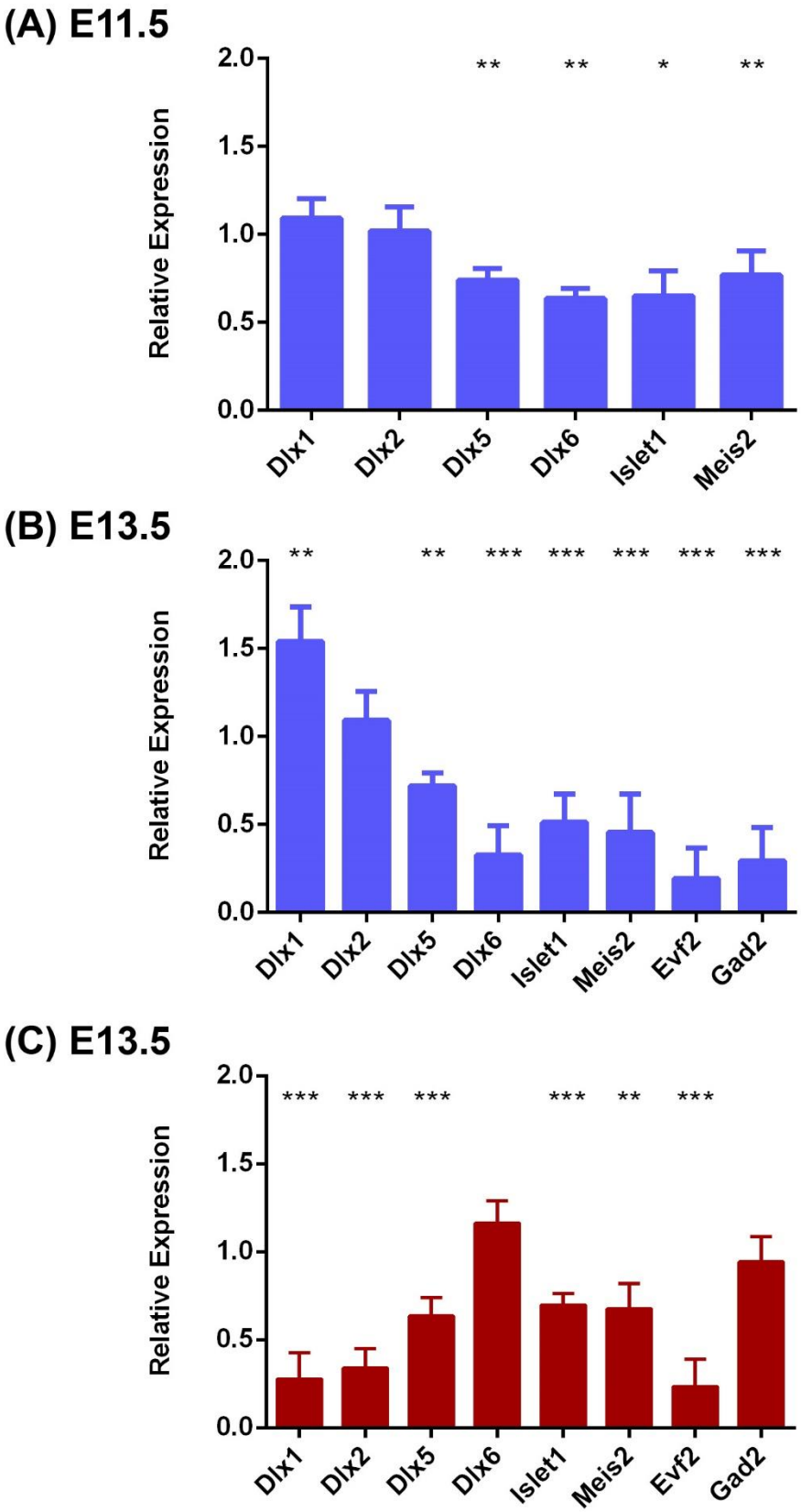
The changes in the transcript levels are exacerbated at E13.5 (Fig. 4.1B). *Dlx1* expression is increased significantly (~55%, $p<0.01$) in the ΔI56ii mutant at E13.5 (Fig. 4.1B); whereas, *Dlx2* expression is not affected as a result of the I56ii enhancer deletion (Fig. 4.1B). Furthermore, the

expression levels of *Dlx5* (~25%, p<0.01) and *Dlx6* (~70%, p<0.001) are significantly reduced in the ΔI56ii mutant mice at E13.5 (Fig. 4.1B). The decreases in the mRNA levels of *Dlx5/Dlx6* are accompanied by a decrease in the expression of *Gad2* (~70%, p<0.001; Fig. 4.1B). In addition to the aforementioned changes in *Dlx* and *Gad2* expression levels, the transcript levels of the striatal markers *Islet1* and *Meis2* are also significantly reduced. *Islet1* and *Meis2* expression levels are decreased by approximately 50% (p<0.001) at E13.5 in the developing forebrain (Fig. 4.1B). Lastly, the expression levels of *Evf2* is also significantly reduced in the developing forebrain at E13.5 (~80%, p<0.001; Fig. 4.1B).

Since the forebrain enhancers showed an overlapping activity pattern in the developing forebrain, which could suggest a functional redundancy between the *Dlx* forebrain intergenic enhancers. Therefore, in addition to investigating the functional consequence of ΔI56ii enhancer deletion on the transcript levels of *Dlx* genes as well as *Islet1, Meis2, Evf2* and *Gad2*, it was important to examine the effect of multiple enhancer deletions on *Dlx* and *Islet1, Meis2, Evf2* and *Gad2* expression levels. In order to address this question, I performed qRT-PCR on mice homozygous for both I56ii and I12b enhancers. As depicted in Fig. 4.1C, the expression levels of *Dlx1,* and *Dlx2* are significantly decreased in the developing ventral telencephalon of the ΔI56ii/ΔI12b mutant mice at E13.5 (~70%, p<0.001; Fig. 4.1C). Furthermore, the expression of *Dlx5* is also significantly decreased when compared to the wildtype littermates (~30%, p<0.01; Fig. 4.1C). As a result of multiple *Dlx* enhancer deletions the expression levels of *Evf2* are significantly decreased in the developing forebrain at E13.5 (~80%, p<0.001; Fig. 4.1C). It is noteworthy that the expression levels of *Dlx6* and *Gad2* are not affected in the ΔI56ii/ΔI12b mutant mice at E13.5 (Fig. 4.1C). Furthermore, the expression of *Islet1* and *Meis2* are significantly reduced (~25%, p<0.001; Fig. 4.1C).

The changes in the expression levels of *Dlx* genes as well as *Islet1, Meis2, Evf2* and *Gad2* are very intriguing when comparing the ΔI56ii to the ΔI56ii/ΔI12b mutant mice at E13.5 (Fig. 4.1B, C). According to the data presented here, *Dlx1* and *Dlx2* expression levels are significantly reduced in ΔI56ii/ΔI12b mutant mice compared to ΔI56ii mutant mice at E13.5. Interestingly, the expression levels of *Dlx5* and *Evf2* do not change significantly (~10%) between the two mutant lines. It is noteworthy that the expression levels of *Dlx6* and *Gad2* are almost restored back to the normal levels when comparing the two mutant lines at E13.5 (Fig. 4.1B, C). Furthermore, *Islet1* and *Meis2* expression levels are significantly increased ~25% ($p<0.001$) in the ΔI56ii/ΔI12b mutant at E13.5 (Fig. 4.1C). These data suggest that the deletion of *Dlx* intergenic enhancer(s) impairs the expression patterns of *Dlx* genes as well as other genes whose expression has been suggested to be regulated or maintained by *Dlx* activity.

Figure 4.1: Impaired *Dlx, Islet1, Meis2, Gad* and *Evf2* expression in the ventral telencephalon mutant mice. Quantitative Real-Time PCR (qRT-PCR) results corresponding to relative expression levels of *Dlx1*, *Dlx2*, *Dlx5*, *Dlx6*, *Gad2*, *Evf2*, *Islet1* and *Meis2* transcripts in the mouse ventral telencephalon of mutant and wildtype littermate embryos from (A) E11.5 ΔI56ii (B) E13.5 ΔI56ii and (C) E13.5 ΔI56ii/ΔI12b mutants. In all cases, gene expression levels were analyzed using five biological replicates each assayed in experimental triplicates (n=5). The error bars represent the standard error of the mean of all replicates for each gene relative to the housekeeping gene (*Ef1α*). Relative expression levels of wildtypes were defined as 1.0. (**P* <0.05) (***P* < 0.01) (****P*<0.001).


(A) E11.5
Relative Expression
2.0
1.5
1.0
0.5
0.0
**
**
*
**
Dlx1
Dlx2
Dlx5
Dlx6
Islet1
Meis2
(B) E13.5
Relative Expression
2.0
1.5
1.0
0.5
0.0
**
**
***
***
***
***
***
Dlx1
Dlx2
Dlx5
Dlx6
Islet1
Meis2
Evf2
Gad2
(C) E13.5
Relative Expression
2.0
1.5
1.0
0.5
0.0
***
***
***
***
**
***
Dlx1
Dlx2
Dlx5
Dlx6
Islet1
Meis2
Evf2
Gad2

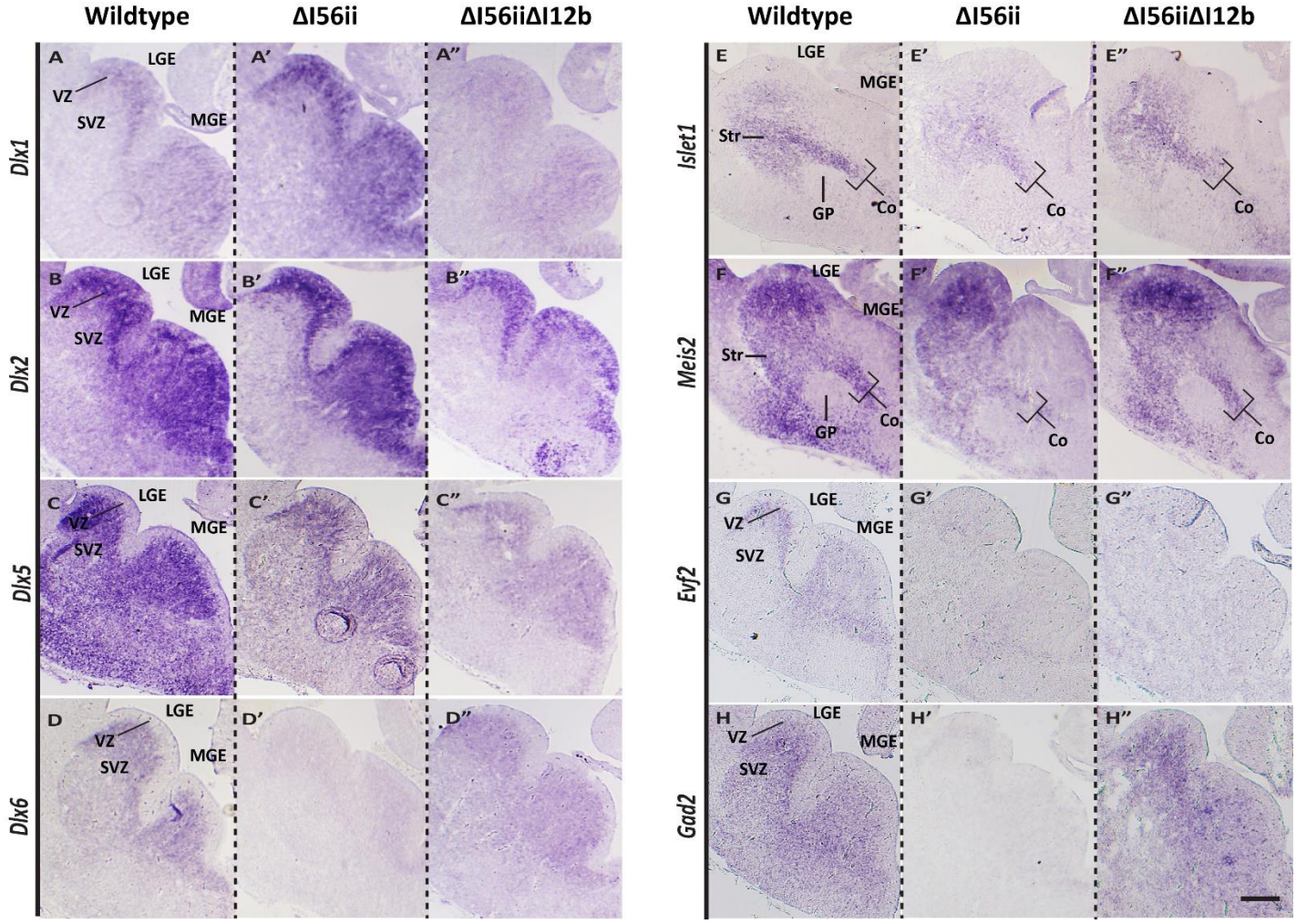
4.3.3 *In situ* hybridization supports the changes observed with qRT-PCR in the ventral telencephalon of mutant mice.

Since qRT-PCR measures a global change in the expression levels of the gene of interest, I also performed *in situ* hybridization to examine the possibility of a spatial change in the expression patterns of *Dlx* genes as well as *Islet1, Meis2, Evf2* and *Gad2* in the developing forebrain. Since the changes that were observed using qRT-PCR analysis were more pronounced at E13.5, *in situ* analyses were carried out on the ventral telencephalon of E13.5 developing mice. The changes that were detected in the mutant mice were compared to the expression patterns of the wildtype littermates. Antisense probes against *Dlx1, Dlx2, Dlx5, Dlx6, Islet1, Meis2, Gad2* and *Evf2* were used for this experiment. The expression patterns that were observed with *in situ* experiments matched the previously published patterns, in which *Dlx1* and *Dlx2* are shown to be expressed in the immature cells of the VZ and SVZ, whereas, *Dlx5* and *Dlx6* are mainly expressed in the SVZ and MZ (Eisenstat et al., 1999). As it is demonstrated in Fig. 4.2, *in situ* hybridization support the changes that are observed in the qRT-PCR experiments (Fig. 4.1).

Accordingly, *Dlx1* expression increase in the ventral telencephalon of the ΔI56ii mutant at E13.5 (Fig. 4.2A, A´). However, *Dlx1* expression greatly decreases in the ΔI56ii/ΔI12b mutant mice (Fig. 4.2A-A″). *Dlx2* expression is not affected in the ΔI56ii mutant; whereas, the expression levels of *Dlx2* are greatly reduced in ΔI56ii/ΔI12b mutant mice and is only detectable in the VZ at E13.5 (Fig. 4.2B-B″). The expression of *Dlx5* is reduced globally in the ΔI56ii mutant at E13.5 (Fig. 4.2C, C´). The expression of *Dlx5* appears to be more severely affected in the ΔI56ii/ΔI12b mutant when comparing to the wildtypes and ΔI56ii mutants (Fig. 4.2C-C″). Interestingly, the changes in the expression patterns of *Dlx6* and *Gad2* are very similar. The expression levels of

Dlx6 and *Gad2* are greatly reduced in ΔI56ii mutants at E13.5 (Fig. 4.2D-D´, G-G´). However, the expression levels of *Dlx6* and *Gad2* are very similar to the wildtype samples in the ΔI56ii/ΔI12b mutant mice (Fig. 4.2 D, D″; G, G″). *Evf2* expression patterns are severely affected as a result of the *Dlx* enhancer deletion, and the signal is undetectable in ΔI56ii and ΔI56ii/ΔI12b mutant mice (Fig. 4.2 G-G″). Furthermore, expression of *Islet1* and *Meis2* is also reduced in the striatum of the developing forebrain (Fig. 4.2 E-E´, F-F´). This decrease is particularly evident in the corridor cells population located in the striatum region of MGE of the developing forebrain at E13.5 (Fig. 4.2E-E´, F-F´). However, the signals corresponding to the expression of *Islet1* and *Meis2* are increased in the ΔI56ii/ΔI12b mutants compared to the ΔI56ii mutants. It is worth noting that despite this increase in the expression of *Islet1* and *Meis2* in the ΔI56ii/ΔI12b mutants, expression levels of these genes are, apparently, still lower than the wildtype samples (Fig. 4.2E-E″, F-F″).

Figure 4.2: *In situ* hybridization on coronal sections of E13.5 embryonic ventral telencephalon tissues from wildtype (A-H), ΔI56ii (A´-H´) and ΔI56ii/ΔI12b mutants (A″-H″). In the ΔI56ii mutants, *Dlx1* expression increases, but then it decreases drastically in the ΔI56ii/ΔI12b mutant (A-A″). No changes are observed in *Dlx2* (B-B″) expression in the ΔI56ii mutant, but signal has decreased in the ΔI56ii/ΔI12b mutants. Conversely, *Dlx6* activity is markedly reduced in ΔI56ii mutant, but has increased in the ΔI56ii/ΔI12b mutant (D-D″). The ΔI56ii and ΔI56ii/ΔI12b mutant mice show a global decrease in *Dlx5* (C-C″), *Islet1* (E-E″), *Evf2* (G-G″), and *Gad2* (H-H″) expression levels in the ventral telencephalon when compared to wildtype littermates. *Meis2* expression in reduced in the corridor cells of ΔI56ii mutant; but is increased in the ΔI56ii/ΔI12b mutants (F-F″). LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence, VZ: ventricular zone, SVZ: subventricular zone, Str: striatum, GP: globus pallidus, Co: corridor cells. Scale bar: 50μm.


Wildtype
ΔI56ii
ΔI56iiΔI12b
Wildtype
ΔI56ii
ΔI56iiΔI12b
Dlx1
Dlx2
Dlx5
Dlx6
Islet1
Meis2
Evf2
Gad2
A
A'
A"
B
B'
B"
C
C'
C"
D
D'
D"
E
E'
E"
F
F'
F"
G
G'
G"
H
H'
H"
LGE
MGE
VZ
SVZ
Str
GP
Co

4.3.4 Immunohistochemistry demonstrates a change in the expression patterns Islet1 and Ctip2 proteins as well as Semaphorin 3A, Slit1, and Ephrin A5 in the ventral telencephalon of the mutant mice.

It has been demonstrated through I56ii-*lacZ* studies that the I56ii enhancer is active in the corridor cells located deep in the mantle zone of the developing forebrain (Ghanem et al., 2008). In order to address the functional importance of the I56ii activity in the corridor cells of the developing forebrain, we performed immunohistochemistry experiments for the transcription factors Islet1 and Ctip2. Islet1 and Ctip2 are striatal markers that are also expressed in corridor cells. As depicted in Fig. 4.3 and 4.4, the expression of ISLET1 and CTIP2 proteins decreases drastically in the mutants at E13.5. More specifically, expression of ISLET1 is greatly reduced in the rostral regions of the developing forebrain of the ΔI56ii mutants, more specifically in the striatal region near the LGE (Fig. 4.3B-B´). It is noteworthy that the striatal expression of ISLET1 is detectable in the ΔI56ii/ΔI12b mutant but is apparently lower than in the wildtype samples (Fig. 4.3A-A´, C-C´). The change in the ISLET1 expression is similar in the caudal regions of the developing forebrain at E13.5 (Fig 4.3D-F´). The expression of ISLET1 is greatly reduced in the striatal regions of the LGE, but is undetectable in the corridor cells of ΔI56ii mutant mice (Fig. 4.3E-E´). Interestingly, the increase in the striatal expression of ISLET1 in the ΔI56ii/ΔI12b mutants is similar in the caudal region as well. However, the ISLET1 expression remains undetectable in the corridor cells of the ΔI56ii/ΔI12b mutants (Fig. 4.3F-F´). The changes observed in the apparent expression levels of CTIP2 are also drastically reduced in ΔI56ii the mutant mice (Fig. 4.4B-B´and are undetectable in ΔI56ii/ΔI12b mutants (Fig. 4.4C-C´). Collectively, the reductions in the levels of ISLET1 and CTIP2 proteins could imply that the I56ii enhancer may

play an important role in maintaining the expression of these proteins in the corridor cells of the developing forebrain.

Upon observing the consequences of I56ii enhancer deletion in the expression levels of striatal markers, I was interested to examine if *Dlx* enhancer deletion(s) in fact affect the expression patterns of the guidance cues that have been shown to be active in the corridor cells. A study conducted by Bielle and colleagues (2011) demonstrated that the concentration gradients of several guidance cues including Slit1, Semaphorin 3A (Sema 3A), Ephrin A5 and Netrin 1 within corridor cells dictate the trajectory of the thalamocortical axons (TCAs) to the cortex (Bielle et al., 2011). Therefore, we performed immunohistochemistry against Slit1, Semaphorin 3A and Ephrin A5 to examine the potential effects of the enhancer deletions on these guidance cues. As depicted in Fig. 4.5A-C´, the expression levels of Sema 3A are seemingly altered in the mutants when compared to the wildtype samples (Fig. 4.5A-C´). The Sema 3A-expressing cells seem to be dispersed in the striatum region of ΔI56ii mutant mice (Fig. 4.5B-B´); however, the expression of Sema 3A is undetectable in the ΔI56ii/ΔI12b mutants (Fig. 4.5C-C´). Interestingly, the expression levels of Ephrin A5 are apparently increased in the ventral telencephalon of ΔI56ii mutant mice at E13.5 (Fig. 4.6B). The expression levels of Ephrin A5 seem to be even higher in the forebrain of ΔI56ii/ΔI12b mutant mice compared to the levels of the wildtype and ΔI56ii mutants (Fig. 4.6C). Considering the fact that both Ephrin A5 and Sema 3A are important for the proper trajectory of TCAs in the developing forebrain, the increase in the expression of Ephrin A5 could suggest a compensatory role for decreased levels of Sema 3A in the ventral telencephalon at E13.5. Furthermore, the striatal expression of Slit1 is not affected in ΔI56ii mutant mice at E13.5 (Fig. 4.5E-E´). However, striatal expression is increased in ΔI56ii/ΔI12b mutant mice compared to the

wildtype samples (Fig. 4.5F-F´). The increases in the levels of Slit1 may alter the trajectory of the TCAs in the developing forebrain more caudally.

Figure 4.3: Mutant mice demonstrate alterations in the expression of ISLET1 proteins in the ventral telencephalon at E13.5. Immunohistochemistry was performed using an antibody against ISLET1 on coronal-sections of E13.5 developing forebrain both rostrally A-A´ (wildtype), B-B´ (ΔI56ii), and C-C´ (ΔI56ii/ΔI12b mutant) and caudally D-D´ (wildtype), E-E´ (ΔI56ii), and F-F´ (ΔI56ii/ΔI12b mutant). Panels A´-F´ are high magnification pictures of the boxes shown in panels A-F. Panels G and H are the schematic representation of the positioning of the corridor cells rostrally (red box) and caudally, respectively. LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence, Str: striatum, GP: globus pallidus, Co: corridor cells. Scale bar: 100 µm in A-F, and 50 µm in A´-F´.

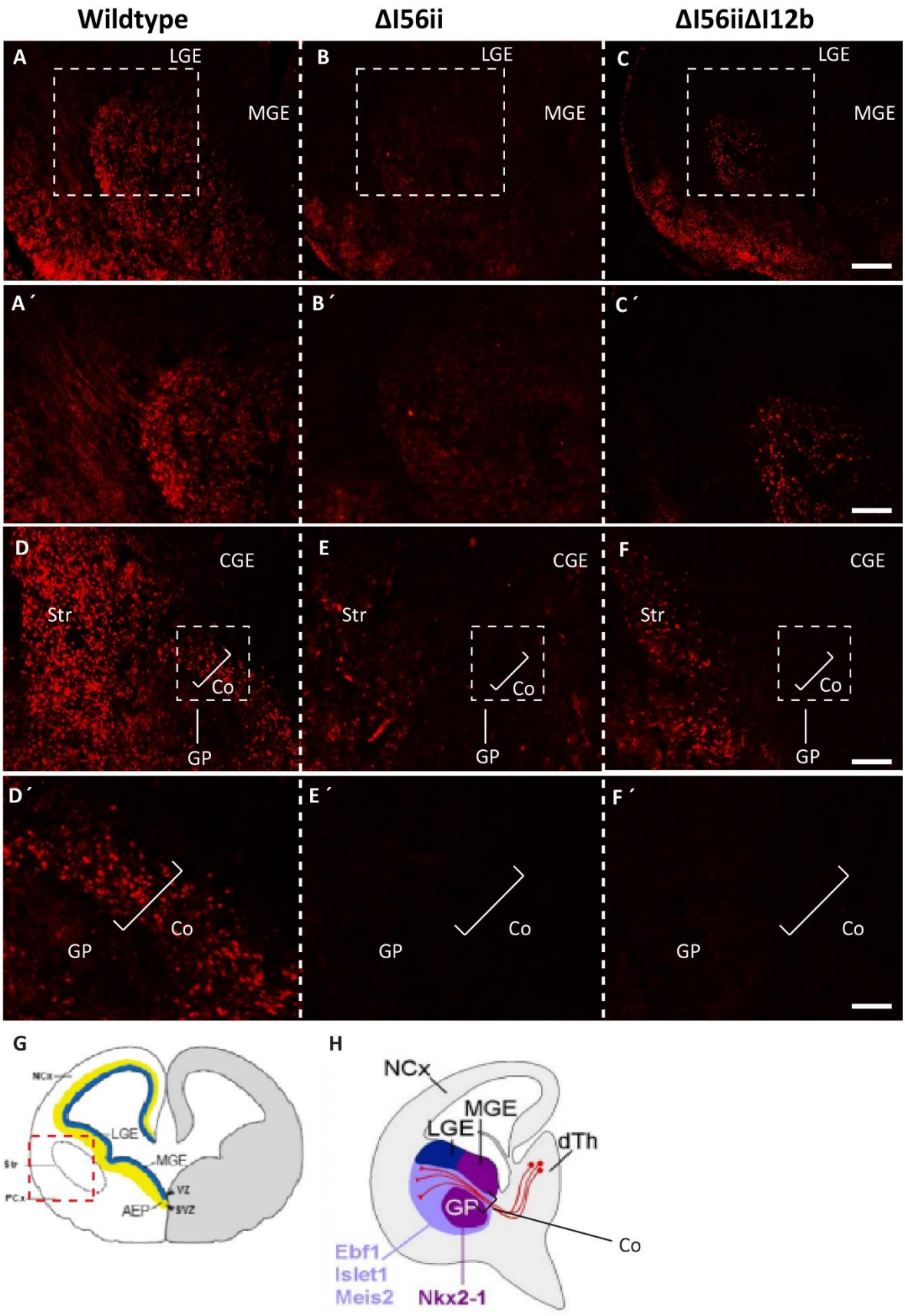

Wildtype
ΔI56ii
ΔI56iiΔI12b
A
LGE
MGE
B
LGE
MGE
C
LGE
MGE
A´
B´
C´
D
CGE
Str
Co
GP
E
CGE
Str
Co
GP
F
CGE
Str
Co
GP
D´
Co
GP
E´
Co
GP
F´
Co
GP
G
NCx
LGE
MGE
Str
VZ
AEP
SVZ
H
NCx
MGE
LGE
dTh
GP
Co
Ebf1
Islet1
Meis2
Nkx2-1

Figure 4.4: Mutant mice demonstrate a change in the expression of CTIP2 proteins in the ventral telencephalon at E13.5. Immunohistochemistry was performed using an antibody against CTIP2 on coronal-sections of E13.5 developing forebrain from wildtype (A, A´), ΔI56ii (B, B´), and ΔI56ii/ΔI12b mutants (C, C´). Panels A´ and B´ are high magnification pictures of the boxes shown in panels A and B. LGE: lateral ganglionic eminence, Co: corridor cells. Scale bar: 100 µm in A-C, and 50 µm in A´-C´.

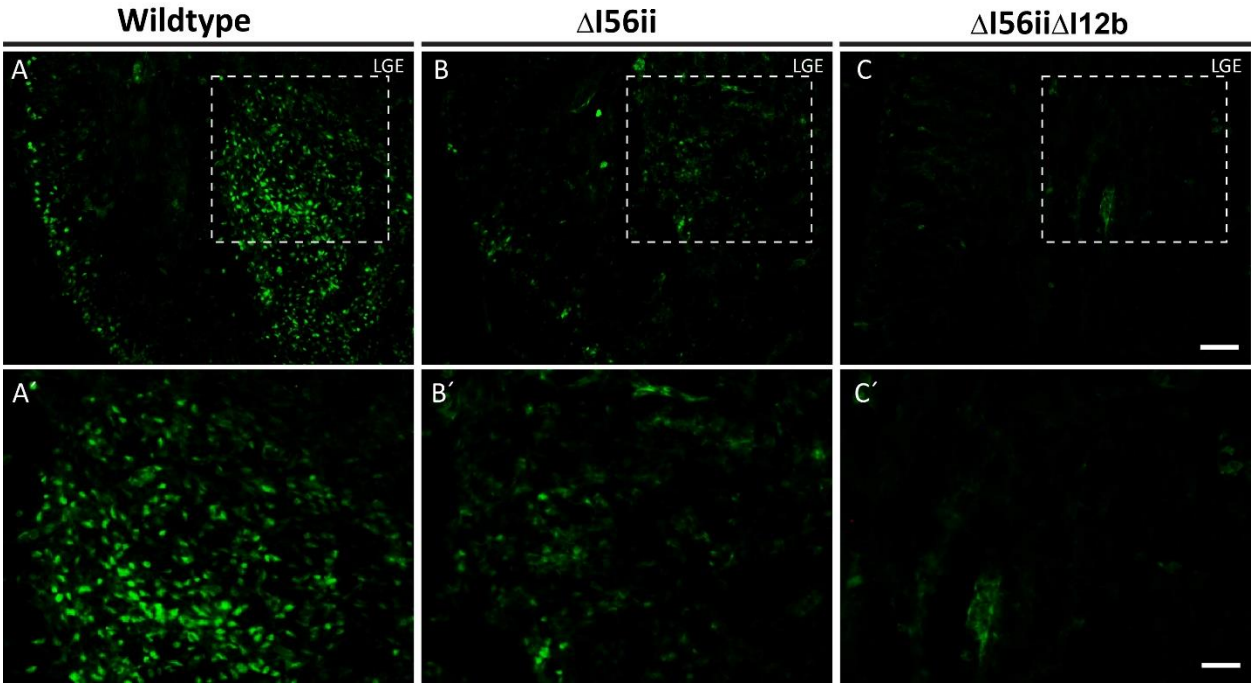

Wildtype
ΔI56ii
ΔI56iiΔI12b
A
LGE
B
LGE
C
LGE
A´
B´
C´

Figure 4.5: Mutant mice exhibit a disruption in the expression of Sema 3A and Slit1 guidance cues in the ventral telencephalon at E13.5. Immunohistochemistry was performed using antibodies against Semaphorin 3A and Slit1 on coronal-sections of E13.5 developing forebrain from wildtype (A, D), ΔI56ii (B, E), and ΔI56ii/ΔI12b mutant (C, F). Panels A´-F´ are high magnification pictures of the boxes shown in panels A-F. Panel G is the schematic representation of the developing forebrain at E13.5, with red box indicating the region shown in panels A-F. LGE: lateral ganglionic eminence, Co: corridor cells. Scale bar: 100 µm in A-F and 50 µm in A´-F´.

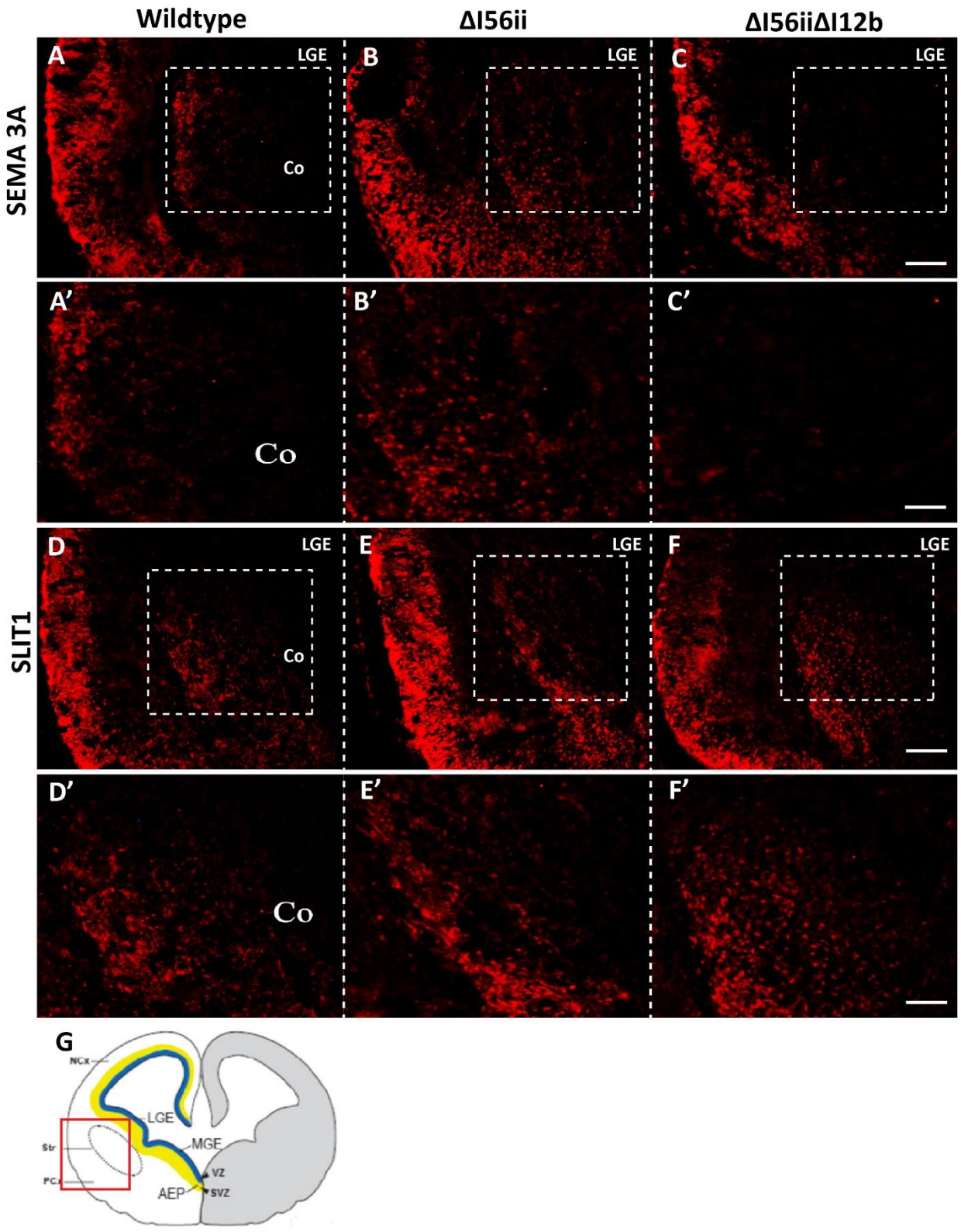
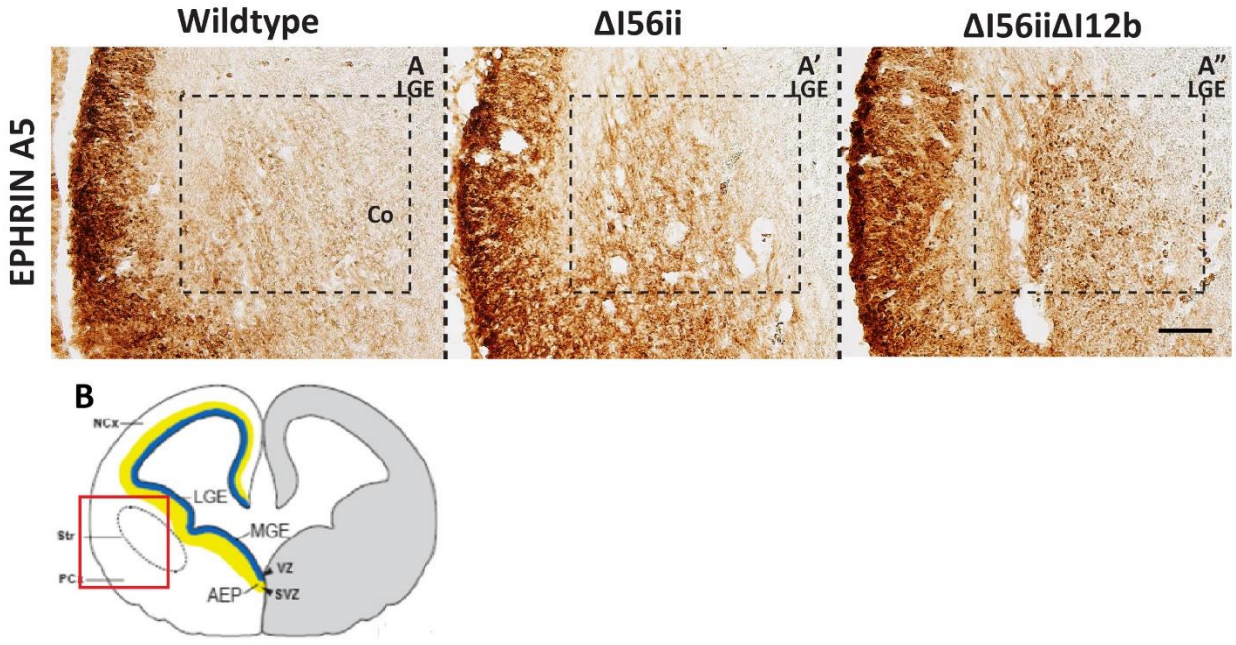

Wildtype
ΔI56ii
ΔI56iiΔI12b
SEMA 3A
SLIT1
A
B
C
A'
B'
C'
D
E
F
D'
E'
F'
LGE
Co
G
NCx
LGE
MGE
Str
PC
VZ
SVZ
AEP

Figure 4.6: Mutant mice show changes in the expression of Ephrin A5 in the ventral telencephalon at E13.5. Expression of Ephrin A5 was examined by using an antibody against Ephrin A5 on coronal-sections of E13.5 developing forebrain from (A) wildtype, (A´) ΔI56ii, and (A″) ΔI56ii/ΔI12b mutant mice. Panel B is the schematic representation of the developing forebrain at E13.5, with the red box indicating the region shown in panels A-A″. The black dash-lined box represents the location of the corridor cells within the striatum. LGE: lateral ganglionic eminence, Co: corridor cells. Scale bar: 100 μm.


Wildtype
ΔI56ii
ΔI56iiΔI12b
EPHRIN A5
A
LGE
Co
A'
LGE
A"
LGE
B
NCX
LGE
MGE
Str
PC
VZ
SVZ
AEP

4.4 Discussion

4.4.1 *Dlx* intergenic enhancers demonstrate functional redundancy in the developing mouse forebrain:

Cis-regulatory elements possess binding domains for different transcription factors which allows them to regulate spatial and temporal expression patterns of genes (Wolpert & Tickle, 2011). It has been suggested that the CREs that are involved in developmental processes possess an extremely high sequence conservation amongst species, which signifies their essential regulatory role (Pennacchio et al., 2006). It has been shown that the *Dlx* intergenic CREs have been highly conserved throughout evolution (Ghanem et al., 2008; Zerucha & Ekker, 2000; Zerucha et al., 2000). Therefore, any mutation in these conserved CREs should have a dramatic impact on the growth and the survival during the developmental stages, with potential morphological complications. This theory has been supported by Sumiyama and Ruddle (2002), in which an intergenic enhancer of the *Dlx3/Dlx4* cluster (known as I37-2) was removed in the mouse (Sumiyama & Ruddle, 2003). These mice demonstrated a failure to produce visceral arches as results of the enhancer removal (Sumiyama & Ruddle, 2003).

In characterizing the functional importance of the I56ii forebrain *Dlx* intergenic enhancer, located in the *Dlx5/6* cluster, I monitored the morphology, viability as well as the fertility of ΔI56ii mutant mice. These mice appear normal and do not possess any overt morphological abnormalities when compared to their wildtype littermates. Previous studies investigated the activity patterns of different *Dlx* intergenic enhancers through *lacZ* reporter genes in the developing forebrain. The overlapping patterns for different forebrain intergenic enhancers suggested that there may exist a functional redundancy between various enhancers (Ghanem et al., 2003; Ghanem et al., 2007; Ghanem et al., 2008; Park et al., 2004; Poitras et al., 2007). With this in mind, I generated a second

mutant line in which both I56ii and I12b enhancers located in the *Dlx5/6* and *Dlx1/2* loci were deleted (also referred to as ΔI56ii/ΔI12b line). Surprisingly, mice homozygous for both I56ii and I12b enhancer deletions were viable, fertile and did not demonstrate any phenotypic abnormalities when compared to the wildtype mice. These observations may suggest that I56ii and I12b enhancers are not essential during the developmental stages as well as the overall well-being of the individual. Alternatively, it can be suggested that other CREs may compensate for the lack of the activity of these enhancers, leading to the lack of any morphological changes in the developing mice.

4.4.2 Changes in *Dlx* expression levels as a result of enhancer deletion(s) may suggest an important regulatory role for *Dlx6* in the developing forebrain.

At the molecular level, the I56ii enhancer deletion significantly impairs *Dlx* expression as well as that of other genes that are thought to be, directly or indirectly, under the control of *Dlx* genes (Fig. 4.1). Previous work has demonstrated that *Islet1* and *Meis2* striatal markers can activate transcription via the I56ii enhancer sequence in co-transfection assays *in vitro*, suggesting that these transcription factors may be upstream regulators of *Dlx* genes *in vivo* (Ghanem et al., 2008). According to the qRT-PCR data presented in Fig. 4.1, the I56ii enhancer deletion significantly reduces the expression levels of *Dlx6* as well as *Islet1* and *Meis2*. Furthermore, an increase in *Dlx6* expression in the ΔI56ii/ΔI12b mutant mice is accompanied by an increase in the transcript levels of *Islet1* and *Meis2*. These changes could suggest the presence of a positive feedback loop between *Dlx6* and *Islet1/Meis2* regulating their expression levels in the developing forebrain (Fig. 4.7A). In addition to the decrease in *Dlx5* and *Dlx6* expression levels, ΔI56ii mutant mice exhibit a significant increase in *Dlx1* expression (Fig. 4.1). It has been hypothesized that *Dlx1* and *Dlx2* might play a regulatory role in activating and maintaining the expression of *Dlx5/6* since *Dlx1* and

Dlx2 expression is initiated prior to *Dlx5/Dlx6* transcription (Panganiban & Rubenstein, 2002). The increase in *Dlx1* expression may be a result of functional compensation for the significant decrease in the expression of *Dlx5* and *Dlx6* through a negative-feedback loop (Fig. 4.7A, B).

Previous studies have suggested that DLX1, DLX2 and DLX5 proteins are capable of binding to the *Gad* enhancer. This interaction is thought to be responsible for initiating and maintaining *Gad* expression (Panganiban & Rubenstein, 2002). Furthermore, it is noteworthy that a decrease in the expression of *Dlx5* and *Dlx6*, coincided with reduced *Gad2* expression levels (~70%). *Gad2* codes for the enzyme GAD, responsible for synthesizing GABA, an inhibitory neurotransmitter in the GABAergic interneurons. Therefore, it can be deduced that the increase in *Dlx1* expression could also be due to a functional compensation to maintain levels of GABA in GABAergic interneurons.

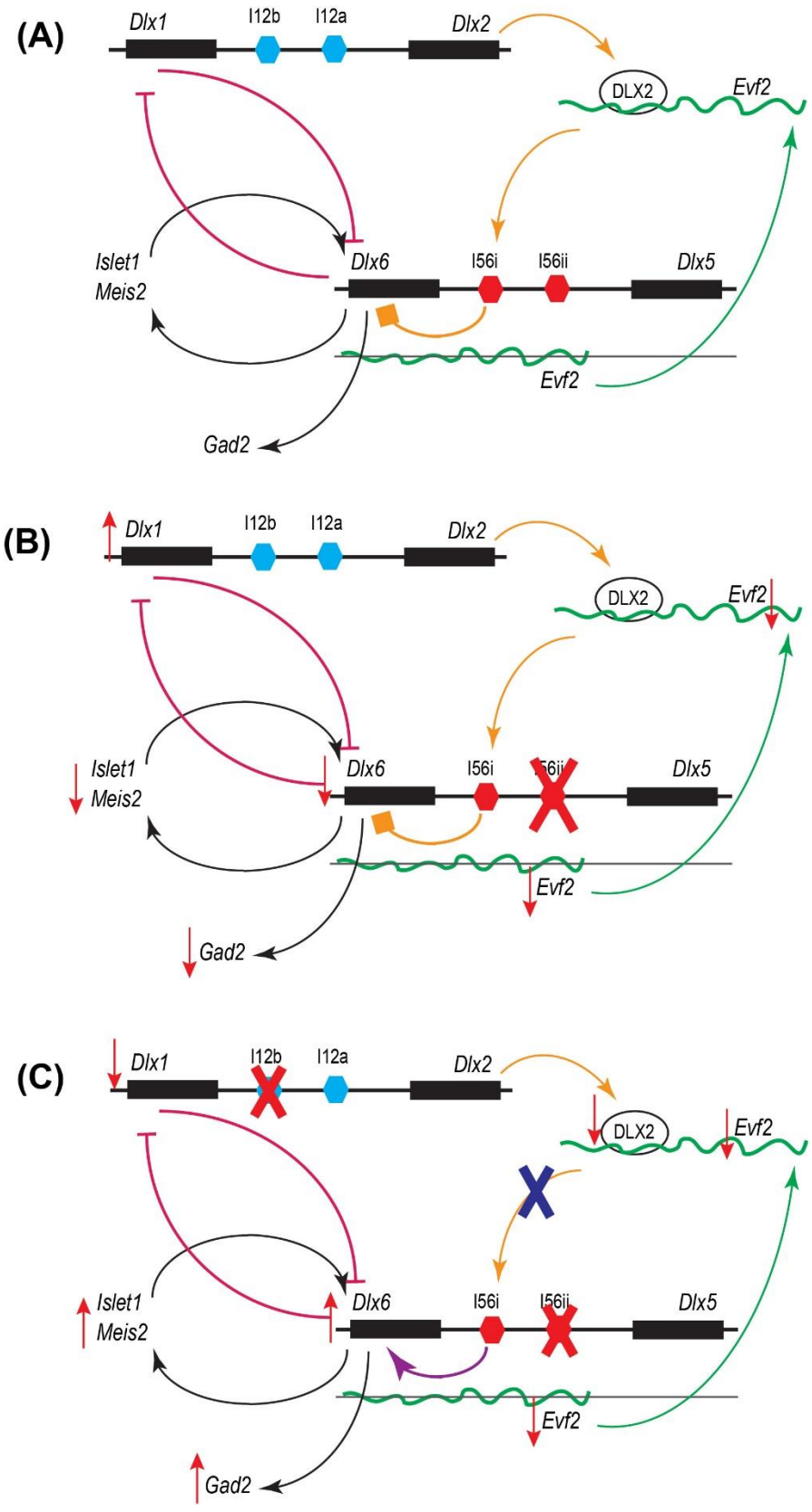
In addition, previous studies investigated the regulatory importance of transcription regulating ultraconserved noncoding RNA (trucRNA), known as *Evf2*, and demonstrated that *Evf2* binds to DLX2 proteins to form a stable complex (Feng et al., 2006). It has been suggested that this complex binds to the I56i enhancer located in the intergenic region of *Dlx5/6* cluster and plays a negative transcriptional regulatory role in controlling the expression of the *Dlx5*/*Dlx6* locus (Fig. 4.7A) (Feng et al., 2006). However, contrary to what would be expected from these studies, a significant decrease in *Evf2* expression (~80%) in the ΔI56ii mutants did not lead to increased *Dlx5* and/or *Dlx6* expression. Since *Dlx2* levels are not affected in the ΔI56ii mutant mice, it can be hypothesized that sufficient amounts of the DLX2 protein in the cell will maintain this regulatory pathway. Therefore, the reduced levels of *Evf2* may still be sufficient to maintain this proposed regulatory pathway in the presence of DLX2 proteins.

The expression changes we observed are much more intriguing when comparing the expression levels of the ΔI56ii mutant mice with those of the ΔI56ii/ΔI12b double-mutants (Fig. 4.1B and 1C). *Dlx1* and *Dlx2* expression levels have significantly decreased in the double mutants. This is particularly interesting since the ΔI12b single mutant showed only 20-25% decreases in the expression levels of *Dlx1* and *Dlx2*, respectively when compared to the wildtype littermates at E13.5 (Yu, 2011). The decreases in *Dlx1* and *Dlx2* are accompanied by an increase in the expression levels of *Dlx6* and *Gad2* in the ΔI56ii/ΔI12b mutant mice. Considering the potential existence of feedback mechanisms, it can be proposed that the decreases in both transcript levels of *Dlx2* and *Evf2* disrupt the regulatory mechanism shown in Fig. 4.7A that negatively regulates the expression of the *Dlx5/6* cluster via the I56i enhancer. As a result, *Dlx6* expression levels are restored back to normal levels (Fig. 4.7C). The increase in the expression levels of *Dlx6* might explain the decrease in *Dlx1* expression through the previously proposed negative-feedback loop (Fig. 4.7C). It is worth noting that even though *Dlx5* expression has remained relatively unchanged between the ΔI56ii and ΔI56ii/ΔI12b mutant mice, the increase in the *Dlx6* levels is paralleled by an increase in *Gad2* expression, in that is almost restored to normal levels (~95%; Fig. 4.7C). These data may suggest an important regulatory role for *Dlx6* in maintaining the expression of *Gad2*. This level of regulation is particularly important since *Gad2*, as discussed previously, responsible for the production of GABA. Therefore, it can be hypothesized that *Dlx6* may play an important role in maintaining the GABAergic phenotype through regulating the expression levels of *Gad2*.

Collectively, this proposed mechanism may highlight an important functional role for *Dlx6* in the genetic regulatory mechanisms that are involved in maintaining GABAergic phenotype as

well as controlling the differentiation and migration of neurons via the activity of the corridor cells in the developing forebrain.

Figure 4.7: Proposed mechanism underlying the *Dlx* regulation in the ventral telencephalon of the developing forebrain of (A) wildtype, (B) ΔI56ii, and (C) ΔI56ii/ΔI12b mutants at E13.5. (A) This model proposes the presence of a negative feedback-loop between *Dlx1* and *Dlx6*; paralleled by a positive feedback-loop between *Dlx6* and upstream striatal markers *Islet1* and *Meis2*. Additionally this model states the previously described regulatory mechanism in which the DLX2 protein and evf2 noncoding RNA form a complex which negatively affects the transcription of the *Dlx5/Dlx6* genes. (B) Upon deletion of the I56ii enhancer, *Dlx6* and *Dlx5* expression levels decrease, which in turn reduces the expression of *Gad*. Reduced levels of *Dlx6* decrease the expression of *Islet1* and *Meis2* via the proposed positive feedback-loop. *Dlx1* expression levels have increased in response to the reduced levels of *Dlx6*, which may support the existence of the proposed negative feedback-loop between these two genes. (C) Once both I56ii and I12b enhancers are deleted, the DLX2/evf2 complex is no longer forming, which disrupts the negative regulatory cascade on the *Dlx5/Dlx6* locus. This leads to an increase in *Dlx6* expression levels, which ultimately reduces *Dlx1* expression through the proposed negative feedback-loop and increases the expression of *Islet1* and *Meis2* through the activity of the proposed positive feedback-loop. In response to increased levels of *Dlx6*, *Gad2* expression is increased and restored back to normal levels. The lack of a drastic change in *Dlx5* levels may suggest an important regulatory role for *Dlx6* in controlling and/or maintaining the expression of *Gad2* in the ventral telencephalon of the developing forebrain.


(A)
Dlx1
I12b
I12a
Dlx2
DLX2
Evf2
Islet1
Meis2
Dlx6
I56i
I56ii
Dlx5
Evf2
Gad2
(B)
Dlx1
I12b
I12a
Dlx2
DLX2
Evf2
Islet1
Meis2
Dlx6
I56i
Dlx5
Evf2
Gad2
(C)
Dlx1
I12b
I12a
Dlx2
DLX2
Evf2
Islet1
Meis2
Dlx6
I56i
I56ii
Dlx5
Evf2
Gad2

4.4.3 *Dlx* enhancer deletion(s) may disrupt the migration pattern of tangentially migrating neurons by affecting the activity of the corridor cells.

The ability of neurons to migrate away from their progenitor zone, where they are born, to their final destination is one of the central features of the developing central nervous system (Gleeson & Walsh, 2000; Lambert de Rouvroit & Goffinet, 2001). This process is essential for the proper development and function of the brain (Wichterle et al., 2003). Most interneurons migrate tangentially to reach their appropriate location in the cortex (López-Bendito & Molnár, 2003). An example of tangential migration is the movement of interneurons from the ganglionic eminence to the cerebral cortex. During this process, the corridor cells located deep in the MZ of the MGE serve as a permissive corridor for the tangentially migrating interneurons by providing various guidance cues to coordinate the proper migration of these interneurons to their final destination in the developing cortex. Although there is no information on the molecular mechanisms underlying the differential cell specification of the tangentially migrating neurons, the expression levels of *Islet1*, *Meis2*, *Ebf1*, and *Ctip2* within corridor cells (Bielle et al., 2012; Molnár et al., 2012) provides an opportunity to further investigate the impact of enhancer deletion on the activity of the corridor cells. In the quest for elucidating the effects of enhancer deletion(s) on neuronal migration by means of altering the activity of the corridor cells a series of immunohistochemistry experiments against Islet1 and Ctip2 transcription factors were performed.

Deletion of the I56ii enhancer leads, indirectly, to apparent reductions in the expression of the ISLET1 proteins both rostrally and caudally (Fig 4.3A-F´). Interestingly, even though *Islet1* and *Meis2* mRNA levels are still detected in the ΔI56ii mutants (at ~50% normal levels; Fig. 4.1B), the nuclear signal pertaining to their protein levels are undetectable in the ΔI56ii mutants (Fig. 4.3B-B´). Since the decrease in the expression levels of *Islet1* and *Meis2* are accompanied by a

significant decrease in *Dlx6* transcript levels, it may be proposed that DLX6 proteins act as a co-activator and/or enable the ISLET1/MEIS2-mediated regulatory mechanism in the corridor cells. This hypothesis can be further supported by the qRT-PCR data observed in ΔI56ii/ΔI12b mutant mice. *Dlx6* mRNA levels are restored to wildtype levels in the ΔI56ii/ΔI12b mutant mice when compared to the ΔI56ii mutant mice. This increase is accompanied by the re-emergence of the ISLET1 nuclear signal in the striatum region of the ΔI56ii/ΔI12b mutant mice at E13.5 (Fig. 4.3C-C´, F-F´). It is important to note that the decreases in the expression of these transcription factors do not imply that corridor cells no longer reside within the mantle zone of the LGE.

Collectively, the decreases in the expression of ISLET1 and CTIP2 proteins in the corridor cells may have consequences on the trajectory of the tangentially migrating thalamocorticol axons (TCAs). To address this hypothesis, I investigated the impact of enhancer deletion(s) on the guidance cues that have been shown to be active in the corridor cells, namely Semaphorin 3A (Sema 3A), Slit1 and Ephrin A5 (Bielle et al., 2011). It seems that the expression patterns of these guidance cues are altered in the mutant mice at E13.5 (Fig. 4.5, 4.6). Sema 3A-expressing cells are dispersed in the striatum of the ΔI56ii mutant mice (Fig. 4.5B-B´). The levels of Sema 3A are undetectable in the ΔI56ii/ΔI12b mutant mice (Fig. 4.5C-C´). Interestingly, the changes in the expression patterns of Ephrin A5 are opposite that of Sema 3A. The expression of Ephrin A5 is increased in the striatum of the ΔI56ii mutant mice when compared to the wildtype sample (Fig. 6B). The ΔI56ii/ΔI12b mutant mice exhibit an apparently more pronounced increase in Ephrin A5 expression. Sema 3A and Ephrin A5 guidance cues are particularly important since they are responsible for controlling the initial positioning of the TCAs by providing necessary information through their gradients in the subpallium and striatum (Dufour et al., 2003). It has been shown that Sema 3A and Ephrin A5 guidance cues prevent the caudal growth of rostral and intermediate TCAs

(Dufour et al., 2003; Wright et al., 2007). Therefore, it can be hypothesized that the increase in Ephrin A5 levels is a compensatory mechanism triggered by the reduced levels of Sema 3A. This hypothesized mechanism could potentially be developed to maintain the proper trajectory of the tangentially migrating TCAs to their final destination in the developing cortex. Studies have investigated the contact-dependent signalling pathways between membrane-linked ligands and receptors including the ephrin family and Eph receptor tyrosine kinases (Marquardt et al., 2005). It has been shown that the Eph/ephrin system which consists of both diffusible and cell-bound signaling molecules regulates and controls migration of the interneurons. For instance, it has been suggested that Ephrin A5 is expressed at the borders of the pathways of MGE-derived interneurons in order to ensure their proper migration and to prevent them from entering inappropriate regions of the brain during their migration (Steinecke et al., 2014). During this process, the ephrin A family acts as an attractant and signals axonal growth to project caudally in the developing forebrain (Bielle et al., 2011; Marquardt et al., 2005).

Furthermore, recent studies have shown that Slit1 plays a dual role in the corridor cells of the developing forebrain. First, it acts as a rostral repellent, which aids the trajectory and positioning of the intermediate axons. Second, Slit1 enables the activity of Netrin1-mediated attraction to coordinate rostral axons (Bielle et al., 2011). Additionally, it has been demonstrated that the combination of Slit1 and Netrin1 provides a very precise and intricate spatial information for the navigation of thalamocortical axons in the developing brain (Bielle et al., 2011). The increased levels of Slit1 in the ΔI56ii/ΔI12b mutant mice (Fig. 4.5D-D´, F-F´) can have several potential outcomes. This increase may affect the trajectory of the rostral axons more caudally due to excessive repulsive force acting on these rostral axons. This excessive repulsive force may also impact the trajectory of the intermediate axons, potentially due to the accumulation of the rostral

axons in this region. In addition, the increase in the Slit1 levels could disrupt Slit1-Netrin1 coordination, which may disrupt the previously published spatial coordination of the TCA navigation in the developing forebrain.

In conclusion, the data presented in this chapter reflect a complex and dynamic regulation of *Dlx* gene expression during the early stages of embryonic development through the activity of various regulatory elements (namely I56ii and I12b) with overlapping and distinct function(s). Furthermore, these data demonstrate an intricate regulatory mechanism, which highlights the important regulatory role of the *Dlx* genes and *Evf2* non-coding RNA in maintaining *Dlx* expression. This process may be essential in maintaining a GABAergic phenotype by regulating the activity of *Gad.* Additionally, this project demonstrates that the changes in gene expression resulting, directly and indirectly, from enhancer deletion, may lead to altered properties of the corridor cells, which may disrupt the guidance and/or coordination of the thalamocortical axon pathfinding in the developing forebrain.

Chapter 5: General Discussion

Proper development of an organism is deliberately controlled via regulation of gene transcription to ensure that necessary information is expressed properly and at a suitable rate (Uchikawa et al., 2003). *Cis*-regulatory elements (CREs) including enhancers, promoters, silencers and insulators play an essential role in directing the proper activity of their target genes during different developmental stages (Bulger & Groudine, 2010; Bushey et al., 2008; Riethoven, 2010). It has been suggested that during embryogenesis, enhancers play a prominent role in spatiotemporal gene regulation (Noonan & McCallion, 2010). Precise spatiotemporal regulation is due to the ability of the enhancers to drive gene expression at a distance independent of their orientation with respect to the transcription start site (Ong & Corces, 2011). In addition, enhancers can also serve as a platform for transcription factor binding in order to activate and maintain transcription of their target (Ong & Corces, 2011).

Given the important transcriptional regulatory role of enhancers during different developmental processes, it is fundamental to address the role of *Dlx* intergenic enhancers during development. This topic is particularly interesting due to the peculiar genomic arrangement of *Dlx* bigene clusters, their genomic and functional conservation throughout evolution, and their important role during development. During the series of experiments presented in this thesis, the regulatory role of the *Dlx* intergenic enhancers were examined by means of chromatin conformation capture to detect enhancer-promoter interactions within and between *Dlx* loci. The phenotypic consequence of intergenic enhancer deletion was also examined in various targeted deletion lines. Chromosome Conformation Capture (3C) data support DNA looping model of enhancer action in the developing forebrain. Furthermore, the functional importance of the I56ii and I12b intergenic enhancers was demonstrated through the phenotypic consequences of the

enhancer removal by generating different targeted-deletion lines, in which I56ii as well as I12b and I56ii enhancers were removed.

5.1 Investigating the functional importance of I12b and I56i CREs suggest a potential functional redundancy between the two intergenic CREs.

There has been a lot of effort focused on discovering the functional importance of *Dlx* intergenic enhancers using *lacZ* reporter constructs in the developing transgenic forebrain (Ghanem et al., 2003; Ghanem et al., 2007; Ghanem et al., 2008; Park et al., 2004). These studies collectively demonstrated that I12b-*lacZ* and I56i-*lacZ* positive cells were detected in the tangentially migrating cell that were originally derived from the LGE, MGE and to a lesser extent from the CGE (Ghanem et al., 2007). Through spatiotemporal analysis of *lacZ* transgenesis, Ghanem et al. (2007) also showed that I12b and I56i enhancers were active in the adult cortical GABAergic interneurons (Ghanem et al., 2007). These data suggest an important regulatory role for I12b and I56i enhancers in the developing forebrain. A high level of regulation via the I12b and I56i intergenic enhancers was further confirmed by utilizing Chromosome Conformation Capture (3C), which demonstrated that I12b and I56i *cis*-regulatory elements (CREs) interact with the promoter regions of *Dlx1/Dlx2* and *Dlx5/Dlx6* clusters, respectively, in order to initiate and maintain the expression of their target gene in each cluster at E13.5. These interactions are shown to persist over different developmental stages when comparing the interactions from E13.5 to E15.5. In addition to the involvement of these enhancers (I12b and I56i) in the tangentially migrating neurons, this high level of regulation at the chromatin level could further explain the phenotype observed in the *Dlx* null mice (Anderson et al., 1997a). Anderson et al (1997a) demonstrated that *Dlx1/Dlx2* null mice had a predominant disruption in differentiation and migration of GABAergic interneuron to the neocortex (Anderson et al., 1997a).

The regulatory roles of I12b and I56i enhancers were further investigated by generating targeted deletion mouse lines in which I12b and I56i enhancers were removed; namely ΔI12b (Yu, 2011) and ΔI56i (Esau, 2013), respectively. In addition to the two aforementioned deletion lines, a third deletion line was also generated to study the effect of the deletion of both I12b and I56i enhancers on forebrain development (also referred to as ΔI56i/ΔI12b). It has been shown that these enhancer deletions impair *Dlx* transcript levels and other GABAergic markers in the developing forebrain (Esau, 2013; Yu, 2011). In addition, I56i enhancer deletion was also shown to cause learning deficits in the mutant mice (Esau, 2013). Notably, the phenotype is much more exacerbated in the ΔI56i/ΔI12b in which the expression of *Dlx* and target genes including *Gad2* are decreased more than 60%, while *Evf2* expression is abolished (see Appendix 2). Collectively, these data suggest a possible functional redundancy between I12b and I56i enhancers and could further strengthen the importance of the I12b and I56i enhancers for regulating and maintaining the proper expression of *Dlx* genes and their targets in the developing forebrain.

This level of functional redundancy may suggest an important regulatory role for both I56i and I12b enhancers. Ghanem et al (2007) through *lacZ* transgenesis demonstrated that the majority of the cells in SVZ and MZ are co-labeled for the activity of both enhancers suggesting that these CREs are mainly active in the overlapping cell populations and could potentially respond to similar regulatory factors (Ghanem et al., 2007). Therefore, it is probably feasible that both I12b and I56i enhancers may form a focal point in order to properly regulate the expression of the *Dlx* genes in the developing forebrain. This hypothesis is supported by the detection of *trans* interactions between *Dlx1/2* and *Dlx5/6* clusters. The presence of a *trans* interaction between the two *Dlx* clusters could suggest a new mode of auto-regulatory (Schoenfelder et al., 2010; Sexton et al., 2009) or cross-regulatory (Dmitriev et al., 2009; Petrov et al., 2008) mechanism for the

transcriptional regulation of *Dlx* and other targets that their expression is under control of the activity of *Dlx* genes in the developing forebrain. These targets may include *Islet1* and *Meis2* striatal markers as well as *Gad*, which encodes for GAD responsible for synthesizing GABA, which serves as an inhibitory neurotransmitter in the developing forebrain. The possibility of the presence of a cross-regulation between *Dlx* genes was previously suggested in the *Dlx1/Dlx2* null mice, in which *Dlx5/Dlx6* expression was lost in the majority of the developing telencephalon, implying that *Dlx1/Dlx2* are potential upstream regulators of *Dlx5* and *Dlx6* (Anderson et al., 1997a; Zerucha et al., 2000).

5.2 I56ii enhancer plays an important regulatory role during forebrain development.

Concomitant to this work, the activity of the I56ii enhancer located in the intergenic region of *Dlx5/Dlx6* cluster was also investigated using *lacZ* reporter transgenesis (Ghanem et al., 2008). Considering the complete overlap between the endogenous expression of *Dlx6* in the developing forebrain (Panganiban & Rubenstein, 2002) with the exogenous activity of the I56ii enhancer (Ghanem et al., 2008), it was suggested that the I56ii enhancer might play an important role in regulating the expression of *Dlx6* in the developing forebrain. This hypothesis is confirmed and further supported by the data obtained from the 3C studies in which I56ii enhancer is shown to interact with the promoter region of *Dlx6* at E13.5. The interaction between the I56ii enhancer and the promoter region of *Dlx6*, could suggest a regulatory role for *Dlx6* in coordinating the proper function and activity of the corridor cells. Following the previously published *lacZ* transgenesis in which enhancer activity was undetectable after E13.5, I56ii interaction with the promoter of *Dlx6* is undetectable at E15.5, meaning that this interaction does not persist at later developmental stages. Such limited activity of the enhancer could signify its functional importance during forebrain development. Ghanem and colleagues (2008) also demonstrated that the I56ii enhancer

was active in a small group of post-mitotic cells that are thought to be derived from the LGE and have tangentially migrated deep into the MZ of LGE and MGE to form a permissive corridor for the tangentially migrating neurons, known as corridor cells. This potentially fundamental regulatory role for the I56ii enhancer in corridor cells was further investigated in a targeted deletion line in which the I56ii enhancer was deleted.

In addition to this mutant line, a second deletion line was also generated in which both I12b and I56ii enhancers were deleted (also referred to as ΔI56ii/ΔI12b) in order to examine the effect of multiple enhancer deletions on neuronal migration and forebrain development. I56ii enhancer deletion impairs *Dlx* activity as well as other targets including striatal markers *Islet1* and *Meis2*. The ISLET1+ corridor cells are undetectable in both mutant lines, suggesting an important regulatory role for I56ii enhancer in regulating the activity of the corridor cells within the developing forebrain. However, there are many interesting changes in *Dlx* expression levels as well as other targets including *Islet1, Meis2, Evf2* and *Gad2* when comparing the ΔI56ii with the ΔI56ii/ΔI12b mutants, which may lead to a potential mechanism relating these factors to one another.

5.3 Potential mechanistic model between *Dlx1/2* and *Dlx5/6* clusters suggests an important regulatory role for *Dlx6* during forebrain development.

It has been suggested that *Evf2* transcription regulating ultraconserved noncoding RNA (trucRNA) and DLX2 proteins bind to each other and form a stable complex (Feng et al., 2006). It has been proposed that this complex binds to the I56i enhancer located in the intergenic region of the *Dlx5/6* cluster and plays a negative transcriptional regulatory role in controlling the activity of the *Dlx5*/*Dlx6* locus (Feng et al., 2006). The significant decrease in the expression levels of

Dlx6 in the developing forebrain of ΔI56ii mutant mice can be justified through two different lines of evidence presented here. First, the significant decrease in *Dlx6* expression in the ΔI56ii mutant mice can be attributed to the fact that I56ii enhancer only interacts with the promoter region of *Dlx6* gene. Second, in addition to the lack of I56ii activity, the significant decrease in *Dlx6* can be due to a negative regulation by the *Evf2*-DLX2 complex on the *Dlx5/Dlx6* cluster through the I56i enhancer. The disruption of the *Evf2*-DLX2 regulatory path due to decreased levels of *Dlx2* and *Evf2* transcripts in the ΔI56ii/ΔI12b mutants might explain why *Dlx6* transcript levels are restored back to normal.

According to previous work by Ghanem and colleagues (2008), it can be deduced that there is a positive feedback-loop between *Dlx6* and *Islet1/Meis2* striatal markers in the developing forebrain. The possibility of such a positive feedback-loop is further strengthened by the changes in expression levels of these genes in the mutants. A decrease in *Dlx6* expression in ΔI56ii mutants is accompanied by a significant decrease in the expression levels of *Islet1/Meis2*. However, as *Dlx6* expression increases in the ΔI56ii/ΔI12b mutant, so do the expression levels of *Islet1/Meis2* at E13.5.

In addition, ΔI56ii mutant mice exhibit a significant increase in *Dlx1* transcripts while *Dlx2* expression remains stable in the developing forebrain. It has been hypothesized that *Dlx1* and *Dlx2* might play a regulatory role in activating and maintaining the expression of *Dlx5/6* since expression of *Dlx1* and *Dlx2* is initiated prior to that of *Dlx5/Dlx6*. The increase in *Dlx1* expression (~54%) may be a result of functional compensation for the significant decrease in the expression of *Dlx5* and *Dlx6* through a negative-feedback loop. This is further supported by the fact that once *Dlx6* expression is restored in the ΔI56ii/ΔI12b mutants, *Dlx1* expression levels decrease. This

decrease can be attributed to a possible negative feedback-loop since *Dlx1* expression only decreases by 25% in the ΔI12b mutant (Yu, 2011).

Furthermore, it is noteworthy that a decrease in the expression of *Dlx5* and *Dlx6*, significantly reduced *Gad2* expression levels, which codes for the enzyme GAD, responsible for synthesizing GABA, an inhibitory neurotransmitter in the GABAergic interneurons. Since *Dlx5* expression levels are approximately the same between ΔI56ii and ΔI56ii/ΔI12b mutants (70% and 60%, respectively), the significant increase in the *Gad2* expression levels in the ΔI56ii/ΔI12b mutant mice can be attributed to an increase in *Dlx6* expression levels. Collectively, this model suggests a very important regulatory role for *Dlx6* during forebrain development.

5.4 Future Directions:

According to the data gathered from the 3C studies, a *trans* interaction may exist between the I12b and I56i enhancers located in the *Dlx1/2* and *Dlx5/6* clusters, respectively, in the developing forebrain at E13.5. However, observing a specific association between two loci does not by itself reveal a function for this interaction. Additional approaches such as knock-down of proteins (e.g. transcription factors) that mediate the interaction or deletion of the regulatory element can reveal causal relations between long-range interactions and gene regulation. Therefore, it would be fascinating to expand our understanding of the function of the *Dlx* intergenic CREs by using chromosome fluorescent *in situ* hybridization (FISH) to further investigate the presence of a *trans* interaction between the two *Dlx* loci. Additionally, it would be interesting to use ΔI56i/ΔI12b mutant line in the FISH experiments to see whether deletion of the enhancers affect the presence of an overlapping signal. This will give us a better understanding of the effect of the enhancer deletion on the DNA looping mechanism between these two loci. In addition, this

work will allow us to investigate if there is a compensatory mechanism by the other enhancer in the intergenic region of *Dlx* clusters; and if so, how this compensation affects the chromatin structure and DNA looping mechanism.

The functional consequences of the I56ii enhancer deletion on the expression levels of *Dlx6* in the developing forebrain raises the provocative question as to how does this mutation affects the trajectory of the tangentially migrating neuron to the cortex? It would be intriguing to perform *ex vivo* experiments by using GFP-expressing dorsal telencephalic explants on the ventral telencephalon preparations of the mutant mice to investigate the potential consequence of the decrease in corridor cells activity on the fate of the thalamocorticol projections in the developing forebrain. It would be interesting to investigate whether the I56ii enhancer deletion impacts the trajectory of the tangentially migrating neurons that would normally migrate from the dorsal telencephalon to the developing cortex through the corridor cells.

5.5 Significance of Study:

Overall, the data presented and discussed in this thesis, presents a functional analysis of *Dlx* intergenic enhancers in the developing forebrain. This intricate level of regulation through the activity of the enhancers is shown to be essential for controlling *Dlx* expression through a DNA looping mechanism. The outcome of this research has also shed more light on the intriguing question of whether the *Dlx6* contributes to the regulation of the tangential migration of "immigrant" neurons in the ventral telencephalon.

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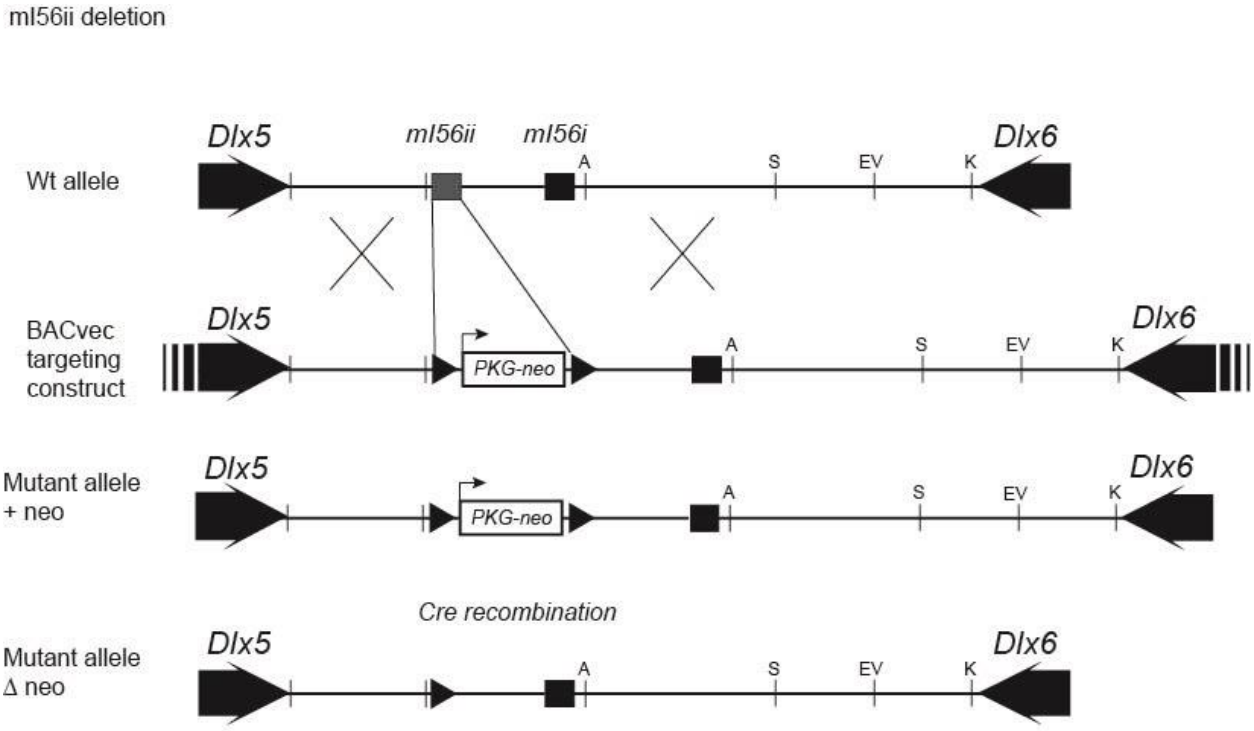
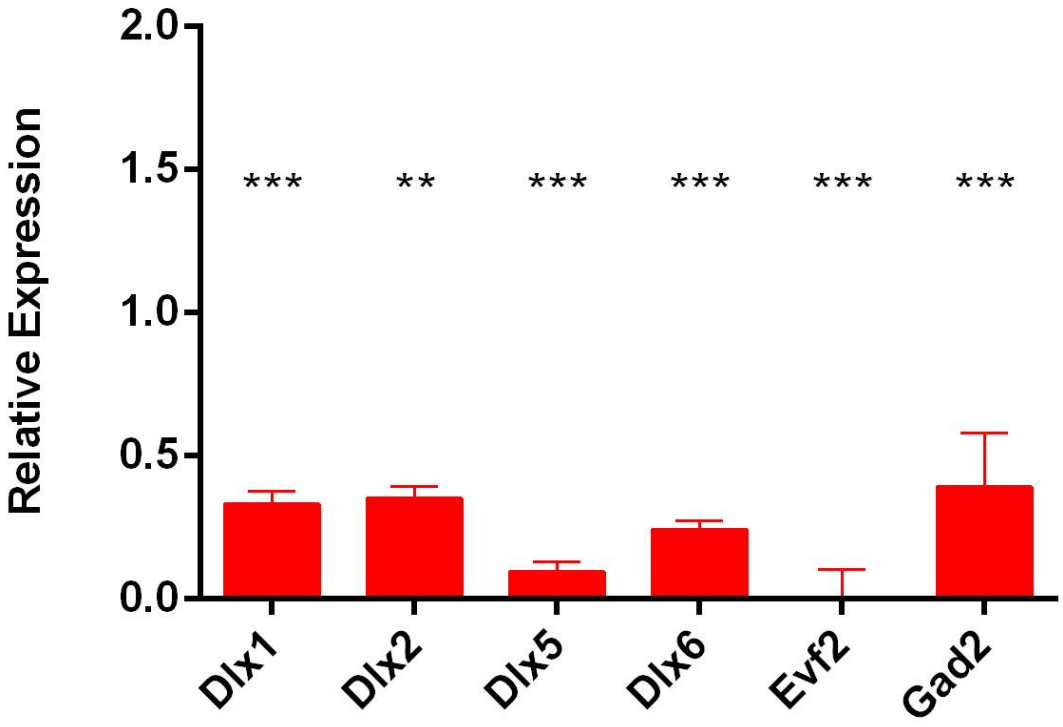
Appendix 1: Targeting strategy for the deletion of the I56ii enhancer in mouse embryonic stem cells. The I56ii BAC targeting vector containing a neomycin cassette flanked by loxP-sites (black triangles) undergoes homologous recombination with one allele of the endogenous I56ii CRE (grey box). Homologous recombination regions, represented by cross lines result in the replacement of the ~500bp enhancer with the neo cassette. The black arrows represent the transcriptional orientation of *Dlx5* and *Dlx6* genes.


mI56ii deletion
Dlx5
mI56ii
mI56i
Dlx6
Wt allele
A
S
EV
K
BACvec
targeting
construct
PKG-neo
Mutant allele
+ neo
Cre recombination
Mutant allele
Δ neo

Appendix 2: qRT-PCR demonstrates an exacerbated decrease in the expression levels of *Dlx*, *Gad2* and *Evf2* in the ventral telencephalon of the ΔI56i/ΔI12b mutant mice at E13.5.


Relative Expression
2.0
1.5
1.0
0.5
0.0
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Dlx1
Dlx2
Dlx5
Dlx6
Evf2
Gad2