Phenotype characterization of mice with targeted deletions of *Dlx* enhancers

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Abstract:

The *Distal-less homeobox (Dlx)* genes encode a group of transcription factors that are involved in early vertebrate development of limbs, sensory organs, branchial arches and the forebrain. In the forebrain, four *Dlx* genes, *Dlx1, Dlx2, Dlx5, Dlx6,* play essential role in the differentiation and proper migration of GABAergic interneurons to the cortex. *Dlx* genes are organized in convergently transcribed bigene clusters and each cluster includes a short intergenic region harboring *cis*-regulatory elements (CREs): specifically, the *Dlx1/2* cluster includes I12b and I12a CREs, while *Dlx5/6* harbors I56i and I56ii. In an effort to determine the regulatory role of the CREs on *Dlx* expression and forebrain development, I characterized mice with an I56i deletion and both I12b and I56i deletions. At late embryonic stage (E18.5) and the adult stage (P35), both mutants had similar expression levels of *Dlx2* and *Gad2* gene, encoding enzyme glutamic acid decarboxylase that is responsible for synthesis of GABA. Mutant mice showed impaired expression levels of *Dlx5*. The expression levels of *Gad1* were decreased in ΔI56i mutants but increased in ΔI12b/I56i mutants at E18.5, and both adult mutants had comparable expression of *Gad1* as wildtype mice. Together with previous *in situ* hybridization results of mice at earlier stages (E11.5, E14.5), my data show that *Dlx* CREs have different levels of activity in regulating the expression of *Dlx* genes at different developmental stages. The mutations of I56i and I12b CREs did not affect the development of two subtypes of GABAergic neurons (calbindin and calretinin expressing neurons) in the forebrain. Compared to wildtype mice, both mutants had hypersociability and deficits of memory and learning ability. This opens the possibility that the deletions of *Dlx* intergenic CREs caused some developmental abnormalities and, therefore affected the behavior of the mice. Through studying the mice lacking *Dlx* intergenic CREs, I gained a better understanding of the role that I56i and I12b play in the regulation of the expression of *Dlx* genes, the development of GABAergic neurons, and the social and cognitive behavior.

Résumé

La famille de gènes à homéoboîte apparentés à *Distal-less*, les gènes *Dlx*, codent pour un groupe de régulateurs transcriptionnels impliqués dans le développement des membres, des organes sensoriels, des arcs branchiaux et du cerveau antérieur. Dans le cerveau antérieur, 4 gènes *Dlx*, *Dlx1*, *Dlx2*, *Dlx5*, et *Dlx6*, jouent un rôle essentiel dans la différenciation et la migration vers le cortex des interneurones GABAergiques. Les gènes *Dlx* sont organisés physiquement en paires de gènes (bi-gène) dont la transcription est convergente et chaque bi-gène comporte une courte région intergénique qui contient des éléments de régulation agissant en cis (CREs). Plus spécifiquement, le bi-gène *Dlx1/2* contient les CRES I12a et I12b alors que le bi-gène *Dlx5/6* contient les CREs I56i et I56ii. Dans le but de déterminer le rôle régulateur des CREs dans l'expression des *Dlx* et le développement du cerveau antérieur, j'ai caractérisé le phénotype de souris portant des mutations qui causent la perte de I56i (ΔI56i) ou une perte combinée deI56i et de I12b (ΔI56i/ΔI12b). A des stades embryonnaires tardifs (E18.5) et au stade adulte (P35), les deux lignées de souris mutantes montrent des niveaux d'expression de *Dlx2* comparables à ceux de souris sauvages ainsi que des niveaux normaux d'expression de *Gad2*, un gène qui code pour la glutamate décarboxylase. Les deux lignées de souris mutantes montrent des niveaux d'expression diminués pour *Dlx5*. Les niveaux d'expression de *Gad1* étaient réduits à E18.5 chez les souris ΔI56i mais augmentés chez les souris ΔI56i/ΔI12b. Les adultes des deux lignées de mutants avaient des niveaux normaux de *Gad1*. Combinés à des études antérieures faites à des stades embryonnaires plus précoces (E11.5, E14.5), mes résultats montrent que les CREs I56i et I12b ont des impacts différents sur l'expression des *Dlx*, selon le stade de développement. La perte des CREs I56i et I12b n'affecte pas le développement de deux populations d'interneurones GABAergiques dans le cerveau antérieur, ceux exprimant la calbindine et la calrétinine. Comparées aux souris sauvages, les souris des deux lignées de mutants montrent des signes d'hyper-sociabilité et des déficits de mémoire et d'apprentissage. Ceci suggère la possibilité que la perte des CREs intergéniques affecte le développement du cerveau antérieur et, par conséquent, le

comportement de la souris. Par cette étude de souris ayant perdu les CREs intergéniques I56i et I12b, nous avons acquis une meilleure connaissance de leur rôle dans le contrôle de l’expression des gènes *Dlx*, dans le développement des neurones GABAergiques et dans le comportement social et cognitif.

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Abbreviations and acronyms

ASD: autism spectrum disorder

BAC: bacterial artificial chromosomes

bp: base pairs

BMP: bone morphogenetic protein

CNS: central nervous system

CGE: caudal ganglionic eminence

Dll: *Distal-less*

Dlx: *Distal-less homeobox*

E18.5: embryonic development day 18.5 after conception

ES: embryonic stem

FBS: fetal bovine serum

FGF: fibroblast growth factor

GABA:γ-aminobutyric acid

Gad: glutamic acid decarboxylase

GE: ganglionic eminence

Gtf2i: general transcription factor 2I

HTH: helix-turn-helix

LGE: lateral ganglioniceminence

Mash1: *mammalian achaete scute homolog-1*

MGE: medial ganglionic eminence

MZ: mantle zone

NPY: neuropeptide Y

nNos: neuronal nitric oxide synthase

PBS: phosphate buffered saline

PFA: paraformaldehyde

PNS: peripheral nervous system

P35: postnatal day 35 after birth

RNA: ribonucleic acid

Shh: sonic hedgehog

SNP: single nucleotide polymorphism

SOM: somatostatin

Spt: septum

Str: stritum

SVZ: subventricular zone

URE1: upstream regulatory element 1

URE2: upstream regulatory element 2

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

VZ: ventricular zone

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1 Introduction

1.1 Vertebrate neurogenesis

Neurons are a type of specialized cells, also known as nerve cells. A typical neuron consists of a cell body, dendrites, and an axon (also known as a nerve fiber). With their axons, neurons are able to transmit information to other cells through electrical or chemical connections, known as synapses. There are hundreds of different types of neurons in the human body and each operates in a different manner. The three most common neurons are (1) sensory neurons, (2) motor neurons and (3) interneurons. As their names suggest, sensory neurons transform stimuli that affect sensory organs into neural signals and send them to the CNS (central nervous system), while motor neurons receive signals from the CNS and activate muscle contractions. In turn, interneurons make connections between neurons within the same region of the brain or spinal cord. Besides neurons, there is another category of cells in the nervous system that are called glial cells. These cells play a variety of functions, including supporting neurons structurally, supplying nutrients to neurons, maintaining homeostasis, guiding axons to the target cells, and producing myelin for more efficient transmission. In the human brain, the number of glial cells is approximately the same as the number of neurons (Allen & Barres, 2009).

The synapse is the junction across which a nerve impulse passes from one nerve cell to another nerve cell, a muscle cell or a gland cell. There are two fundamental types of synapses: (1) electrical synapse and (2) chemical synapse. In an electrical synapse, the presynaptic neuron and the postsynaptic neuron are connected by gap junctions, which pass an electric current and induce voltage change in the postsynaptic cell. This type of synapse represents a rapid way to transmit signals (Ruppert, Fox & Barnes, 2004). Meanwhile, in a chemical synapse, the presynaptic neuron contains a large number of synaptic vesicles which are packed with a chemical called a neurotransmitter, and once the presynaptic neurons are electrically stimulated, the neurotransmitters are released and bind to the receptors located on the postsynaptic neuron. In principle, the type of activated state that a neuron exerts usually depends on the type of receptors it activates (Siegelbaum & Hudspeth, 2000). However, in some cases, the final result also depends on the type of neurotransmitter produced by the neurons. For instance, glutamate always causes excitatory or modulatory effects, while gamma-amino butyric acid or GABA consistently leads to inhibitory effects. For these reasons, glutamatergic cells which are neurons that produce glutamate, are then usually referred to as "excitatory neurons", while GABAergic cells which are neurons that produce GABA, are often referred to as "inhibitory neurons".

In vertebrates, the embryonic neural development starts from stem cell precursors producing new neurons, followed by the differentiation of these nerve cells. As development proceeds, these nerve cells migrate from their birthplace to their final destinations. Once the neurons become mature, they are capable of generating synapses with other neurons through their axons, and finally lead to the formation of mature neural networks (Siegelbaum & Hudspeth, 2000).

1.2 The nervous system of vertebrates

Nervous systems are found in all animals that are more evolved than sponges. It is an important network that is used to control the body by transmitting signals between different cells or body parts. At the very early embryonic stage, all bilaterians (a major group of animals with a two-fold symmetry that gives them front and rear, and left and right body structures) form a gastrula, consisting of three layers of cells, named, from the inner layer to the outer layer, as endoderm, mesoderm and ectoderm (Sanes, Reh & Harris, 2006). In order to initiate the differentiation of neural tissue, inhibition of the gene for bone morphogenetic protein (BMP) especially BMP4 is required. The two proteins (Noggin and Chordin) which are secreted by the mesoderm are able to induce the ectoderm by inhibiting the gene for BMP4, and transform it into neural tissue, which will eventually give rise to the skin and the nervous system. In vertebrates, the development of the nervous system begins with the formation of the neural plate, which is assembled by ectodermal cells along the center of the dorsal surface of the embryo. As development proceeds, the inner part of the neural plate becomes the central nervous system (CNS), and the outer part becomes the peripheral nervous system (PNS). The CNS encompasses the brain and spinal cord, while the PNS includes mainly axon bundles (nerves), which connect the CNS to every other part of the body (Sanes et al., 2006).

The main role of the nervous system is to make communication between different body parts by sending signals in a very unique way, more specifically, from neurons in the nervous system that radiate their axons to the appointed zones and connect with the appointed target cells. The great advantage of this signaling mode is the high speed, which can be as fast as 100 meters per second (Siegelbaum & Hudspeth, 2000).

The nervous system can also act as a stimulus-response associator (Sherrington, 1906), as described above in the introduction of sensory neurons and motor neurons. However, since the nervous system has become more complex as animals evolved, a series of complicated behaviors have been achieved, such as social interactions, precise coordination of different organs, and handling of simultaneous signals. In humans, the nervous system has given us even more complex and precise abilities, such as the skills to solve mathematical problems, summarize concepts, and learn different languages (Siegelbaum & Hudspeth, 2000).

1.3 Development of the mammalian brain

As a critical organ that acts as the center of the nervous system in all vertebrate animals, the brain has been studied extensively since the last century, especially in the field of neuroscience. In vertebrates, after the formation, the neural plate then folds inward and a hollow cord-like structure emerges, named the neural tube which eventually produces the top three segments of the brain: (1) forebrain, (2) midbrain and (3) hindbrain (Fig. 1.1). During the next set of divisions, the forebrain splits into the telencephalon and the diencephalon which contains the thalamus and hypothalamus. Meanwhile, the hindbrain divides into the metencephalon and the myelencephalon (Purves & Lichtman, 1985). Instead of splitting into other brain structures, the midbrain remains undivided during the neural development (Fig. 1.1).

The current thesis is focusing on the telencephalic region, which is responsible of high-level neural functions, including communication, learning, voluntary movement and memory (Roth & Wullimann, 2001; Zaki, Quinn & Price, 2003). During brain development, the telencephalon subdivides into different regional territories after the closure of the anterior neuropore when telencephalic cells have migrated from the anterior zone to more ventral zones. Two discrete proliferative zones with their own regionally-expressing markers have been identified: (1) dorsal telencephalon and (2) ventral telencephalon. The dorsal telencephalon expresses *Emx-1*, *Emx-2* and *Pax-6* genes while the ventral telencephalon expresses *Dlx* and *Nkx* genes, to name only a few. Previous studies have shown that these genes play an important role in patterning the regional territories as well as maintaining the segregation between the two parts of the telencephalon (Fishell, 1997). Ultimately, the two portions of the telencephalon will turn into five domains: (1) cortex, (2) striatum, (3) pallidum, (4) septum and (5) limbic system (Fig. 1.2 A). The current study mainly focuses on the first two.

The ventral telencephalon (ventricular zone) is generally referred to as the lateral and medial ganglionic eminences (Fishell, 1997; Fig. 1.2 A). A ganglionic eminence is a temporary brain structure that guides cell migration and is only present during the embryonic and fetal stages (Encha-Razavi & Sonigo, 2003). Right next to the ventricular zone (VZ), there is a permanent structure called the subventricular zone (SVZ) that can persist throughout a lifespan, and act as a site of neurogenesis and self-renewing neurons in the adult brain (Lim & Alvarez-Buylla, 1999) (Fig. 1.2 A). During embryonic development, cell migration is an important basic process for all cells, including neurons that ultimately reside in a different location from where they originated after the formation of the CNS. More specifically, the birth of most neurons generally happens near the ventricle, but the neurons will eventually reside in the outer surface of the CNS. There are two ways for neurons to attain their final destination: (1) radially or (2) tangentially (Fig. 1.2 B).Those that originate from the ventricular zone of the pallium will migrate radially, in parallel to radial glial fibers, and will finally get integrated into the glutamatergic pyramidal neurons. However, those that eventually give rise to GABA-producing local circuit neurons, migrate tangentially, orthogonal to the radial axis of the neural tube, from the ventricular zone of the subpallium to the dorsal cortex and hippocampus (Ghanem et al., 2007; Marín & Rubenstein, 2001) (Fig. 1.2B). Since the ganglionic eminences facilitate the tangential migration during the development, they play a key role in building up the final GABAergic cortical cell population (Encha-Razav i& Sonigo, 2003; Ghashghaei, Lai & Anton, 2007; Hernández-Miranda, Parnavelas & Chiara, 2010).

Figure 1.1: Schematic representation of the developing mammalian brain.

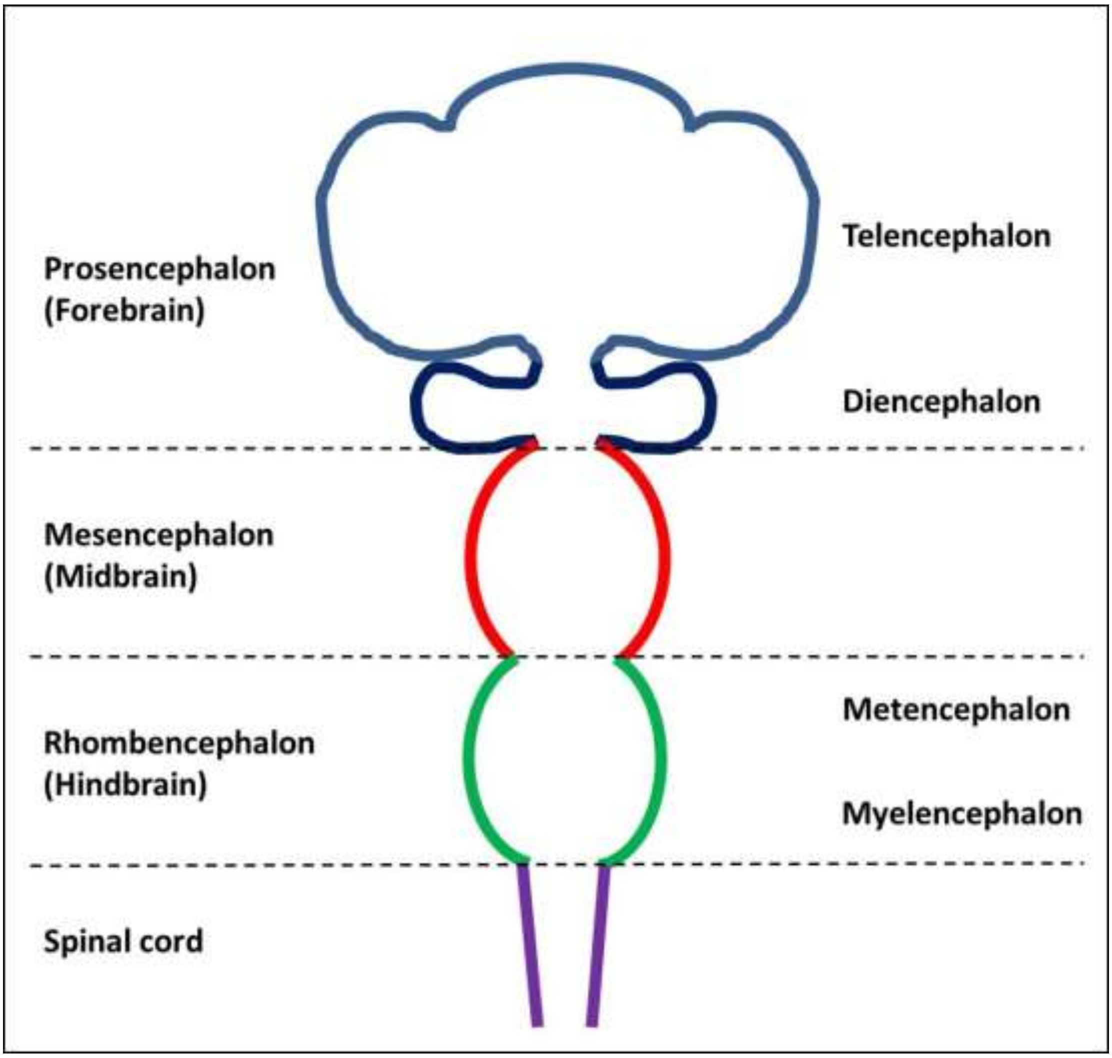
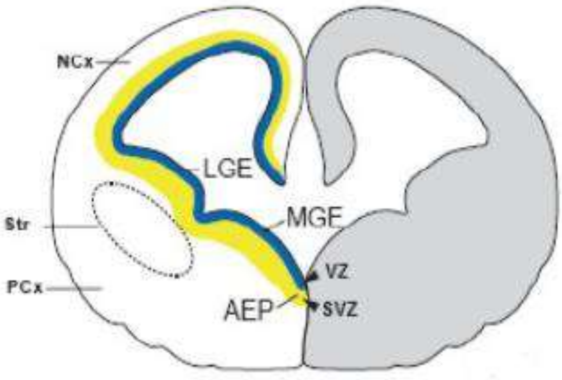

Prosencephalon
(Forebrain)
Telencephalon
Diencephalon
Mesencephalon
(Midbrain)
Rhombencephalon
(Hindbrain)
Metencephalon
Myelencephalon
Spinal cord

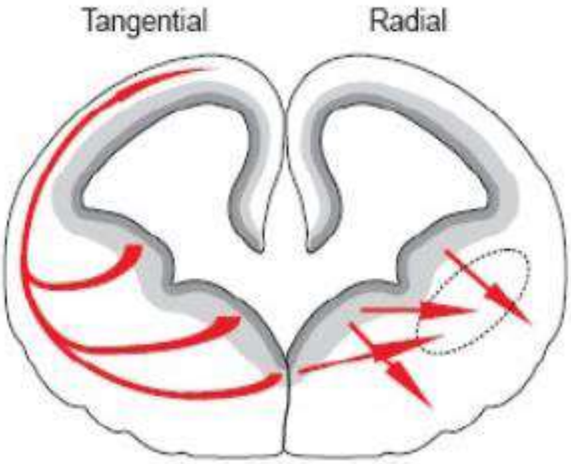
Figure 1.2: Coronal cross sections of a basal telencephalon of an E12.5 mouse embryo. (A) Structures of the telencephalon **(B)** Radial and tangential neuronal migration pattern during brain development (Courtesy of Cindy Pelletier 2011).

A)



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

B)



1.4 GABAergic neurons

GABAergic neurons, a type of inhibitory interneurons that use GABA as their neurotransmitter, are found in the mature neocortex along with the cooperation of glutamatergic pyramidal neurons (Marín & Rubenstein, 2001). Interestingly, before the brain enters into its mature stage, GABA works as an excitatory neurotransmitter until the polarity shift from depolarizing to hyperpolarizing as a result of a change in the concentration of intracellular chloride ions (Ben-Ari, 2002; Ben-Ari, Khaliov, Kahle & Cherubini, 2012). As a neurotransmitter, GABA is synthesized by the enzyme glutamic acid decarboxylase (Gad), which has two isoforms: (1) Gad67 and (2) Gad65, also known as Gad1 and Gad2 respectively. GABAergic neurons are derived from the ganglionic eminences, later migrate to the olfactory bulb, cortex, hippocampus and striatum. They make up about 20% of the neuron population within the cerebral cortex and hippocampus and 95% of the neuron population within the striatum (Anderson, Eisenstat, Shi & Rubenstein 1997a; Potter et al., 2009). Due to their remarkable diversity in morphology, synaptic connectivity, electrophysiology and neurochemistry, GABAergic neurons can be classified into many different groups.

For instance, according to their synaptic profiles, there are three categories of GABAergic neurons: (1) the basket cells, which have axo-somatic inhibitory synapses and the name originate from the basket-shaped arrangement of their terminal boutons. These neurons together with neurogliform cells, are the only cortical interneurons that have been found to receive direct thalamic inputs. (2) Chandelier cells have axo-axonal inhibitory synapses and their axonal branches extend vertically from the cell body to form "candles". Finally, (3) double bouquet cells and axon tuft cells have axo-dendritic inhibitory synapses that provide inhibitory input to both basket neurons and chandelier cells. GABAergic neurons can also be categorized based on their expression of neurochemical markers, including calcium-binding proteins, (e.g., calbindin(CB), calretinin(CR) and parvabumin(PVB)), neuropeptides(e.g., somatostatin(SOM) and neuropeptide Y(NPY)), and neurotransmitters(e.g., neuronal nitric oxide synthase(nNos)) (Markram et al., 2004).

Functionally, at their most basic level, GABAergic neurons manage neuronal activity within the nervous system. However, at a more integrative level, these neurons also play a critical role in the formulation of complex behaviors (Benes & Berretta, 2001; Potter et al., 2009). Dysfunction of GABAergic neurons has been associated to a number of neurodevelopmental diseases, such as epilepsy, schizophrenia and autism spectrum disorder, Tourette syndrome and anxiety.

1.5 *Dlx* genes

1.5.1 *Homeobox* genes

Homeobox genes are a group of genes containing a unique structure called the *homeobox*, which is a 180-bp stretch of DNA, encoding for a homeodomain that consists of a 60-amino acid helix-turn-helix (HTH) structure. The *Homeobox* was first identified in *Drosophila* species and later proved to be conserved in many other animals, including vertebrates (Gehring, 1992; Schofield, 1987; Scott, Tamkun & Hartzell, 1989). Homeodomain proteins are transcription factors that can interact with DNA and thereby regulate the expression of target genes. During embryogenesis, *homeobox* genes are critical in the organization of the body plan and the differentiation of individual tissues (Alberts et al., 2007).

1.5.2 *Dll* gene

The *Dll* (*Distal-less*) gene is the earliest gene expressed in the developing insect limb, and its expression persists throughout the entire embryonic development (Dey, Zhao, Popo-Ola & Campos, 2009). In *Drosophila*, the homeodomain-containing transcription factor that is encoded by the *Dll* gene is required for the formation of the proximal-distal axis pattern and the ventral appendages, including the distal segments of the legs and the antenna (Dey, Zhao, Popo-Ola & Campos, 2009). *Drosophila Dll* mutants die before birth because of the deficiency of the rudimentary larval limbs (Cohen & Jürgens, 1989). Besides these two limb-derived structures, the *Dll* gene also regulates mouthparts (Cohen and Jürgens, 1989).

Dlx (*distal-less homeobox*) genes, which are found in vertebrates, are paralogous genes that have high degree of similarities with the *Dll* gene. They both have major roles in the development of the nervous system. The *Dll* gene is a critical element for the formation of peripheral nervous system, whereas *Dlx* genes are required for the development of the forebrain (Panganiban & Rubenstein, 2002). So far, only one *Dll* gene has been found in the invertebrates, whereas, six *Dlx* genes have been identified in most vertebrates. They are *Dlx1*, *Dlx2*, *Dlx3*, *Dlx4*, *Dlx5* and *Dlx6* (Zerucha & Ekker, 2000).

1.5.3 *Dlx* genomic organization

Dlx genes have been identified in all chordate phylae and have been shown to play a key role in the development of the forebrain, branchial arches, sensory organs and limbs (Zerucha & Ekker, 2000). In mammals, the six *Dlx* genes have been found to be arranged in three pairs: (1) *Dlx1* /*Dlx2*, (2) *Dlx3* / *Dlx4*, (3) *Dlx5* / *Dlx6* (Fig. 1.3). Each *Dlx* gene has a common structure: 3 exons and 2 introns, and the *homeobox* is split between exons 2 and 3 (Panganiban & Rubenstein, 2002). Each pair is located on a distinct chromosome that also includes a *Hox* cluster. In humans, the *Dlx1*/*Dlx2*, *Dlx3/Dlx4, Dlx5/Dlx6* pairs are located on chromosome 2, 11 and 7 respectively, with the *HOX-D*, *HOX-B* and *HOX-A* cluster nearby respectively (Merlo et al., 2004). This *Hox* cluster-containing arrangement has been found to be the result of an initial tandem gene duplication event that has happened prior to the emergence of vertebrates, and during which *Hox* genes were implicated (Amores et al., 1998; Stock et al., 1996). There is a short intergenic (3.5-16kb) region between each pair of *Dlx* genes that harbors various *cis*-regulatory elements (Ghanem et al., 2003).

Because of its highly conservative genomic organization, the *Dlx* gene family has become the ideal model to study gene regulation and divergent development between paralogs (Ellies et al., 1997).

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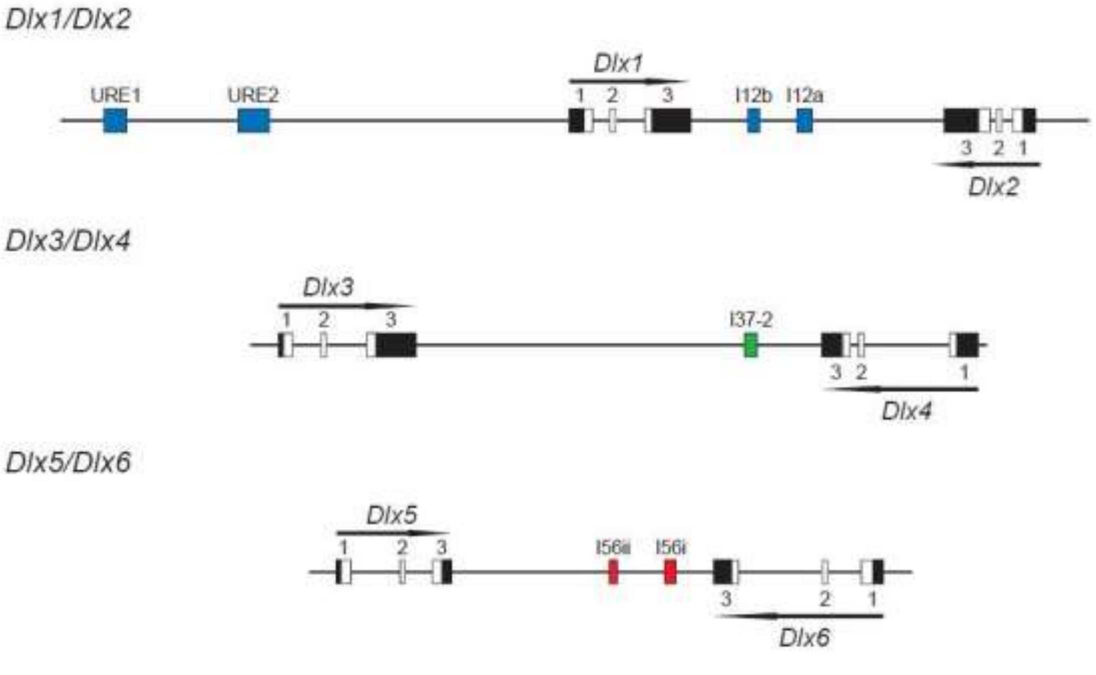
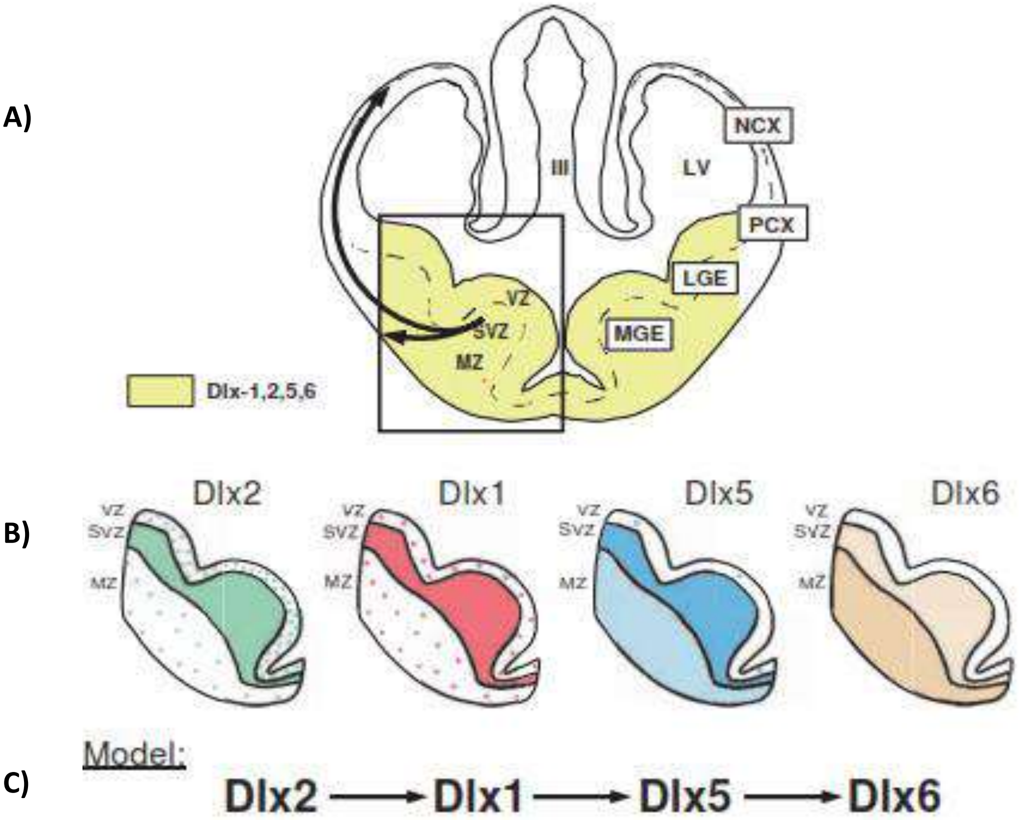
Figure 1.3: Genomic organization of the vertebrate *Dlx* genes. The numbers represent the exons, coding regions are shown in white boxes while non-coding regions are represented by black boxes. *Dlx cis*-regulatory elements (CREs) between each pair are shown in colored boxes (Courtesy of Dr. Luc Poitras).


Dlx1/Dlx2
Dlx1
URE1
URE2
1
2
3
I12b
I12a
3
2
1
Dlx2
Dlx3/Dlx4
Dlx3
1
2
3
I37-2
3
2
1
Dlx4
Dlx5/Dlx6
Dlx5
1
2
3
I56ii
I56i
3
2
1
Dlx6

1.5.4 The expression of *Dlx* genes

In the mouse, *Dlx* genes are expressed in spatially and temporally restricted patterns in the craniofacial primordia, the distal regions of extending appendages, the CNS and the skeletal tissues. Most of the expression happens during embryonic stages (Merlo et al., 2004). Specific cells, derived from the neural crest express the six *Dlx* genes either during their migration or after migration, and eventually comprise the facial skeleton and connective tissue in branchial arches (Depew & Rubenstein, 2002). Within this region, they are expressed in overlapping patterns along the proximal-distal axis. The expression of *Dlx1* and *Dlx2* covers the entire branchial arches, while that of *Dlx5* and *Dlx6* occurs in the intermediate and distal regions. Meanwhile, *Dlx3* and *Dlx4* are only expressed in the distal regions (Depew et al., 1999; Qiu et al., 1995; Qiu et al., 1997). Besides the branchial arches, four of the six *Dlx* genes (*Dlx1*, *Dlx2*, *Dlx5* and *Dlx6*) are also expressed in the basal telencephalon and diencephalon of the developing forebrain, at different stages of differentiation (Anderson et al., 1997a; Liu, Ghattas, Liu, Chen & Rubenstein, 1997) (Fig. 1.4). Within the basal telencephalon, the expression of *Dlx* genes is found in the lateral and medial ganglionic eminence (LGE and MGE), the preoptic area and the parts of amygdala. More specifically, *Dlx2* is the first *Dlx* gene to be expressed in most undifferentiated proliferative cells found in the ventricular zone (VZ) and also in some cells found in the subventricular zone (SVZ) and mantle zone (MZ). This is followed by the similar expression of *Dlx1* in the same areas. *Dlx5* is mainly expressed in post-mitotic differentiating cells in the SVZ and MZ. Meanwhile, the strongest expression of *Dlx6* is detected in the MZ (Eisenstat et al., 1999; Liu et al., 1997).

Figure 1.4: A) Schematic drawing of a transverse section from an E12.5 mouse telencephalon. The boxed region shows the expression domains of *Dlx* genes in the forebrain. Arrows represent neuronal migration from the subpallium to the cortex (pallium). **B) *Dlx* expression domains in the forebrain**. *Dlx* genes are mainly expressed in uniform colored regions and also in scattered cells. **C) Sequential expression pattern of *Dlx* genes**. *Dlx2* is expressed first, followed by the expression of *Dlx1*, *Dlx5* and finally *Dlx6* (Adapted from Panganiban and Rubenstein 2002).


A)
NCX
III
LV
PCX
LGE
MGE
VZ
SVZ
MZ
Dlx-1,2,5,6
B)
Dlx2
Dlx1
Dlx5
Dlx6
VZ
SVZ
MZ
C)
Model:
Dlx2 → Dlx1 → Dlx5 → Dlx6

1.5.5 *Dlx* function

The homeobox genes in the *Dlx* family have been demonstrated to play a central role in the development of basal forebrain and craniofacial morphogenesis. The analyses of targeted deletions of *Dlx* genes in mice have provided evidences for this (Eisenstat et al., 1999).

Dlx1 null mice are viable at birth, but they are smaller in size compared to their wildtype counterparts and all die within one month. Newborn mice with a *Dlx2* deletion (*Dlx1/Dlx2*$^{+/-}$ and *Dlx1/Dlx2*$^{-/-}$) do not feed, and keep accumulating air in their stomachs and intestines once they are born, and eventually die within a few hours after birth (Qiu et al., 1995; Qiu et al., 1997). All these types of mutants have abnormalities found in bones and cartilage of both the splanchnocranium and dermatocranium. The splanchnocrnium is the part of the skull that consists of the visceral arches composed of cartilage or cartilage replacement bone, while the dermatocranium is the part of the skull composed of plates of dermal bone that cover the head and protect the brain and gills. Both *Dlx1* and *Dlx2* mutations affect the morphogenesis of splanchnocranial structures in the first two arches.

Abnormalities have been observed, for example, in the maxillary derivatives of the first arch and in Reichert's cartilage of the second arch. However, mutations of *Dlx1* and *Dlx2* only affect the proximal regions, which suggest that the other *Dlx* genes that are expressed in the distal parts may show compensation, therefore proving a redundant function among these *Dlx* genes. Meanwhile, only mutants without *Dlx2* have shown alterations of the dermatocranial structures, such as temporal skull wall. These results suggest that *Dlx1* and *Dlx2* genes still have unique functions even though their expression patterns in the branchial arches are similar (Qiu et al., 1997).

Mutations of *Dlx1* and *Dlx2* have also shown some abnormal phenotypes in the forebrain. The dendrite-innervating cortical interneurons have differentiation abnormalities in *Dlx1*$^{-/-}$ mutants (Cobos et al., 2005) while the expression of tyrosine hydroxylase in periglomerular neurons of olfactory bud was also disrupted in *Dlx2*$^{-/-}$ mutants (Qiu et al., 1995). A block in the differentiation of late-born neurons (i.e.

projection neurons that express GABA) is also noticeable within the subcortical telencephalon of *Dlx1/2*$^{-/-}$ mutants. As a result, these partially differentiated neurons fail to migrate, and eventually lead to a massive reduction of the GABAergic neurons in the cerebral cortex. More specifically, a 75% reduction in the neocortex and a virtually complete loss in the hippocampus have been observed (Marín & Rubenstein, 2001; Panganiban & Rubenstein, 2002). These reductions together with the fact that the expression of *Dlx* genes coincides with the location of all neurons using GABA as their neurotransmitter are the evidences of *Dlx* genes involvement in the development of GABAergic neurons (Anderson et al., 1997a; Anderson et al., 1997b; Stühmer, Anderson, Ekker & Rubenstein, 2002).

As for *Dlx5/6*$^{+/-}$ new born mice, they are viable, fertile, and no obvious physical abnormalities seem to exist. However, *Dlx5*$^{-/-}$ and *Dlx5/6*$^{-/-}$ mutants are smaller in size compared to their wild-type littermates when they are born, and they shortly die after birth. *Dlx5*$^{-/-}$ and *Dlx5/6*$^{-/-}$ mutants also exhibit exencephaly, a type of cephalic disorder wherein the brain is located outside the skull. More specifically, these mice lack the calvaria (or skullcap; the upper part of the skull), which is caused by the absent fusion of the dorsal midline of the rostral neural tube (Depew et al., 1999; Depew & Rubenstein, 2002; Robledo, Rajan, Li & Lufkin, 2002). Moreover, the ablation of either *Dlx5* or *Dlx6* also causes some obvert craniofacial defects. In *Dlx5*$^{-/-}$ mutants, defects include the absence of a coronoid process at the proximal end of dentary, the shortening of Meckel's cartilage and abnormal morphogenesis of the olfactory pit and inner ear. Similarly, *Dlx6*$^{-/-}$ mice have abnormalities in the structures derived from the proximal end of the mandibular arch and also show other defects such as a deviated Meckel's cartilage and the absence of an extended superior cornu (Depew, Simpson, Morasso & Rubenstein, 2005). Likewise, mice with deletion of both *Dlx5* and *Dlx6* demonstrate severe limb, craniofacial, and axial skeletal defects. For example, they have smaller eyes, kinked tail vertebrae, and a transformation of the lower jaws to the upper jaws (Depew & Rubenstein, 2002; Robledo et al., 2002). Additionally, *Dlx5/6*$^{-/-}$ mutants have split distal limb defects that are the equivalent of the split-hand/foot malformation (SHFM) in humans. More specifically, all combinations of the central digits are absent from the disrupted development of apical ectodermal ridge (AER) of the limbs. This abnormal limb phenotype represents the first evidence of mammalian *Dlx* genes are involved in limb development (Robledo et al., 2002).

Within the telencephalon, the loss of *Dlx5* gene expression reduces the number of interneurons that express tyrosine hydroxylase and GABA in the olfactory bulb as well as in mature neurons that express parvalbumin. Meanwhile, *Dlx6* mutants have some molecular defects, such as the reduced expression of *Dlx5* in the central nucleus of the amygdala. In addition, *Dlx5/6*$^{-/-}$mutants not only show a reduction in the number of mature parvalbumin$^+$ neurons, but also a reduced efficiency in the tangential migration of interneurons (Anderson et al., 1997a; Marín & Rubenstein 2001; Wang et al., 2010; Wang, Lufkin & Rubenstein, 2011). All of these examples prove that *Dlx* genes are critical for the development of the CNS.

1.5.6 *Dlx* regulation

Various molecular factors have been identified in regards to the mechanisms responsible for the regulation of *Dlx* gene expression in vertebrates. For instance, the Bone Morphogenetic Protein 2 (BMP2) can induce the expression of *Dlx2* in chondrocytes (Xu, Harris, Rubenstein, Mundy & Harris, 2001), while the Bone Morphogenetic Protein 4 (BMP4) can induce the expression of *Dlx1* and *Dlx2* in dental mesenchyme (Bei & Maas, 1998) and the expression of *Dlx5* in osteoblasts (Miyama et al., 1999). Additionally, treatments with retinoic acid (RA) can transiently induce the expression of the human *Dlx5* and *Dlx6* genes. Other molecules such as sonic hedgehog (Shh) and fibroblast growth factors (FGFs) have also shown their roles in maintaining *Dlx* expression (Ferrari, Harrington, Dealy & Kosher, 1999; Gaiano, Kohtz, Turnbull & Fishell, 1999; Mullen et al., 1996; Zerucha & Ekker, 2000).

As mentioned in sections above, there are also some cis-acting regulatory elements (CREs) located in the intergenic regions between the two linked *Dlx* genes that are capable of regulating *Dlx* expression once they are associated with promoters. The fact that the two members from the same *Dlx* bigene cluster share the same cis-regulatory elements may in fact contribute to their overlapping patterns of expression (Ghanem et al., 2003; Zerucha & Ekker, 2000). In the mouse, two enhancers, I12a and I12b, are identified as the two cis-regulatory elements of the *Dlx1/2* bigene cluster (Fig. 1.5). I12a is about 550 bp in length and the percentage of similarity between the mouse and human sequences is 99%. It can drive the expression of the *lacZ* reporter gene in the mandibular component of the first arch and in the hyoid arch, starting at E9.5 and finishing at around E16. I12b is mainly active in the forebrain (Fig. 1.6). It is 400 bp in length and show 97% sequence identity between human and mouse. Similarly, the *Dlx5* and *Dlx6* cluster harbors two enhancers as well (Fig. 1.5), named as I56i and I56ii. Their sizes are 440 bp and 310 bp respectively, and the percentages in sequence identity between mice and humans are 99% for both enhancers (Ghanem et al., 2003; Zerucha & Ekker, 2000). Additionally, *lacZ* reporter gene constructs using these enhancers are expressed in the telencephalon and diencephalon starting at E10 and persisting in adulthood (Ghanem et al., 2003; Fig. 1.6). All of the four well-conserved enhancers (i.e., I12a, I12b, I56i, and I56ii) have a really high degree of sequence identity among different vertebrate species, even between two distantly related species, such as human and zebrafish. However, the sequence similarity is much more limited when they are compared to each other. For instance, although three CREs, I12b, I56i and I56ii have highly overlapping patterns of activity in the forebrain, I12b has almost no similarity in DNA sequence to the two enhancers, I56i and I56ii. The *lacZ* reporter genes they drive show indistinguishable expression in some regions (including the SVZ, mantle zone (MZ), the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE)) of the subpallial telencephalon and diencephalon at E11.5 and E12.5 (Fig. 1.6). These reporter transgenes are also coexpressed in tangentially migrating cells, which suggest that even though their DNA sequence are largely different, I12b and I56i may respond to similar transcriptional factors (Ghanem et al., 2003; Ghanem et al., 2007).

Other than these four enhancers, another two CREs that control *Dlx1* and *Dlx2* expression have also been identified: URE1 and URE2 (Fig. 1.3). They are respectively located at around 17 kb and 12 kb upstream of the *Dlx1* transcriptional start site. Similar to I12b, I56i and I56ii, URE2 is mainly active in the forebrain (Fig. 1.6). In transgenic animals, *lacZ* reporter expression driven by URE2 is restricted to the subpallial telencephalon, the prethalamus, and the hypothalamus, but is also present in the hyoid arch, the somites, and the apical ectodermal ridge of the limbs. Unlike URE2, URE1 has activity in the retina only, but not in the forebrain (Ghanem et al., 2007).

Via these CREs, *Dlx* genes are able to perform some cross-regulatory interactions (Fig. 1.5). For example, *Dlx1* and *Dlx2* proteins can induce the expression of *Dlx5* and *Dlx6* through the I56i enhancer. This regulating pattern has been shown in Dlx1/2$^{-/-}$ mutants. In these mice, most of *Dlx5* and *Dlx6* expression in the telencephalon is lost (Zerucha et al., 2000). On the other hand, *Dlx5* and *Dlx6* products act as regulators to activate downstream targets including glutamic acid decarboxylase *(Gad)* through interactions with the *Gad* enhancer region. *Gad* is the enzyme responsible for the synthesis of GABA (Anderson et al., 1997a).

To gain a better characterization of the roles that intergenic CREs have in the development of forebrain, previous members of the Ekker laboratory produced mice that had mutations on the I12b, I56i and I56ii enhancer. They undertook the phenotype characterization of these mice using a series of experimental methods, including q-RT PCR, *in situ* hybridization, immunohistochemistry and behavior tests. At E13.5, Δ I12b mutants (mice without the I12b enhancer) had decreased levels of expression of *Dlx1* and *Dlx2*, but seemingly normal expression of *Dlx5*, *Dlx6* and *Gad2* in the telencephalon. The number of GABAergic interneurons in the somatosensory cortex did not seem to be affected compared to wildtype mice (Darbandi, 2014). At the same stage, deleting I56ii reduced the expression levels of *Dlx5*, *Dlx6* and *Gad2* but had no clear impact on the expression of *Dlx2*. It also caused a reduction in the number of GABAergic neurons in the somatosensory cortex at E13.5 (Darbandi, 2016).

The vI56i mutants had an adenine-to guanine single-nucleotide polymorphism (SNP) at position of 182 of I56i. This SNP location was found to be part of a completely conserved 8 bp motif between 40 vertebrate genomes. Since the I56i enhancer is an ultraconserved element between mouse and human (99% of sequence similarity), this 182-SNP is possible to affect the transcriptional regulation of *Dlx5/Dlx6* through impacting the activity of I56i enhancer. Lesage-Pelletier (2011) tested this possibility. At E11.5 and E13.5, she observed a decrease in the expression of *Dlx5* and *Dlx6* in the telencephalon of vI56i homozygous mice compared to wildtype mice (Lesage-Pelletier, 2011). Additionally, Poitras et al. (2010) found that the 182-SNP caused a reduced affinity of Dlx proteins binding to I56i enhancer. In an affinity purification experiment using the central portion of the I56i enhancer, the most abundant protein isolated was Gtf2i, which was identified as a new regulator of *Dlx5/Dlx6* cluster (more information about Gtf2i is in the introduction below) (Poitras et al., 2010).

Mice with both 182-SNP on I56i enhancer and deletion of I12b enhancer (vI56i/ △I12b) had nearly half reduction in the expression of *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* in the telencephalon at E14.5 (Esau, 2013).

Another mutant that has been characterized is ΔI56i. At E13.5, the absence of I56i significantly decreased the expression of *Dlx5*, *Dlx6*, *Gad1* and *Gad2* in the telencephalon, but caused an increase in the expression of *Dlx1* and no observable change in the expression of *Dlx2*. At this stage, ΔI56i mutants had a marked reduction of number of GABAergic neurons in the somatosensory cortex, but at P35, mutants had comparable expression of GABAergic neuron markers as wildtype mice. Among all these mutants, only the deletion of I56i led to some statistically significant behavioral consequences, such as hypersociability, impaired learning ability and memory (Esau, 2013).

Besides *cis*-regulatory elements, some *trans*-acting factors have also been demonstrated as critical modulators in the expression of *Dlx* genes. For instance, the *Mammalian achaete scute homolog-1(Mash1)* (Fig. 1.5), which is located upstream of *Dlx1/Dlx2*, have been shown to regulate the transcriptions of *Dlx1/Dlx2* through interacting with I12b enhancer (Poitras, Ghanem, Hatch & Ekker, 2007; Porteus et al., 1994).

Another potential regulator of *Dlx* genes is general transcription 2i (Gtf2i). Previous studies have demonstrated that it can synergize with *Dlx2* and *Dlx5* to activate transcription through the I56i enhancer. Co-transfection of Gtf2i with *Dlx2* and *Dlx5* expression plasmids increased the transcriptional activity by 38% and 24% respectively (Poitras et al., 2010). Gtf2i is one of candidate genes for Williams-Beuren syndrome (WBS), which is a neurodevelopmental disorder caused by a recurrent deletion of 1.55Mb including 26-28 genes at chromosomal region 7q11.23 (Antonell et al., 2010). Notably, three of these genes are from the GTF2 family: Gtf2i, Gtf2RD1 and Gtf2iRD2. WBS patients have specific facial features and multiple problems that affect many parts of the body, such as teeth, heart, and supravalvular aortic stenosis. WBS patients are not able to do visual spatial tasks such as drawing or communicate normally because of their intellectual disability (Morris et al., 2013). Socially, these patients are often overly friendly and trust strangers easily. Interestingly, this lacking-shyness behavioral phenotype was also observed in Gtf2i heterozygous mice (Sakurai et al., 2011). These mice showed singularly active interactions with others and lacked typical social habituation processes, suggesting the lower-dosage of Gtf2i gene expression would lead to an increase in social interactions. However, even though they are highly sociable, nearly half of WBS patients have severe social skills problems as seen in people with autism spectrum diseases (ASDs), finally leading to social isolation and loneliness (Kaplan, Wang & Francke, 2001; Malenfant et al., 2012). Further studies have supported evidence that Gtf2i is indeed involved in the etiology of ASDs (Kaplan, Wang & Francke, 2001).

Figure 1.5: Proposed schematic diagram of the *Dlx* gene regulatory network. *Mash1* regulates transcription of *Dlx1/Dlx2* through its interaction with I12b enhancer. By binding to I56i enhancer, *Dlx1*/*Dlx2* proteins initiate the transcription of *Dlx5/Dlx6. Dlx5/Dlx6* transcripts initiate the transcription of *Gad* via binding to the *Gad* enhancer.

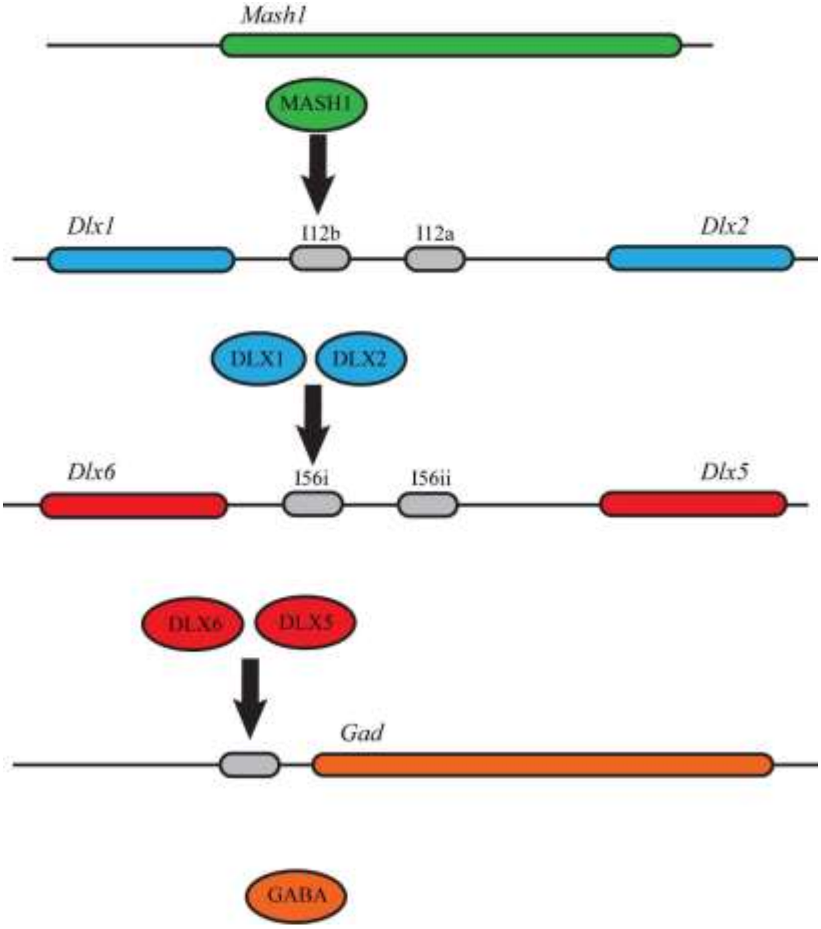
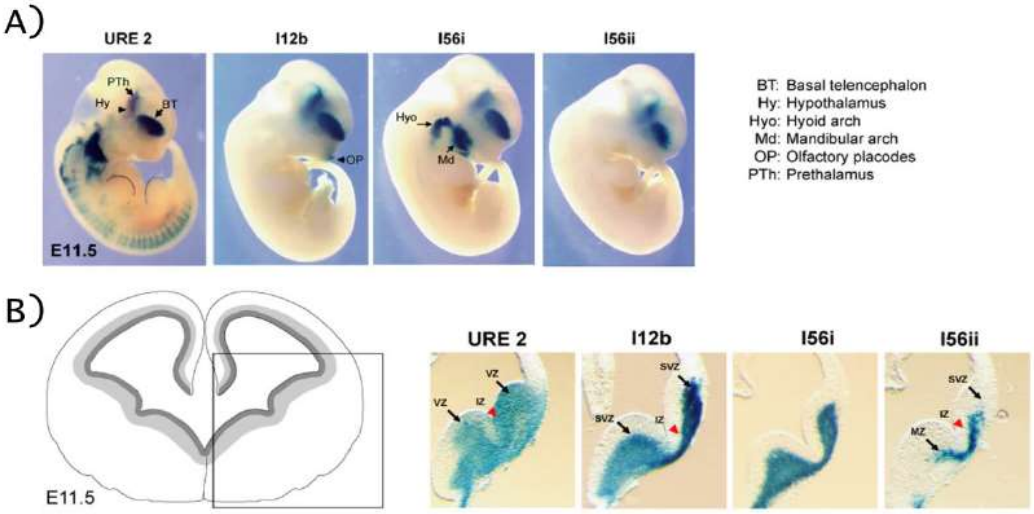

Mash1
MASH1
Dlx1
I12b
I12a
Dlx2
DLX1
DLX2
Dlx6
I56i
I56ii
Dlx5
DLX6
DLX5
Gad
GABA

Figure 1.6: Regions where *Dlx* enhancers are active. (A) The expression of the *lacZ* reporter gene in E11.5 transgenic mouse embryos under the control of URE2, I12b, I56i and I56ii. (**B)** Coronal sections from E11.5 transgenic mouse forebrain show the active domains of URE2, I12b, I56i and I56ii (Ghanem et al. 2008).


A)
URE 2
I12b
I56i
I56ii
PTh
Hy
BT
OP
Hyo
Md
E11.5
BT: Basal telencephalon
Hy: Hypothalamus
Hyo: Hyoid arch
Md: Mandibular arch
OP: Olfactory placodes
PTh: Prethalamus
B)
E11.5
URE 2
I12b
I56i
I56ii
VZ
VZ
IZ
SVZ
SVZ
IZ
SVZ
IZ
MZ

1.6. Statement of Inquiry

In vertebrates, *Distal-less homeobox* (*Dlx*) genes have been demonstrated to play key roles in the embryonic development of the forebrain, branchial arches, sensory organs and limbs (Zerucha & Ekker, 2000). The dysfunction of *Dlx* genes in the forebrain has been linked to disturbed development of GABAergic neurons, which could become one reason that cause some neurological diseases, such as autism spectrum disorder (ASD), epilepsy and schizophrenia (Poitras et al., 2010).

Several molecular factors including BMP, Shh, FGF and some elements, such as CREs, *Mash1* and Gtf2i have been identified as regulators of the expression of *Dlx* genes in the forebrain. The particularly high degree of sequence similarity of four intergenic CREs (I12a, I12b, I56i and I56ii) among different vertebrate species indicates their importance in the evolution of developmental mechanisms. In order to explore the regulatory mechanisms of these intergenic CREs in the expression of *Dlx* genes, previous members of the Ekker laboratory have generated and begun the characterization of mice with mutations in the intergenic regions, such as ΔI12b, ΔI56ii, vI56i, vI56i/ΔI12b and ΔI56i (as mentioned in section 1.5.6). Even though we did gain a better understanding of the regulatory roles that these CREs play in regulating the expression of *Dlx* genes, their works mainly focused on only two specific developmental stages: the early embryonic stage (E11.5-E14.5) and the adult stage (P35), which left us some peculiar results. For example, former student Crystal Esau has found the deletion of I56i enhancer caused reductions in the number of GABA-expressing cells in the somatosensory cortex compared to wildtype mice at E13.5. However, the adult ΔI56i mutants (P35) had generally comparable expression of GABA in the somatosensory cortex as their wildtype counterparts (Esau, 2013). These CREs have their activity during different developmental stages, for example, I12a is active from E9.5 to E16, while activity of I56i and I56ii is observed starting at E10 and persists in adulthood (Ghanem et al., 2003; Zerucha & Ekker, 2000). In order to learn more about the activity that these CREs have at different developmental stages, I characterized mice with I56i enhancer deletion (ΔI56i) and mice with both I12b and I56i deletions (ΔI12b/I56i), at two specific developmental stages: late embryonic stage (E18.5) and adult stage (P35).

To explore the impact that the enhancer deletions may have on the expression of *Dlx* and *Gad* genes, I performed *in situ* hybridization with antisense RNA probes on forebrain sections of three genotypes of mice (i.e., wildtype, ΔI56i and ΔI12b/I56i mutants) at E18.5 and P35. Immunohistochemistry on the forebrain sections of these mice was applied to test the possibility that enhancer deletions may affect the development of GABAergic neurons at E18.5 and P35, I used antibodies to mark two subpopulations of GABAergic neurons: those that express calbindin and calretinin. Lastly, I carried out juvenile interaction and fear conditioning behavioral tests on adult mice (around three-month old) to examine the impact that developmental abnormalities resulting from enhancer deletions may have on behavior.

2 Materials and methods

2.1 Animal maintenance

Mice were kept in Roger Guindon Hall and Vanier Mouse Facilities at the University of Ottawa, and taken care of by people who have received Animal User Care training program. Their living environment was sterilized and controlled with 12 hour light/dark cycle. All mice were provided with enough water and standard mouse chow. The experimental protocols we used were approved by the University of Ottawa's Animal Care Ethics Committee.

2.2 Generation of mutant mice

The ΔI56i and ΔI12b/I56i mutants were generated at the Transgenic Mouse Core Facility at the McGill Cancer Center. Via homologous recombination, a LoxP-flanked PGK-neomycin-resistant cassette replaced the enhancer sequence of I56i or both I56i and I12b on Bacterial Artificial Chromosomes (BAC) which harbors *Dlx5/Dlx6* and *Dlx1/Dlx2* locus. The BACs with a successful recombination were selected by screening and sequencing, and the desired ones were electroporated into 129SV mouse embryonic stem (ES) cells. The cells containing the mutant BAC were detected using gentamicin, and ES cells were screened for the presence of the neomycin cassette by quantitative real-time PCR. Positive embryonic stem cells were injected into host C57BL/6 blastocysts to generate chimeric mice. These chimeric mice were mated with C57BL/6 mice. Meanwhile, we did genotyping to verify germ-line transmission of the mutant allele was successful. Lastly, mutant mice were mated with Sox-Cre mice in order to remove the neomycin cassette. The homozygous mice with the I56i enhancer deletion were generated by mating heterozygous mice. The mice with both I56i and I12b deletion were generated by mating mice heterozygous for each deletion or mice heterozygous for one deletion but homozygous for another deletion.

2.3 Weaning and ear-tagging

Mouse pups were separated from their mother 21~28 days after birth, and then, individuals of the same gender of pups were housed in the same cage, with a maximum number of mice per cage set at 4. Each pup had an ear tag with a specific number. Before performing genotyping, we cut the tip of their tails to prepare DNA.

2.4 Genomic DNA extraction

A small portion (~0.5 µm) of the tail was clipped as tissue sample, and placed in the solution mix which is comprised of 20 µL of dilution buffer and 0.5µL of DNA release (F140WH, Thermo Fisher) samples were incubated at room temperature for 5 minutes, followed by incubation at 98 ℃ for 2 minutes, and then kept on ice for preparation of genotyping.

2.5 Genotyping

Mice with the I56i enhancer or both I56i and I12b enhancer deletions were screened by polymerase chain reaction (PCR) using a group of primers flanking the enhancer region. Besides 4% DNA template, the master mix for each PCR reaction includes 20% 5X PCR buffer, 2% dNTPs, 2% Taq polymerase, 4% forward and reverse primer and 68% ddH_2O. PCR reaction starts with the denaturation step: 95℃ for 5 min, followed by 95℃ for 30 sec, 58℃ for 45 sec and 72℃ for 45 sec, the three steps above were cycled 39 times, finishing with an extension step at 72℃ for 45 sec and a cool-down step at 4℃. The PCR products were run on a 1% agarose gel and analyzed under an ultraviolet light. The sizes of four enhancers (I12a, I12b, I56i, I56ii) are around 550bp, 400bp, 440bp, 310bp respectively. Without any enhancers deleted, a band that was around 800bp showed in wildtype mice. Homozygous △I56i mutants showed a band that was about 310bp, while a band that was around 550bp was shown in homozygous △I12b mutants. Combined together, △I12b/I56i mutants were homozygous for both I12b and I56i enhancer. The primer sequences used for genotyping are as follows:

ΔI56i.for	CCCCAATGTCTGCTTCAAAT
ΔI56i.rev	GGAAGCCCCATACTGTGAGA
ΔI12b.for	TGAGTCTGTAATGGCAAAATGC
ΔI12b.rev	CAGGTGCAGATTCCCTGAAG

2.6 Tissue collection and sectioning

2.6.1 E18.5 mice brain tissue collection

Mice were arranged to mate in the afternoon, and the female mice were checked for vaginal plugs in the following four mornings. Once a plug was observed, the date was marked as E0.5 day. Female mice that had babies were euthanized using CO_2 followed by cervical dislocation at E18.5. Embryos were removed and placed in cold 1 X PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH=7.4). Tails were cut from embryos and prepared for genotyping, and brains of embryos were dissected and fixed in 4% PFA at 4℃ overnight. On the next day, brains were washed 3 times for 5 min using 1 X PBS and soaked in 20% sucrose in 1 X PBS at 4℃. After 2~3 days, the brains were calibrated in sucrose and ready to be sectioned.

2.6.2 P35 mice brain tissue collection

At 35 days after birth, mice that have the desired genotype were euthanized. We first injected 10μL/g of Euthanyl to the mouse, and then a needle was inserted into the left ventricle once the mouse was determined to be unreactive. 20 mL of 1 X PBS were pumped by syringe into the heart after the right atrium of the heart was punctured, followed by injection of 20 mL of 4% PFA through the same syringe, this operation made brain fixed. Lastly, the brain was dissected from the skull and fixed in 4% PFA at 4℃overnight, and washed 3 times of 5 min in 1 X PBS on the next day, stored in 20% sucrose at 4℃.

2.6.3 Sectioning

Before sectioning, the brains were removed from sucrose and dried gently with a Kimwipe, transferred into a cryomold and gently adjusted to the center with right position, and covered with OCT. Subsequently, we kept the crymold floating on liquid nitrogen until the OCT froze completely, and then the brain was incubated in liquid nitrogen for 2 minutes. Molds were stored in -80℃ until ready for sectioning. Before sectioning, the cryostat (Leica CM1850) was set to -20℃ in order to optimize the quality of sections. Brain samples were allowed to acclimatize to the chamber temperature for 1 hr. Forebrain samples were sectioned to a thickness of 20 µm and collected on Fisher Superfrost slides. These slides were stored in -80℃ until further use.

2.7 *In situ* hybridization (ISH)

2.7.1 Probe synthesis

Antisense probes we used for *in situ* hybridization were synthesized from vector containing cDNA clones. First, vectors need to be linearized using an appropriate enzyme. The full list of vectors and enzymes can be found in table 2.1. A 100 µL of solution containing 5 µg of plasmid DNA, 10µL of the appropriate buffer, 1 µL of enzyme was incubated at the optimal temperature for 1 hour. Then the same volume of enzyme was added and the solution was incubated for another 1 hour. Finally the plasmid was run on 1% agarose gel to check the degree of digestion.

After linearization, DNA was extracted by adding a solution which has 0.1 X volume 3M NaoAc and 1 X volume of phenol-chloroform. This solution was vortexed for 2 min and then centrifuged at maximum speed for 10 min. The upper phase was transferred to a new tube and 1 X volume of chloroform was added, followed by another 2 min of vortexing and 10 min of centrifugation. In order to precipitate the DNA, 0.1 X volume of 3M NaoAc, 2.5 X volume of cold 100% ethanol were added and incubated at -20℃ overnight. On the following day, the DNA was centrifuged at maximum speed (14800 rpm) at 4℃ for 25 min and then washed with 1 mL of cold 70% ethanol. After another centrifugation at 4℃ for 10 min, the ethanol was discarded. The linearized DNA was dissolved in 30 µL of nuclease free water and quantified using the nanodrop spectrophotometry.

Synthesis of digoxygenin-11-UTP-labelled RNA probes was done using 1µg of linearized plasmid that was mixed with 2µL 10X transcription buffer, 2µL DIG-dNTP, 0.5 µL RNase inhibitor, and 1 µL RNA polymerase. Incubation was done at 37℃ for 1 hour, followed by addition of another 1 µL RNA polymerase and incubation for one more hour at 37℃. After the two hours of incubation, 2µL of recombinant DNAse I was added to the solution that was then incubated at 37℃ for 15 min. The probe was purified using 4µL of LiCl as well as 3X volume of cold 100% ethanol, incubated at -20℃ overnight. On the next day, the mix was centrifuged at 4℃ for 20 minutes and washed with 1 mL fresh 70% ethanol. After centrifugation at maximum speed (14800 rpm) at 4℃ for 5 min, the pellet was air dried on ice for a maximum of 4 min and resuspended in 20µL of nuclease-free water. After a 10-min incubation at -20℃, the probes were stored in aliquots at -80℃. Probe quality was verified by running 1µL on agarose gel, and the concentration of probe was measured using a nanodrop spectrophotometer.

Table 2.1 Vectors containing cDNA clones for probe synthesis for *in situ* hybridization.

	Vector	Probe	Linearizing	RNA
cDNA Clone	Backbone	length(kb)	Enzyme	Polymerase
Mouse *Dlx2*	pBS-SK	1.7	*Hind*II	T3
Mouse *Dlx5*	pBS-SK	1.6	*Sma*I	T3
Mouse *Gad1*	pBS-KS	1.7	*Bam*HI	T3
Mouse *Gad2*	pCMV-sport6	2.2	*Hind*III	T7

2.7.2 Hybridization and staining

Slides with forebrain sections were removed from -80℃ and thawed at room temperature for 2 hours. Probes were diluted to a concentration of 0.5μg/μL in 1 mL hybridization buffer which consists of 100 μL of 10 X salt solution (0.2 M MaCl, 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 yeast tRNA (10mg/mL), 20 μL of 50X Denhardts and 80 μL of DEPC treated dH2O. The probes were denatured at 70℃ for 10 minutes. About 300 μL of hybridization solution were added to each slide and a cover slip was placed on the slide to protect sections Slides were incubated in 70℃ overnight.

On the second day, slides were taken out of the humid chamber and placed in Coplin jars, washed 2 X 30 minutes at 70℃ with solution A (5% 20 X SSC, 50% Formamide, 0.1% Tween-20), followed by 2 X 30 minutes with 1 X TBST (140 mM NaCl, 2 mM KCl, 25 mM Tris-HCl pH=7.5, 0.1% Tween-20) at room temperature. Slides were then dried and a line was drawn around the perimeter of the sections, which were then, covered by 300 μL of blocking solution (10% FBS in 1 X TBST) and incubated in a moist chamber at room temperature for 2 hours. Next, 300 μL of blocking solution with 1:1000 dilution of anti-Digoxygenin AP Fab fragment antibody were added on each slide, and then these slides were incubated at 4℃ overnight.

The last day of *in situ* hybridization started with 4 X 20 min washes in 1 X TBST, followed by 2 X 10 min washes in NTMT buffer (100 mM NaCl, 100 mM Tris-HCl pH=9.5, 50 mM $MgCl_2$, and 0.1% Tween-20). After the washes, 300 μL of NTMT staining buffer that contains 1.05 μL of 50 mg/mL BCIP and 2.03 μL of 50 mg/mL NBT were added on each slide, that were then stained 2.5 hours. In order to stop the reaction, slides were washed 2 X 5 min in distilled water. The following step is fixing by adding 300 μL of 4% PFA in 1 X PBS for 20 min at room temperature. Slides were then rinsed with ddH_2O and mounted with aquamount mounting media. Finally slides were stored at 4℃ until imaging.

2.8 Immunohistochemistry (IHC)

Slides were taken out from -80℃ and thawed for 1 hr before use. Slides were placed in a moist chamber and a line around each slide was drawn using a hydrophobic pen. In order to rehydrate the sections, 1X PBST (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.1% Tween 20, pH=7.4)was added on slides for 10 min. Then, they were placed in a blocking solution made of 10% FBS in 1X PBST for 2 hours at room temperature. This was followed by incubation with the primary antibody in 1% FBS (fetal bovine serum) in 1X PBST at 4℃ overnight. On the second day, the slides were removed from the incubation chamber and placed in Coplin jars to be washed 3 times for 15 minutes and then one time for 5 minutes in 1X PBS at room temperature. Next, the secondary antibody, diluted in 1% FBS in 1X PBST, was added to the slides that were then incubated in a moist chamber for 2 hours at room temperature. After incubation, the slides were placed again in Coplin jars and washed 3 times for 15 minutes in the dark with 1X PBST, followed by a wash of 10 minutes in 1X PBS. After the washes were completed, the slides were rinsed quickly with autoclaved water, dried completely, then mounted with mounting media and covered with a cover slip. After about one hour of incubation at room temperature, the slides were stored at 4℃ until imaging. All primary and secondary antibodies used in this work as well as their dilutions are listed in Table 2.2.

Table 2.2: Primary and secondary antibodies and their dilution ratios used for immunohistochemistry on coronal sections of the developing mouse forebrain at E18.5 and P35.

Primay antibody	Source	Dilution	Company	Catalogue number
Anti-Calbindin	Rabbit	1:500	Swant	CB-38a
Anti-Calretinin	Rabbit	1:500	Swant	6B3

Secondary antibody	Dilution	Company	Catalogue number
Goat anti-rabbit	1:1000	Invitrogen	G-21234
Goat anti-rabbit	1:1000	Invitrogen	G-21234

2.9 Behavioral tests

We carried out all behavioral tests at the Mouse Behavioral Core Facility at the University of Ottawa. All the protocols we used are based on the standard operating procedures (SOPs) that have been approved by Animal Care and Veterinary Services (ACVS) at the University of Ottawa (Crawley, 2007; Know et al., 2006; Tabuchi et al., 2007). 8-10 week-old mice were used in each behavioral test. There were four tests, mice were allowed to take a rest for 2-3 days after one test.

2.9.1 Beam break

Beam break is applied to measure each animal’s locomotor activity. Mice were individually placed in a cage, which was surrounded by a metal frame that was connected to numerous infrared emitters and receptors. These emitters and receptors created a series of invisible infrared light beams so that when a mouse moved through a beam, the beam path was broken and a beam break was recorded. The mice were placed in the dark and monitored 4 hours in total. Numerous parameters can be measured, such as the number of times the animal breaks the beam, time spent mobile/immobile and ambulatory activity (Crawley, 2007). The last parameter was analyzed for this study.

2.9.2 Juvenile interaction

The purpose of this test is to assess the sociability of mice. In order to reduce any external disturbances, a red light and white noise were used in the test room. Before starting the test, all mice had about fifteen minutes to acclimate with the new testing environment. For the test, the tested adult mice were placed simultaneously in a new cage with a juvenile of the same gender. They were then allowed to explore and move freely for two minutes and, during this period, the time that the adult mouse spent on interacting (e.g., sniffing, touching) with the juvenile mouse was recorded. Three days later, the same test adult mouse and the same juvenile partner were again placed in a new cage and the time that the test mouse spent on interacting with the juvenile mouse was again recorded during a two-minute period. Finally, the interaction time of the adult mouse with the juvenile from the first exposure and the second exposure as well as the difference between the two times was then compared between the three genotypes (Crawley, 2007; Know et al., 2006; Tabuchi et al., 2007).

2.9.3 Startle threshold test

The startle test was a measure of the sensorimotor gating of mice. Each mouse was placed in a chamber that was connected to a special motion sensor called electrostatic sensor. The space of the chamber was just big enough for a mouse to fit in. The response of mice to acoustic stimuli was a whole body flinch, which was quantified by recording the amplitude of the mouse movement using the sensor. Before the test, mice had an acclimation period of five minutes, then a loud 120db tone was provided and their responses were recorded. The maximum amplitude and average amplitude of mice in the presence of 120db tone was used to measure their hearing ability (Crawley, 2007).

2.9.4 Fear conditioning

The fear conditioning test was designed to measure learning and memory of mice. Basically, the mice were trained to associate a neutral stimulus (tone) with an unpleasant consequence (foot shock), once they learned from the association between two stimuli, the tone alone should be enough to elicit a state of fear. The whole experiment took three days in total. Mice were trained to associate the tone with the shock on the first day. Each mouse was individually placed in a cage, and had two minutes to explore. After the first two minutes, a cue was played that lasted thirty seconds long, which was immediately followed by 2 sec shock at 0.5 mA. These mice had one min to take a break and another round of cue and shock were provided. There was no cue or shock during the last two minutes. The freezing behavior of mice was recorded over six minutes of test.

The second day is contextual fear testing, each mouse was sent back to the same cage as day one with the same room conditions such as light, sound and smell.

Freezing behavior was recorded during six minutes. The contextual test was used to investigate the memory of mouse to the same environment where the shock was given.

On the last day of test, these mice were placed in a new house with a novel smell, no stimulus was played during the first three minutes, by which time we can make sure these mice didn’t associate the new cage with the cage used on day one or day two. The following step was playing the same cue as on day one for three minutes to measure the ability of mice associate the cue and the shock. Freezing behavior of mice was recorded. During this test, all freezing behavior was recorded using a video camera mounted inside the cage and was measured using the video tracking software from Noldus (Crawley, 2007).

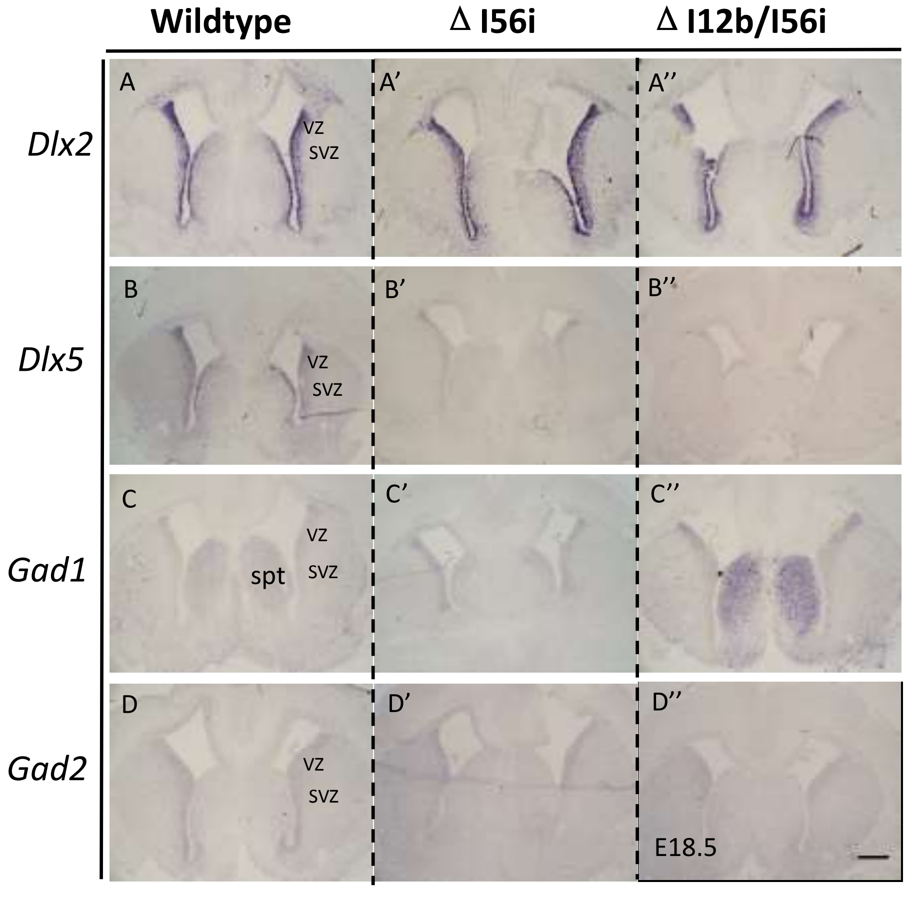
3. Results

3.1 Effect of enhancer deletion on *Dlx* and *Gad* mRNA expression at E18.5 and P35.

In order to characterise the impact of mutations in the intergenic region on the expression of *Dlx* genes and *Gad* genes, ISH were performed using antisense RNA probes on coronal ventral telencephalon sections of mouse embryos at E18.5 and on forebrain sections of P35 adult mice from all three genotypes (i.e., wildtype, ΔI56i and ΔI12b/I56i mutants).

At E18.5, the deletion of the intergenic enhancers affected the expression of *Dlx* genes and of other genes in variable ways. We detected the expression of *Dlx5* in the SVZ and MZ of E18.5 mutants but with obviously reduced levels compared to wildtype mice (Fig. 3.1B, B’, B’’). These mutants seemingly had nearly indistinguishable expression of *Dlx2* in the VZ and SVZ (Fig. 3.1A, A’, A’’) compared to wt. Expression of *Gad2* in the SVZ resembled that of their wildtype counterparts (Fig. 3.1D, D’, D’’). *Gad1* transcripts were detected in the SVZ and Spt with apparently decreased levels in ΔI56i mutants but with increased levels in ΔI12b/I56i mutants (Fig. 3.1C, C’, C’’).

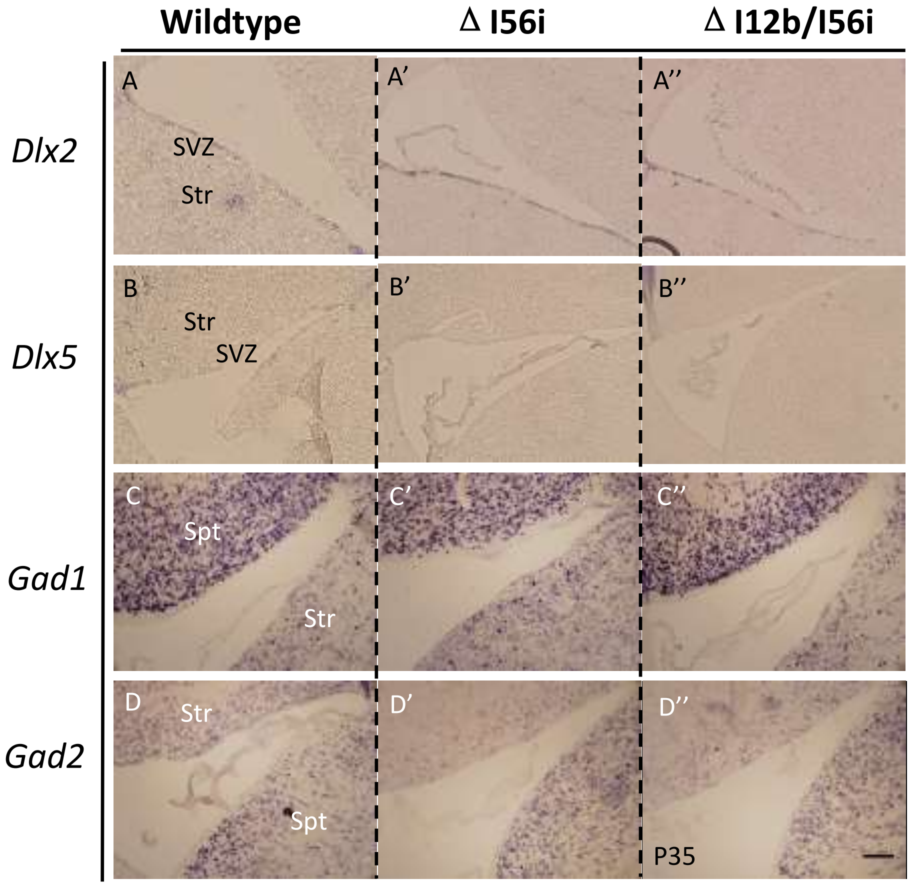
Figure 3.1: *In situ* hybridization on coronal sections of ventral telencephalon of E18.5 wildtype (A-D), ΔI56i mutants (A’-D’) and ΔI12b/I56i mutants (A’’-D’’). No differences were observed in *Dlx2* and *Gad2* expression levels for all genotypes. A decreased in the expression level of *Dlx5* was observed in both mutants compared to the wildtype. ΔI56i mutants showed a decreased *Gad1* expression while ΔI12b/I56i mutants displayed an increase. These are the representative images from six mice of each genotype. **Scale bar**: **175 µm.**


Wildtype
Δ I56i
Δ I12b/I56i
Dlx2
Dlx5
Gad1
Gad2
A
A’
A’’
B
B’
B’’
C
C’
C’’
D
D’
D’’
VZ
SVZ
spt
E18.5

We also performed *in situ* hybridization on forebrain sections from P35 mice to determine changes in the expression of *Dlx* genes and *Gad* genes in adult ΔI56i mutants and ΔI12b/I56i mutants compared to their wildtype counterparts. The expression of *Dlx2* was similar among all three types of mice as demonstrated by the same degree of staining on forebrain sections (Fig. 3.2A, A', A''). *Dlx5* was mainly expressed in the SVZ in P35 wildtype mice, but the expression was barely observable in the same area of P35 ΔI56i mutants and ΔI12b/I56i mutants (Fig. 3.2B, B', B''). This is further evidence that the expression of *Dlx5* is significantly affected by the deletion of the enhancers.

As observed at E18.5, the deletion of the enhancers did not have an impact on the expression of *Gad2* (Fig. 3.2D, D', D''). Finally, while the mutations had variable impact on the apparent levels of *Gad1* expression at E18.5, very similar levels of *Gad1* were observed in the SVZ for both mutants and wildtype mice at P35 (Fig. 3.2C, C', C'').

Figure 3.2: ***In situ*** **hybridization on coronal sections of ventral telencephalon of P35 wildtype (A-D), ΔI56i mutants (A’-D’) andΔI12b/I56i mutants (A’’-D’’).** Both mutants had similar expression of *Dlx2*, *Gad1* and *Gad2* as wildtype mice. However, a clear decrease in the expression of *Dlx5* was observed in both ΔI56i mutants and ΔI12b/I56i mutants. These are the representative images from six mice of each genotype. **Scale bar: 70 µm.**

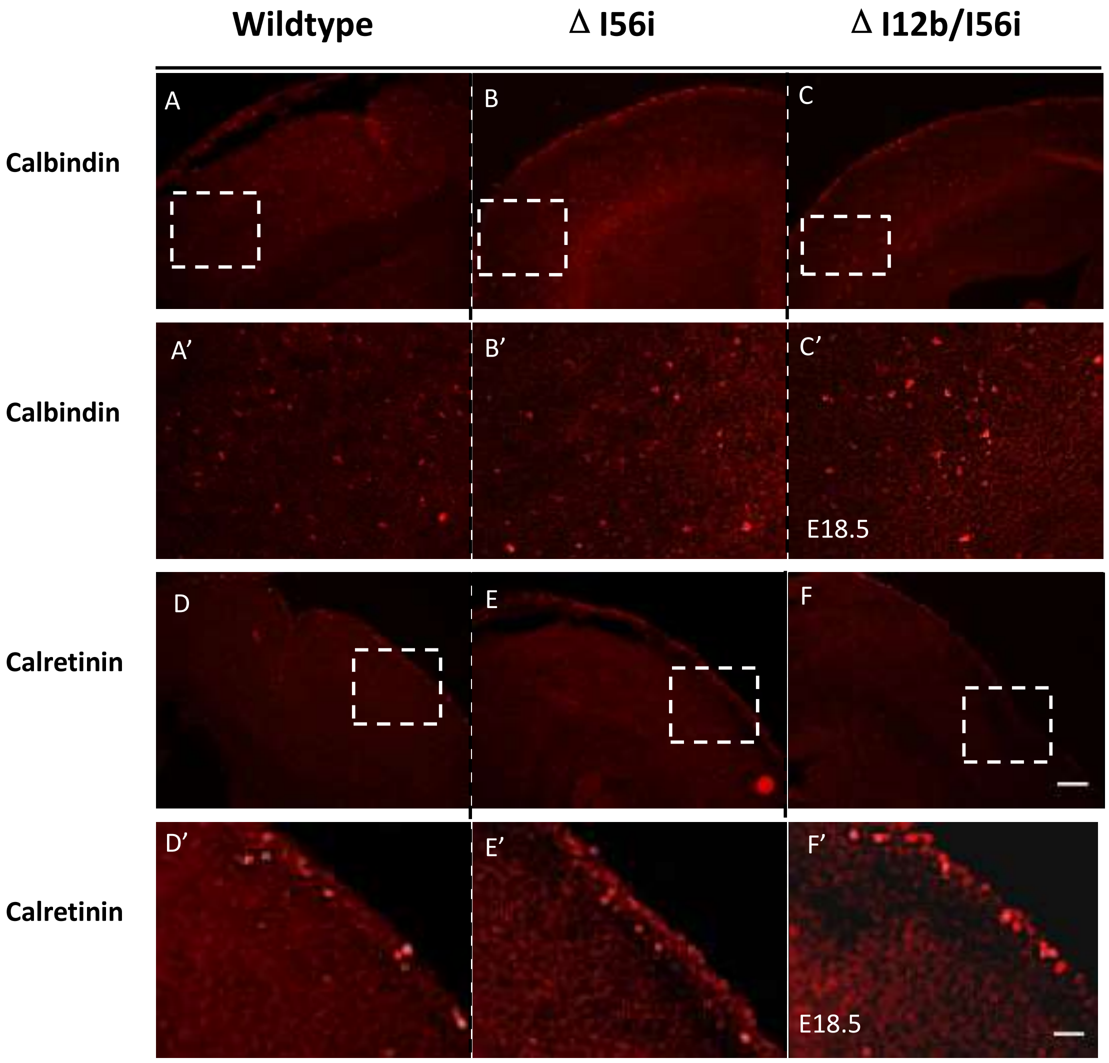

Wildtype
Δ I56i
Δ I12b/I56i
Dlx2
Dlx5
Gad1
Gad2
A
SVZ
Str
A'
A''
B
Str
SVZ
B'
B''
C
Spt
Str
C'
C''
D
Str
Spt
D'
D''
P35

3.2 Impact of intergenic enhancer deletion on the expression of GABAergic neuron markers at E18.5 and P35

Previous studies have shown that *Dlx* genes are expressed in locations where virtually all neurons use GABA as their neurotransmitter and *Dlx5* and *Dlx6* are the regulators of *Gad*, the gene that encodes for glutamic acid decarboxylase, the enzyme synthesizing GABA (Anderson et al., 1997a; Liu et al., 1997). Therefore, in order to further investigate how the deletion of the enhancers influences GABAergic neurons via *Dlx* genes at different developmental stages, we performed IHC on coronal telencephalon sections at E18.5 and P35 from wildtype mice and from the two mutants. We used antibodies directed against calbindin- and calretinin-expressing neurons. These are two neurochemical markers stand for two subpopulations of GABAergic neurons.

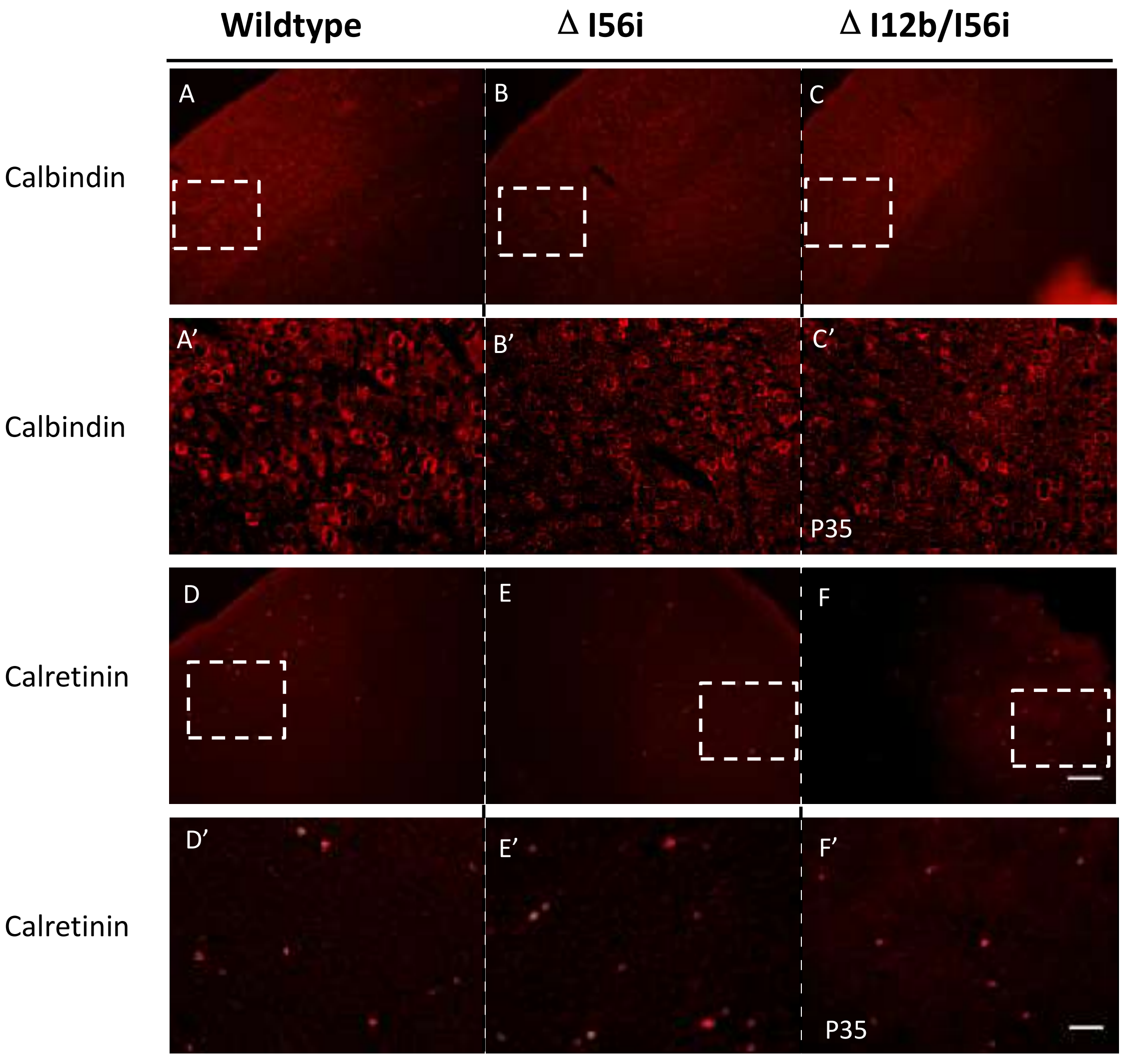
At E18.5, the deletion of the enhancers did not cause any apparent change in these two GABAergic neuron subpopulations in the cortex when compared to wildtype mice. As seen in Figure 3.3, the expression of calbindin and calretinin in the somatosensory cortex of the forebrain appeared to be similar between wildtype mice and mutant mice.

Figure 3.3: Calbindin- and calretinin-expressing neurons in the somatosensory cortex of E18.5 wildtype mice, ΔI56i andΔI12b/I56i mutants. The expression of calbindin (B, C) and calretinin (E, F) in both mutants is comparable to that of wildtype mice (A, D). These are the representative images from three mice of each genotype. **Scale bars: (A-F) 70 µm, (A'-F') 35 µm.**


Wildtype
∆ I56i
∆ I12b/I56i
Calbindin
A
B
C
Calbindin
A’
B’
C’
E18.5
Calretinin
D
E
F
Calretinin
D’
E’
F’
E18.5

At the adult stage, no apparent differences were observed in the expression of calbindin and calretinin on the somatosensory cortex when comparing the two mutant mice and wildtype mice (Fig. 3.4).These results from both E18.5 and P35 would suggest that the deletion of the enhancers I56i or I12b/I56i did not obviously affect the development of GABAergic neurons, at least for the two sub-populations studied here. In other words, I can’t rule out the possibility that other sub-populations of GABAergic neurons may be affected by the deletion of enhancers.

Figure 3.4: Calbindin- and calretinin-expressing neurons in the somatosensory cortex of P35 wildtype mice, ΔI56i andΔI12b/I56i mutants. The expression of calbindin (B, C) and calretinin (E, F) in both mutants is comparable to wildtype mice (A, D).These are the representative images from three mice of each genotype. **Scale bars: (A-F) 70 µm, (A'-F') 35 µm.**

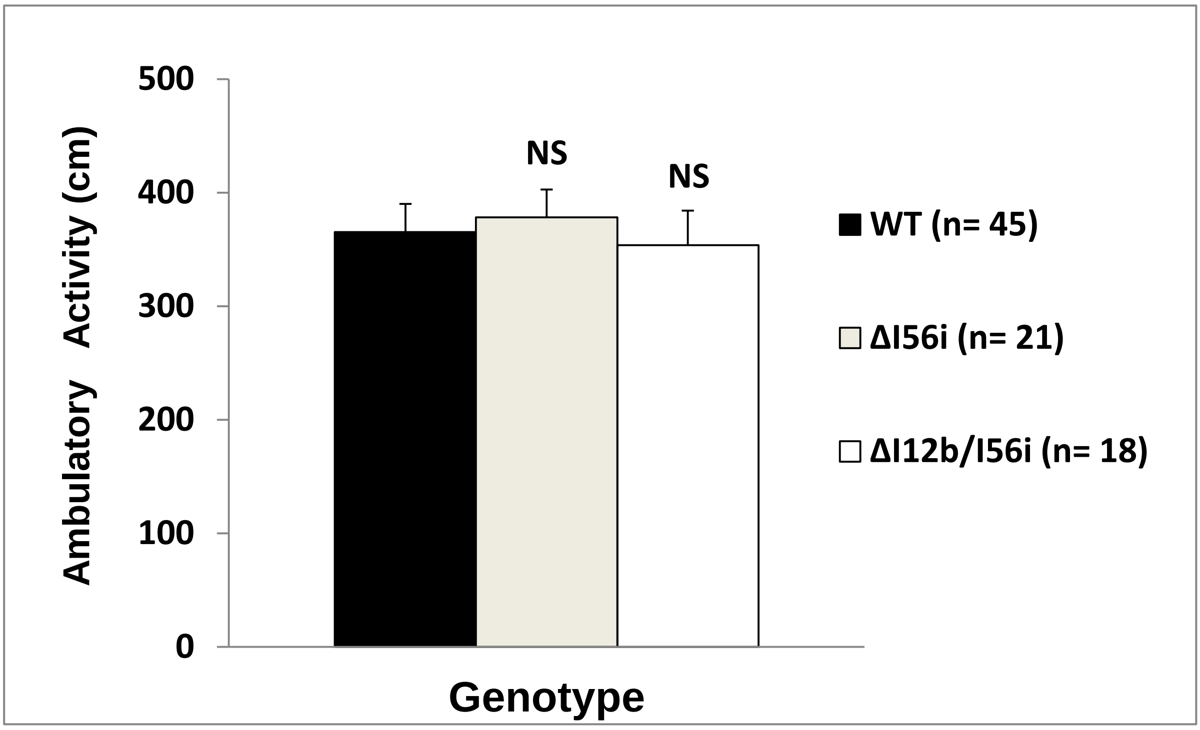

Wildtype
Δ I56i
Δ I12b/I56i
Calbindin
Calbindin
Calretinin
Calretinin
A
B
C
A’
B’
C’
P35
D
E
F
D’
E’
F’
P35

3.3 Behavioral phenotype of ΔI56i and ΔI12b/I56i mutant mice

Since the disturbed development of GABAergic neurons has been linked to some common neurodevelopmental diseases (e.g., epilepsy, schizophrenia and autism spectrum disorder, Tourette syndrome and anxiety; Poitras et al., 2010), and *Dlx* genes play critical roles in the migration and differentiation of GABAergic neurons (Anderson et al., 1997a; Liu et al., 1997), it is important to explore the indirect relation between *Dlx* genes and these disorders. Additionally, the three intergenic enhancers I12b, I56i and I56ii are active in the forebrain and involved in the expression of *Dlx* genes. Previous studies carried out in the Ekker laboratory indicated that the mutation or the deletion of the I56i, I56ii and I12b enhancers affected the expression of *Dlx* genes in the forebrain and the expression of markers for GABAergic neurons at early embryonic stage (Darbandi, 2014; Darbandi et al., 2016; Esau, 2013; Lesage-Pelletier, 2011). Lastly, previous studies revealed abnormal behaviors in some of the mutants (Esau, 2013), raising the possibility that the mutations of intergenic enhancers may affect cause some developmental abnormalities, therefore impact behavior.

In order to gain more information about the relation between the intergenic enhancers and behavioral development, four behavioral tests (i.e., beam break, juvenile interaction, startle threshold test and fear conditioning) were carried out on three genotypes: wildtype, ΔI56i, and ΔI12b/I56i. For each genotype, about twenty mice were tested, with generally an equal number of males and females. The beam break was done first in order to verify the locomotor activity of the mice of the three genotypes (wt, ΔI56i, and ΔI12b/I56i). No significant differences were found among all three genotypes (Fig. 3.5, 1-way ANOVA, $p = 0.78$).This would suggest that the two mutants had no obvious locomotor dysfunction that could affect their performance for the other tests.

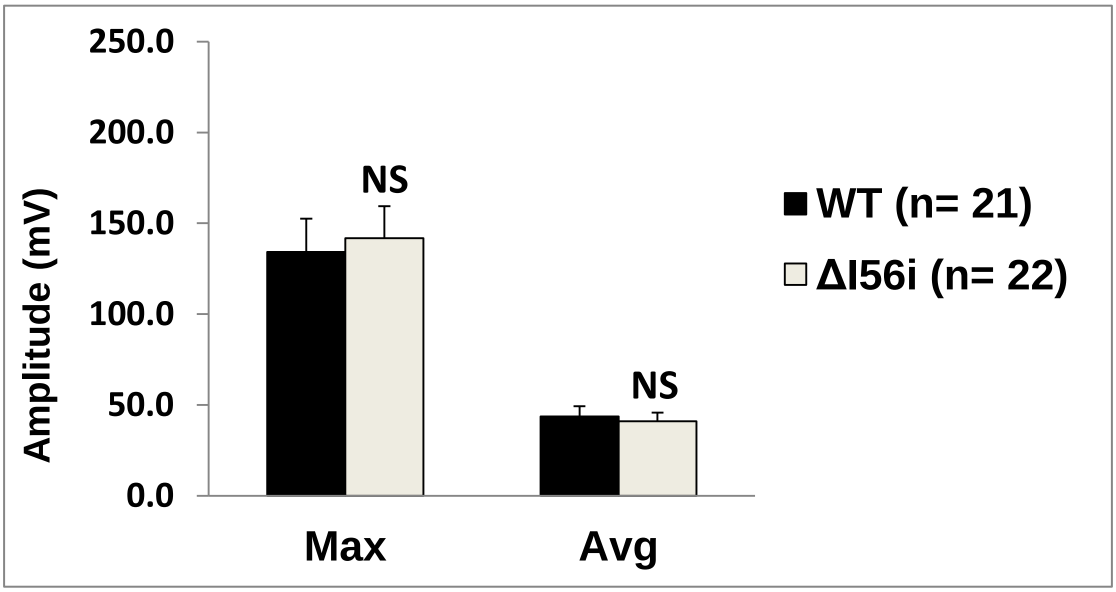
Figure 3.5: Beam break test shows no significant differences in locomotor activity between ΔI56i mutants, ΔI12b/I56i mutants and wildtype mice. The ambulatory activity of mice during test is shown. Total number of mice from each genotype is shown directly in the figure.

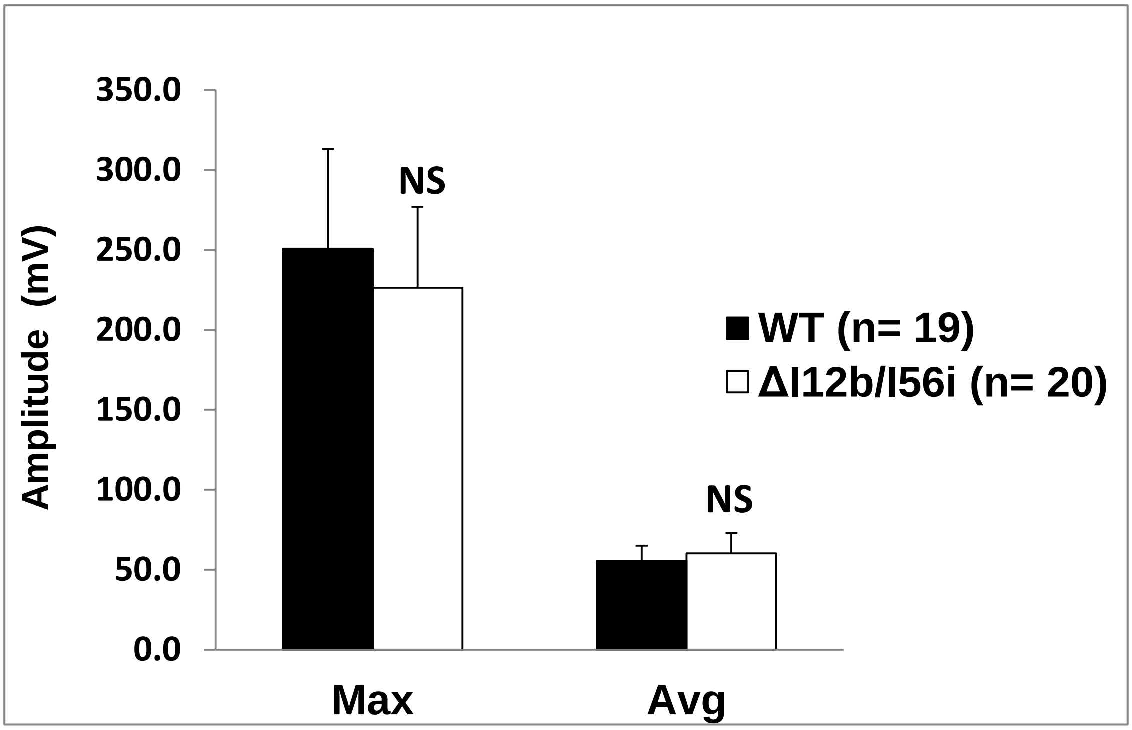


Ambulatory Activity (cm)
500
400
300
200
100
0
NS
NS
■ WT (n= 45)
□ ΔI56i (n= 21)
□ ΔI12b/I56i (n= 18)
Genotype

The acoustic startle test was performed in order to examine the auditory ability of mice, a condition that is needed to do the fear conditioning test (see below). As shown in Figure 3.6, both ΔI56i and ΔI12b/I56i mutant mice displayed similar hearing capabilities as the wildtype mice (1-way ANOVA, p's > 0.68).

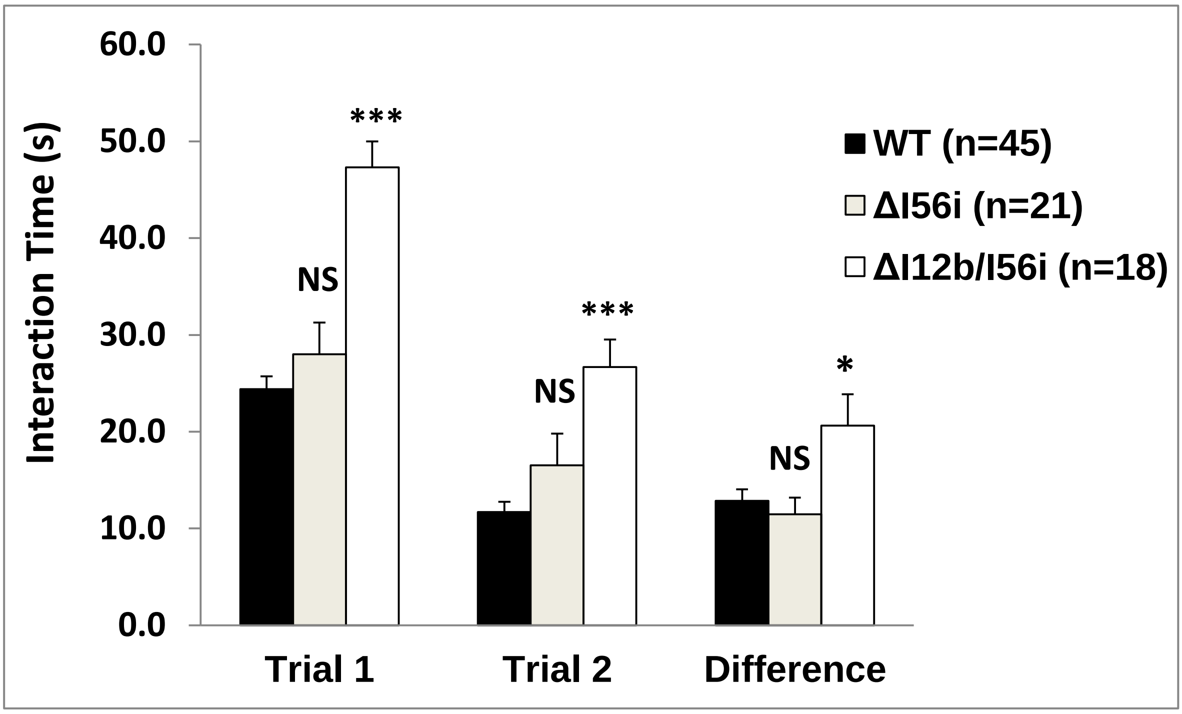
Figure 3.6: No statistical differences in acoustic reaction between mutants (ΔI56i and ΔI12b/I56i) and wildtype mice. The amplitude of the mouse movement was recorded in millivolts and its maximum and average recorded during the test was shown.


250.0
200.0
150.0
100.0
50.0
0.0
Amplitude (mV)
NS
NS
Max
Avg
■ WT (n= 21)
□ ΔI56i (n= 22)


350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
Amplitude (mV)
NS
NS
Max
Avg
■ WT (n= 19)
□ ΔI12b/I56i (n= 20)

The juvenile interaction test was done in order to investigate if the deletion of the enhancers I56i or I12b/I56i would result in some abnormal social behaviors that could be related to ASDs or WBS patients. For this test, an adult test mouse was allowed to socialize for two minutes with the same juvenile partner on one day and again three days later. In principle, the adult mouse should spend less time interacting with the juvenile mouse in the second trial as they have already met in the first trial. For all three genotypes, the adult mice spent less time interacting with the juveniles on their second trial (Fig. 3.7). The time difference between the two trial days was similar between the wildtype adult mice and the ΔI56i adult mutants (Tukey's test $p = 0.70$), but slightly higher in ΔI12b/I56i adult mutants (1-way ANOVA, $p = 0.02$; Tukey's test p's = 0.04). Interestingly, on both the first and second trials, the ΔI12b/I56i mutant adult mice spent significantly more time socializing with the juveniles than the wildtype adult mice (1-way ANOVA, $p < 0.001$; Tukey's test p's < 0.01). Although not significant statistically, the same trend was also observed with the ΔI56i adult mice (Tukey's test $p = 0.21$). This more sociable behavior is reminiscent of the overly-friendly behavior that is observed with WBS patients.

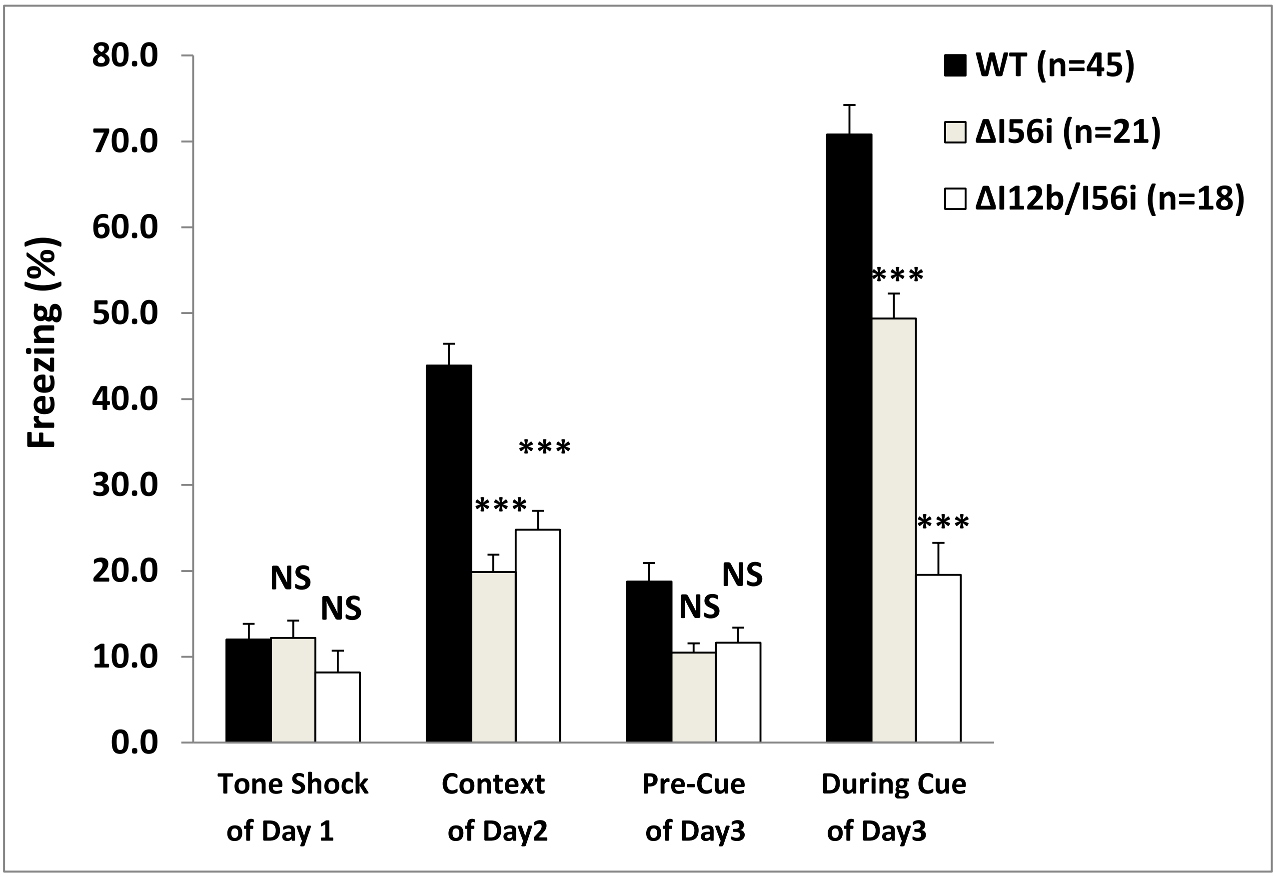
Figure 3.7: Juvenile interaction test shows a trend that mutant mice are more sociable than wildtype mice. Total interaction time that the test adult mouse spent on socializing with the juvenile mouse in each trial is shown as seconds. The time difference between two trials in each group of mice is also shown.


60.0
50.0
40.0
30.0
20.0
10.0
0.0
Interaction Time (s)
***
NS
***
NS
*
NS
■ WT (n=45)
□ ΔI56i (n=21)
□ ΔI12b/I56i (n=18)
Trial 1
Trial 2
Difference

Fear conditioning test was used as a measure of the learning abilities and memory of mice. This test was performed over a period of three days. The first day was referred to as baseline training, during which the mice were trained to associate a tone with a foot shock. The second day was called the contextual training which was used to verify if they can remember the environment where the foot shock was delivered. The third day examined the ability of the mice to associate the cue with the foot shock.

As the data seen in Fig. 3.8, during the context training (the second day), both ΔI56i andΔI12b/I56i mutant mice had a remarkably lower freezing reaction compared to wildtype mice (Fig. 3.8 Context, 1-way ANOVA, $p < 0.001$; Tukey's test p's < 0.00). This would indicate that these mutants may have some memory impairment. On the last day of test, once the same cue as the first day was played, all mice displayed the freezing behaviour, but the both mutant mice showed a lower freezing response, especially the ΔI12b/I56i mutants, compared to the wildtype mice (Fig. 3.8 During Cue, 1-way ANOVA $p < 0.001$; Tukey's test p's < 0.001).These results would suggest that the mutants may have some learning deficits.

Figure 3.8: Fear conditioning demonstrates the memory and learning deficits in mice with enhancer deletions. The reduced freezing behavior in the two mutants during context (day 2) and cue (day 3) suggests some defects in memory and/or learning.


80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Freezing (%)
■ WT (n=45)
☐ ΔI56i (n=21)
☐ ΔI12b/I56i (n=18)
NS
NS
***
***
NS
NS
***
***
Tone Shock of Day 1
Context of Day2
Pre-Cue of Day3
During Cue of Day3

4. Discussion

4.1 *Dlx* enhancers have distinct roles in regulating the expression of *Dlx* genes at different developmental stages

To test the possibility that enhancer deletions may impact the expression of *Dlx* and *Gad* genes in the forebrain, we performed *in situ* hybridization on the forebrain sections of wildtype, and of ΔI56i and ΔI12b/I56i mutants at E18.5 and P35. From the ISH experiments, the expression of *Dlx5* in the ventral telencephalon of both mutants decreased compared to that of wildtype mice, at both E18.5 and P35. These findings are suggesting that the deletion of the I56i enhancer significantly impaired the expression of *Dlx5*. Previous studies with mice that had mutations within the I56i enhancer have also shown this decrease in *Dlx5* expression during earlier embryonic stages, including a study in which there was a single nucleotide polymorphism in I56i (Esau, 2013; Darbandi, 2014; Lesage-Pelletier, 2011). However, the *in situ* hybridization results for *Dlx2* at E18.5 and P35 showed similar levels of expression in the forebrain between the mutant mice and wildtype mice. This is surprising, since I12b plays a critical role in regulating the expression of *Dlx1/2* cluster in the forebrain (Ghanem et al., 2003; Zerucha & Ekker, 2000). Moreover, previous members of the Ekker laboratory (Esau, 2013; Darbandi, 2014) observed a decrease in *Dlx2* expression at earlier embryonic stages (E13.5 for ΔI12b mutants and E14.5 for vI56i/ΔI12b mutants). Based on these results, it is possible that I12b may be the only active regulator of *Dlx2* at early embryonic stages, but not at later developmental stages. This prediction may be consistent with another study in which the I12b enhancer sequence drove GFP gene expression in the forebrain from E10 to E16 (Ghanem et al., 2003). Therefore, there may be other not yet known regulators that play a fundamental role in regulating *Dlx2* expression at later developmental stages.

Two common genes that are known to be involved in the regulation of *Dlx2* are *Mash1* and URE2. *Mash1* is a transcriptional regulator that has been demonstrated to bind to the enhancer I12b and regulate the expression of *Dlx1* and *Dlx2* (Poitras, Ghanem, Hatch & Ekker, 2007; Porteus et al., 1994). However，since the I12b enhancer was absent in one of the mutants in this study, *Mash1* may be able to bind to another potential regulator to compensate for the lack of I12b. Another potential regulator, URE2 has been shown to be the only CRE directing expression of *Dlx2* into the VZ, between E11.5 to E15.5 (Ghanem et al., 2007), tellingly, it is quite important for the normal expression of *Dlx2*.

Furthermore, substances like BMP2, Shh and FGFs (Xu, Harris, Rubenstein, Mundy & Harris, 2001; Bei & Maas, 1998; Miyama et al., 1999; Ferrari, Harrington, Dealy & Kosher, 1999; Gaiano, Kohtz, Turnbull & Fishell, 1999; Mullen et al., 1996), should not be ignored as they have been shown to play an important role in the expression of *Dlx* genes. In summary, I12b would appear to have various roles in the regulation of *Dlx* genes in the forebrain during the developmental stages

4.2 *Dlx* enhancers do not seem to play a major role in regulating the expression of *Gad* genes in the forebrain at late embryonic stage and adult stage

In the forebrain, the expression patterns of *Dlx* genes have been shown to highly overlap that of *Gad* genes (Stühmer et al., 2002), suggesting that *Dlx* genes may have an important role in regulating *Gad* expression. Electroporation of *Dlx2* and *Dlx5* expression vectors induced the expression of *Gad1* and *Gad2* in the mouse telencephalon at E12.5 (Stühmer et al., 2002), *Dlx1/2* null mutants had reduced expression of *Gad* genes compared to wildtype mice (Long et al., 2009), and as mentioned before, *Dlx5* and *Dlx6* are upstream regulators of *Gad* genes (Anderson et al., 1997a). In order to further to explore the regulatory mechanisms between *Gad* and *Dlx* genes, Crystal Esau, a former member of the Ekker laboratory, did research on ΔI56i mutant mice and observed lower expression levels of *Gad1* and *Gad2* in the forebrain of these mutants at E11.5 and E14.5, in conjunction with lower expression of *Dlx5* and *Dlx6*. However, levels of *Dlx1* and *Dlx2* were unchanged. Thus, according to this study, the expression of *Gad* genes seems to depend more on that of *Dlx5* and *Dlx6* than that of *Dlx1* and *Dlx2*. Intriguingly, in the current study, the expression levels of *Gad2* at E18.5 did not seem to change in the forebrain of both ΔI56i and ΔI12b/I56i mutants, but the expression of *Dlx5* was reduced to almost no expression in both mutants (Fig. 3.1, 3.2). Considering the similar expression of *Dlx2* in the forebrain between two mutants and wildtype mice at E18.5, *Dlx2* may play a more important role than *Dlx5* in regulating *Gad2* expression at this stage.

As for *Gad1*, at E18.5, its expression decreased in conjunction with that of *Dlx5* in ΔI56i mutants, but its expression increased in ΔI12b/I56i mutants (Fig. 3.1C, C′, C′′, B, B′, B′′). Meanwhile, at P35, both ΔI56i and ΔI12b/I56i mutants had comparable expression levels of *Gad1* and *Gad2* in the forebrain when compared with wildtype mice. There was almost no expression of *Dlx5* in the mutants (Fig. 3.2). These findings are suggesting little *Gad1/Gad2* expression depends on *Dlx5*, at least at late developmental stages, and the possibility of other regulators being involved in the expression of *Gad* genes at later embryonic stages and in adults.

Previous studies have demonstrated that *Mash1* (Long, Cobos, Potter & Rubenstein, 2009) and Evf-2 (Feng et al., 2006), an ultraconserved noncoding RNA, regulate the expression of *Gad1*. However since *Evf-2* performs its activity by forming a complex with Dlx proteins and binding to the I56i enhancer, it could not have been able to upregulate *Gad1* this way in both ΔI56i and ΔI12b/I56i mutants as they lacked the I56i enhancer. Furthermore, Long (2009) showed that, *Gad1* was still expressed in *Dlx1/2-/-*;*Mash1-/-* mutants, while *Dlx5/6* were downregulated, suggesting that the expression of *Gad1* was not fully dependent on *Dlx* genes and *Mash1* (Long, Cobos, Potter & Rubenstein, 2009). In summary, all of these findings seem to indicate that the mechanisms involved in the expression of *Gad* genes are more complex and may involve other factors or regulators in addition to *Dlx* genes, *Evf-2* and *Mash 1*.

4.3 *Dlx* genes play a role in the development of GABAergic neurons

In the cortex of adult mice, virtually all GABAergic interneurons are derived from cells that express the *Dlx* genes, implying a tight relation between *Dlx* gene function and the development of GABAergic neurons (Anderson et al., 1997a).

More than 20 different subtypes of GABAergic interneurons have been found in the cortex so far, and each of them express distinct subsets of neurochemical markers including, but not limited to, calcium binding proteins (Kelsom & Lu, 2013).

The majority of GABAergic neurons are born in the ganglionic eminences of the subpallium, where the four *Dlx* genes (*Dlx1*, *Dlx2*, *Dlx5* and *Dlx6*) are also expressed. After their formation, GABAergic neurons undergo two main additional sequential steps during their development: (1) their neuronal fate specification and (2) their differentiation. Then, they migrate from the subpallium to their ultimate destinations within the cerebral cortex in order to form synaptic contacts with their targets (Achim, Salminen & Partanen, 2014; Kelsom & Lu, 2013).

Dlx genes have been demonstrated to play a vital role throughout the whole developmental process of GABAergic neurons. For instance, *Dlx1* and *Dlx2* are indispensable for the production of postmitotic GABAergic interneurons in the SVZ (Achim, Salminen & Partanen, 2014). Marín and Rubenstein (2011) found that both *Dlx1/2* and *Mash1* are important for the differentiation of GABAergic neurons, since loss of either of them caused a reduced number of GABAergic neurons in the cortex at the time of birth (Marín & Rubenstein, 2001). Because *Dlx5* and *Dlx6* are expressed after *Dlx1* and *Dlx2*, they have been pointed to be candidate regulators of later differentiation of GABAergic neurons (Marín & Rubenstein, 2001). Additionally, *Dlx5* and *Dlx6* proteins are upstream regulators of *Gad* genes, which produce enzyme that is responsible for the synthesis of neurotransmitter GABA (Anderson et al., 1997a).

In this study, no differences were observed in the expression of both calretinin and calbindin, two GABAergic interneuron markers, in the cortex of the two types of mutants (ΔI56i andΔI12b/I56i) at E18.5 and P35 (Fig. 3.3, 3.4). Thus, the absence of I56i enhancer or both I12b and I56i enhancers seems not to affect the differentiation and migration of the two subtypes of GABAergic interneurons from the embryonic stage to adult, since the failure of developing process of GABAergic neurons is irreversible based on the mutants examples I mentioned above (Anderson et al., 1997b; Marín & Rubenstein, 2001).

With more than 20 different subtypes of GABAergic neurons present in the cortex, their diversity is influenced by the time and location of their birth (Kelsom & Lu, 2013), it is then possible that other subtypes of GABAergic interneurons besides calbindin and calretinin may have been affected by the deletion of I56i and I12b/I56i. In summary, to learn better about the roles of *Dlx* genes in the developmental process of GABAergic neurons, it is necessary to compare the expression of more subpopulations of GABAergic neurons (e.g. parvalbumin, somatostatin and neuropeptide) in the somatosensory cortex as well as the expression of *Dlx1* and *Dlx6* in the telencephalon of two types of mutants (ΔI56i and ΔI2b/I56i) to wildtype mice at E18.5 and P35.

4.4 Behavioral aspects of adult mice are affected by the absence of *Dlx* enhancers

Both ΔI56i andΔI12b/I56i mutants showed abnormalities in their social, learning and memory skills (Fig.3.7, 3.8). More specifically, based on the juvenile interaction test, both mutant types had a tendency to be more sociable than wildtype mice, a typical behavioral phenotype seen in WBS patients (Antonell et al., 2010). Even though no studies so far have shown that the absence of *Dlx* enhancers may cause a WBS-related phenotype in mice, it should be interesting to explore if there are more similar features of WBS patients on *Dlx* enhancers-loss-function mutants. For example, levels of oxytocin, a hormone that plays a role in social bonding and sexual reproduction (Yang, Wang, Han & Wang, 2013), are usually heightened in WBS patients (Donaldson and Young 2008; Dai et al. 2012). Therefore, comparing the levels of oxytocin in mutants (ΔI56i and ΔI12b/I56i) to that of wildtype may provide us more information of probably existing relation between *Dlx* enhancers and the WBS-related behaviors in the mouse.

More importantly, Shuldiner et al. (2017) recently found that the key behavioral feature of WBS, hypersocialibity also acted a central role in domestication that distinguished dogs from wolves (Shuldiner et al., 2017). As mentioned before, Lesage-Pelletier (2011) has done research on mutant mice with a 182-SNP in I56i enhancer and observed a trend that these mutants (vI56i) seemed more sociable compared to wildtype mice, even though the data did not reach statistical significance (Lesage-Pelletier, 2011). The 182-SNP is located in a region of I56i enhancer that is identical between mouse and dog, but this region is only a small part (26 bp) of the I56i enhancer (Poitras et al., 2010). Therefore, it is important to compare the whole genomic sequence of I56i enhancer of dog to that of mouse, the possibility of a genomic difference may suggest that different regions of sequence may be linked with the domestication of dog, therefore the SNP in these regions may contribute to hypersocial features of WBS.

In the fear conditioning test, both ΔI56i andΔI12b/I56i mutant mice had significantly less freezing time during the contextual test (i.e., when they were put in their old environment again with no cue provided) and also during the cue-associated test (i.e., when they were put in a new environment, but provided the same cue). From these results, it would seem that the mutants may suffer from memory loss. Mao (2009) also observed a similar reduced response from the *Dlx1* null mutants in the fear conditioning test (Mao et al., 2009). Memory is a complex process and past experiences or previously learned information are usually remembered with a specific emotional state instead of being retained indiscriminately from our environment (McEwen & Sapolsky, 1995). The hippocampus and the amygdala are two important regions of the brain that are involved in the memory of emotionally charged experiences. They have different contributions to the behavior that are the object of the fear conditioning test (Phillips & LeDoux, 1992). More specifically, the hippocampus has been reported to transmit complex signals (i.e., make the association of the environment with the foot shock), while the amygdala impacts the learning ability of mice to connect the cue tone with the foot shock (Richter-Levin & Akirav, 2000). Therefore, the deletions of the intergenic enhancers, especially I56i, may have impacted both the hippocampus and the amygdala function in this study.

5 Conclusion

In summary, this study showed that the deletion of intergenic enhancers, mainly I56i, resulted in an almost total loss of *Dlx5* expression, with no changes in the expression of *Dlx2* during late embryonic stages (E18.5) as well as in adults at P35. This would indicate that I56i still regulates the expression of *Dlx5* at late developmental stages, while I12b may be not the only regulator of *Dlx2* expression at late embryonic stage. *Dlx* genes have been demonstrated to modulate the expression of *Gad* genes during the early embryonic stages of mice, but the normal expression level of *Gad1* and *Gad2* in the adult mutant mice indicates *Dlx* proteins, especially Dlx5 protein, does not play a significant role in the regulation of *Gad* genes at the adult stage. As for the development of GABAergic neurons, the deletion of I56i or I12b/I56i had no obvious effects on two subtypes of GABAergic neurons: those that express calbindin and calretinin in the somatosensory cortex of both embryos at E18.5 and adults at P35. However, it is possible that other subtypes of GABAergic neurons may have been impacted by the enhancer deletions through affecting the expression of Dlx genes. Finally, the deletion of the *Dlx* enhancers, in particular I56i, impacted some behaviors in mice. More specifically, ΔI56i and ΔI12b/ΔI56i mutant mice became more social and displayed loss in memory and learning skills. In conclusion, this study demonstrated the importance of *Dlx* intergenic enhancers, in particular I56i, on the expression of *Dlx* genes (mainly *Dlx5*) during the late embryonic and adult stages of mice as well as on the behavioral phenotypes of adult mice.

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