Functional analysis of the *cis*-regulatory elements I56i, I56ii and I12b that control *Dlx* gene expression in the developing forebrain of mouse and zebrafish

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Statement of contribution

My PhD thesis entitled "Functional analysis of the *cis*-regulatory elements I56i, I56ii and I12b that control *Dlx* expression in the developing forebrain of mouse and zebrafish" is composed of three independent but highly relevant studies.

I have co-authored with Dr. Noël Ghanem on the first study entitled "Characterization of a distinct subpopulation of striatal projection neurons expressing the *Dlx* genes in the basal ganglia through the activity of the I56ii enhancer". This study has been published in *Developmental Biology* 2008; 322: 415-424. The following individuals contributed to this work: Dr. Noël Ghanem prepared most of the transgenic constructs and generated lines of transgenic mice reflecting the activity of different *Dlx* forebrain enhancers, with the assistance of Gary Hatch, a senior research assistant in the laboratory. Dr. Noël Ghanem also completed the characterization and comparison of different enhancer activities at various time points. I completed the rest of the experiments shown in this chapter, mainly including double immunohistochemistry, *in situ* hybridization, sequence analysis of transcription factor binding sites within the I56ii enhancer, co-transfection and chloramphenicol acetyltransferase (CAT) assays. Dr. Luc Poitras, a former postdoctoral research associate in the laboratory, has provided important technical advice and resources for the design and analysis of the co-transfection and CAT assays. The creation and regular maintenance of transgenic mice were performed by Adrianna Gamborotta and Gary Hatch, respectively. Dr. John Rubenstein (University of California at San Francisco) reviewed this study. The article was collaboratively written by Dr. Noël Ghanem and me, and was corrected by Dr. Rubenstein and Dr. Ekker.

The second study, "Roles of distinct *cis*-regulatory elements, I56i and I56ii, from the *dlx5a/dlx6a* intergenic region, during zebrafish GABAergic neuron development and their cross-regulatory interaction", was wholly conducted and completed in Dr. Ekker's laboratory. This manuscript has been accepted for publication by *International Journal of Developmental Neuroscience*. The transgenic zebrafish line, *tg(1.4kbdlx5a/dlx6a:GFP)*, was previously established by Dr. Qiaoming Long, a former postdoctoral fellow in Dr. Ekker's laboratory. The transgenic line, *tg(1.1kbI56i:GFP)*, was originally established by me, with a tremendous technical assistance from Yanwei Xi, a PhD student in Dr. Ekker's laboratory. Ryan MacDonald, a former PhD student in Dr. Ekker's laboratory, generated the *300bpI56ii:GFP* transgene construct and tested its activity in primary zebrafish embryos. The daily maintenance of these transgenic zebrafish lines was performed by me, with the technical assistance from Vishal Saxena, a research assistant in Dr. Ekker's laboratory. I have wholly designed and performed all the comparative analyses as well as the morpholino knockdown experiments described in this chapter and wrote the article, which was reviewed and corrected by Dr. Ekker.

I have wholly completed a vast majority of the phenotypic analyses shown in the third study entitled "Targeted deletion of a *Dlx* enhancer I12b reduces *Dlx1/Dlx2* expression and inhibits cell proliferation in the developing mouse forebrain". This manuscript is under preparation for final submission. Dr. Luc Poitras and Lisa Tran, a summer student in Dr. Ekker's laboratory, collaboratively designed and completed the real-time RT-PCR assays. The mutant mice carrying a targeted deletion of I12b enhancer was generated and maintained by Dr. Luc Poitras and me, with technical assistance from the colleagues at McGill Cancer Center on the work of genetic targeting in embryonic

stem cells. Gary Hatch and Sylvie Emond also offered tremendous technical supports in regular maintenance of I12b mutants. The article was written by me and corrected by Dr. Poitras and Dr. Ekker.

Abstract

The vertebrate *Dlx* gene family generally consists of multiple convergently transcribed bigene clusters and encodes a group of homeodomain-containing transcription factors crucial for the development of forebrain, branchial arches, sensory organs and limbs. During embryogenesis, the expression patterns of various *Dlx* genes display a high degree of overlaps in many tissues and their expression is well conserved among distantly related vertebrates, such as mammals and zebrafish, probably due to shared regulatory mechanisms. At least four *cis*-regulatory elements (CREs) are responsible for *Dlx* expression in the forebrain: URE2 and I12b in the *Dlx1*/*Dlx2* (zebrafish *dlx1a/dlx2a*) locus, and, I56i and I56ii in the *Dlx5*/*Dlx6* (zebrafish *dlx5a/dlx6a*) locus. Here, we first show that unlike the other three forebrain enhancers, mouse I56ii CRE does not label interneuron progenitor cells in the subpallial telencephalon; instead, I56ii-positive cells are a group of GABAergic projection neurons expressing striatal markers *Meis2* and *Islet1*. Meis2 and Islet1 proteins can bind and activate reporter gene transcription via the I56ii CRE, suggesting that they may be potential upstream regulators of *Dlx* genes *in vivo*. To determine whether there exists a *dlx*-mediated regulatory pathway during zebrafish GABAergic neuron formation similar to that was previously found in mice, we establish two independent lines of transgenic fish in which the GFP reporter gene is controlled by a 1.4kb *dlx5a/dlx6a* intergenic sequence (encompassing zebrafish I56i and I56ii) and a 1.1kb fragment containing only I56i CRE, respectively. Our observations reveal that *dlx5a/dlx6a* regulatory elements exhibit a fairly specific activity in the zebrafish forebrain and may be essential for GABAergic neuron generation, while I56i and I56ii are likely to play distinct roles in modulating this process in different

subpopulations of cells. Additionally, disruption of *dlx1a/dlx2a* or *dlx5a/dlx6a* function leads to a marked decrease of enhancer activity in the diencephalon and midbrain as well as a comparatively lesser extent of reduction in the telencephalon, indicating that a possible functional divergence of *dlx* genes or their CREs may have occurred in the telencephalon between zebrafish and mammals during evolution. In order to define the specific contribution of various individual CREs to overall *Dlx* regulation as well as to proper GABAergic neuron production, we also generate a mutant mouse model in which I12b CRE is selectively deleted. Despite that mice homozygous for I12b loss develop normally and harbor no overt morphological defects in the forebrain, targeted deletion of this enhancer results in a statistically significant reduction of *Dlx1*/*Dlx2* transcript levels and seemingly perturbs cell proliferation in the subpallial telencephalon, particularly in the ventricular and subventricular zones of ganglionic eminences. Taken together, these data illustrate a complex and dynamic *Dlx* regulation in the early developing forebrain through the implications of multiple *Dlx* CREs with overlapping and diverse functions. Further study of *dlx* CRE activity in zebrafish or other related vertebrate species may eventually contribute to our understanding of the evolution of the *Dlx* gene family.

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List of abbreviations

AEP: anterior entopeduncular area

AER: apical ectodermal ridge

AH: anterior hypothalamus

AP: alkaline phosphatase

A/P: anterior/posterior

BAC: Bacterial Artificial Chromosome

BCIP: 5-bromo-4-chloro-3-indolyl-phosphate

BF: basal forebrain

bHLH: basic helix-loop-helix

bp: base pair

BT: basal telencephalon

CAT: chloramphenicol acetyltransferase

CB: calbindin

CCK: cholecystokinin

cDNA: complementary deoxyribonucleic acid

CGE: caudal ganglionic eminence

ChIP: chromatin immunoprecipitation

CNS: central nervous system

CR: calretinin

CRE: *cis*-regulatory element

cRNA: complementary RNA

DEPC: diethylpyrocarbonate

Di: diencephalon

DIG: digoxigenin

Dl: lateral zones of the dorsal telencephalon;

dLGE: dorsal lateral ganglionic eminence

Dll: *distal-less* gene

Dlx: *distal-less* related mouse homologue

dlx: *distal-less* related zebrafish homologue

DLX: *distal-less* related human homologue

Dm: medial zones of the dorsal telencephalon

dMGE: dorsal medial ganglionic eminence

DP: dorsal pallium

Dp: posterior zones of the dorsal telencephalon;

dpf: day post fertilization

D/V: dorsal/ventral

E: embryonic day

EMSA: electrophoretic mobility shift assay

ES cells: embryonic stem cells

FGF: fibroblast growth factor

FN: frontonasal prominence

GABA: γ-aminobutyric acid

Gad: glutamic acid decarboxylase

GAPDH: *glyceraldehyde-3-phosphate dehydrogenase*

GE: ganglionic eminence

GFP: green fluorescent protein

GP: globus pallidus

h: hour

hb: hindbrain

HGF/SF: hepatocyte growth factor/scatter factor

Hy: the second branchial arch

hy: hypothalamus

IZ: intermediate zone

Kb: kilobase

L: limbs

LGE: lateral ganglionic eminence

LP: lateral pallium

LV: lateral ventricle

MAP2: microtubule-associated protein 2

Mb: midbrain

Md: mandibular component of the first branchial arch

MGE: medial ganglionic eminence

min: minutes

MOs: morpholino oligonucleotides

MP: medial pallium

μm: micron

MZ: mantle zone

NBT: nitroblue tetrazolium chloride

NCX: neocortex

neo: neomycin

NPY: neuropeptide Y

nt: nucleotide

O/N: overnight

OP: optic eminences

OPCs: oligodendrocyte precursor cells

P: post-natal day

PB: phosphate buffered saline

PBS: phosphate buffered solution

PBST: phosphate buffered solution and Tween-20

PCNA: proliferating cell nuclear antigen

PCR: polymerase chain reaction

PCX: palliocortex

P/D: proximal-distal

PEP: posterior entopeduncular area

PFA: paraformaldhyde

PH3: phospho-histone H3

PLAP: placental-like alkaline phosphatase

POA: preoptic area

POP: posterior preoptic area

PT: pretectum

pTh: prethalamus (ventral thalamus)

PTU: 1-phenyl-2-thiourea

PV: parvalbumin

RA: retinoid acid

RT: room temperature/reverse transcription

s: second

SCH: suprachiasmatic nucleus

Sdd: dorsal subdivision of the dorsal subpallium

Sdv: ventral subdivision of the dorsal subpallium

Shh: Sonic hedgehog protein

SNP: single-nucleotide polymorphism

SOM: somatostatin

Sp/Sep: septum

ST: subpallial telencephalon

St: somites

Str: striatum

Sv: ventral subpallium

SVZ: subventricular zone

tec: tectum

tel: telencephalon

TBST: Tris buffered solution and Tween-20

TH: tyrosine hydroxylase

TU: tuberal region

URE2: upstream regulatory element 2

VIP: vaso-active intestinal peptide

vLGE: ventral lateral ganglionic eminence

vMGE: ventral medial ganglionic eminence

VP: ventral pallium

VZ: ventricular zone

III: third ventricle

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1. Introduction

1.1. Overview of the mammalian central nervous system

The central nervous system (CNS) has complex morphology and structural organization and is composed of hundreds to thousands of different cell types, which makes it arguably the most intricate organ system throughout the body of vertebrates (Kandel et al., 2000). The CNS of mammals comprises two main subdivisions, the brain at the anterior end and the spinal cord posteriorly. During embryonic development, the process named neurulation by which the CNS is established as well as the diverse basic structures of the CNS appear to be largely similar among different mammalian species and thus are thought to be highly conserved over evolutionary time (Ghysen, 1992; Reichert, 2002). The CNS is originally derived from the ectoderm of the early embryo as a single uniform sheet of cells that subsequently evolve into the neural plate (Lumsden and Krumlauf, 1996; Stern, 2005). During the process of neurulation, the lateral edges of the neural plate edges fold up, roll bilaterally, and close at the dorsal midline along the anterior-posterior (A/P) axis and then form the neural tube, which contains the primordia of different structures of the CNS (Colas and Schoenwolf, 2001; Gilbert, 2006). Specifically, the posterior parts of the neural tube will eventually develop into the spinal cord, whereas the anterior end of the neural tube by itself will be temporarily transformed into three consecutive enlargements (also called primary vesicles), namely the forebrain (prosencephalon), the midbrain (mesencephalon) and the hindbrain (rhombencephalon) (Moore, 1993; Puelles and Rubenstein, 1993; Gilbert 2006). These three primary vesicles can be further regionalized during development and turn into a total of five subdivisions (also as secondary vesicles). The prosencephalon is divided into two major domains, the

more anterior telencephalon and the diencephalon, while the rhombencephalon becomes subdivided into the metencephalon and the myelencephalon (Moore, 1993; Gilbert, 2006). After a series of complex morphological transitions, each of these embryonic structures will ultimately develop into their respective corresponding adult structures with distinct cellular composition and physiological function (Moore, 1993; Gilbert, 2006). For example, the telencephalon will develop into the cerebral hemispheres (cerebrum), the hippocampus and the olfactory lobes, and, the diencephalon will give rise to the thalamus and the hypothalamus (Gilbert, 2006).

1.2. Origin and general structures of the subpallial telencephalon

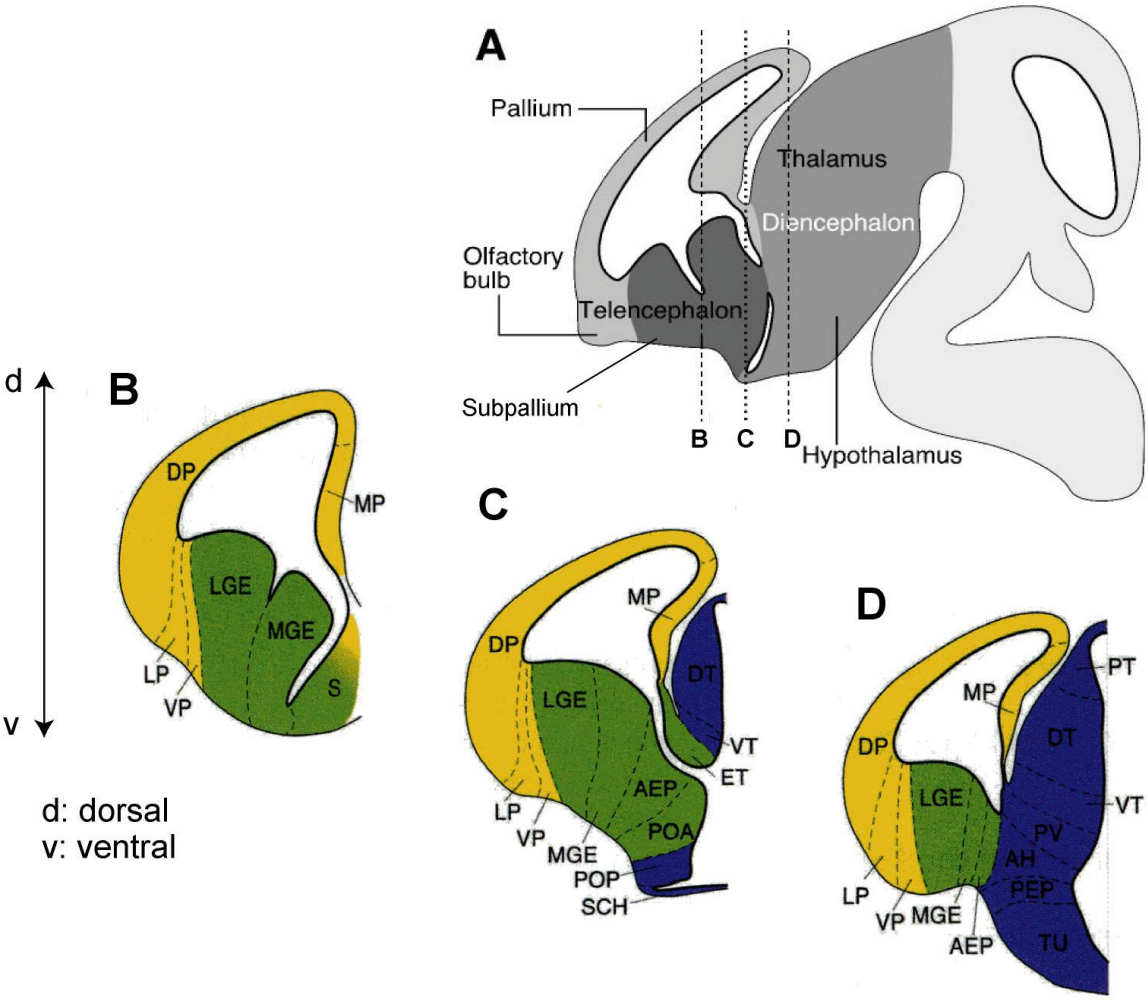
Despite the fact that the general anatomical morphologies of the adult telencephalon across distinct mammalian species are highly variable, the overall organization and morphological structures of the telencephalon appear to be well conserved in the early developing forebrain (Puelles et al., 1999; Puelles et al., 2000). The telencephalon is basically formed as two major subdivisions along the dorsal-ventral (D/V) axis in the alar plate of the embryonic forebrain, namely the pallium (dorsal) and the subpallium (ventral) (Figure 1.1A) (Kallen, 1951; Striedter, 1997; Nieuwenhuys, 1999). On the other hand, the basal plate (undersurface) of the early developing forebrain contains parts of diencephalic structures, such as the mammillary bodies (Kallen, 1951; Striedter, 1997; Nieuwenhuys, 1999). Hence, the pallial and subpallial compartments have been respectively described as the roof and floor of the developing telencephalic vesicle. Herein, I will briefly outline the various anatomical structures within the mammalian subpallium that is of particular relevance to my research. As delineated in

Figure 1.1B-D, the subpallium is comprised of three principal subregions at different rostral, medial and caudal levels: the striatal, pallidal, and telencephalic stalk domains (Puelles et al., 1999; Puelles et al., 2000; Rossant and Tam, 2002; Marin and Rubenstein, 2003; Lindsay et al., 2005). The striatal domain lies lateral to the subdivision of the ventral pallium (VP) and is populated and established by progenitor cells originally generated from an area called the lateral ganglionic eminence (LGE) (Rossant and Tam, 2002; Marin and Rubenstein, 2003). As development progresses, the striatal domain can produce several structures including the caudoputamen nucleus, nucleus accumbens, several parts of the septum and amygdala (Rossant and Tam, 2002; Marin and Rubenstein, 2003). The pallidal domain mainly derived from progenitors born in the medial ganglionic eminence (MGE) is situated right below the striatal domain and its derivatives include the globus pallidus, ventral pallidum, some central parts of the bed nucleus of the stria terminalis and septum. (Rossant and Tam, 2002; Marin and Rubenstein, 2003). Moreover, the telencephalic stalk domain, primarily consisting of the anterior entopeduncular area (AEP) and preoptic area (POA), is located further underneath the pallidal domain (Rossant and Tam, 2002). Apart from its implication in regulating neuron generation, it is also an important subpallial area through which a set of major fiber tracts, such as the medial and lateral forebrain bundles and the anterior commissure, travel when moving in or away from their different source regions (Marin et al., 2000; Rossant and Tam, 2002; Marin and Rubenstein, 2003).

1.3. Regional patterning and specification in the subpallial telencephalon

Figure 1.1. Anatomical organization of the mouse developing forebrain

(**A**) Schematic representation of a sagittal section of an E13.5 mouse brain showing various major domains of the telencephalon and diencephalon. The mouse telencephalon is composed of two primary structures: the pallium (dorsal portion), the subpallium (ventral portion) and olifactory bulb, while the diencephalon contains two main subregions, thalamus and hypothalamus. In the telencephalon, the pallium is demonstrated in lighter gray color than the subpallium. (**B-D**) Serial transverse sections at different levels illustrating the major subdivisions of the pallium, subpallium and diencephalon. The planes of these sections are indicated in panel A. The subdivisions of the pallium include medial pallium (MP), dorsal pallium (DP), lateral pallium (LP) and ventral pallium (VP). The subpallium is composed of the following structures: lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), septum (S), anterior entopeduncular area (AEP), anterior preoptic area (POA) and eminentia thalami (ET). The major diencephalic subdivisions are dorsal thalamus (DT), ventral thalamus (VT), posterior preoptic area (POP), suprachiasmatic nucleus (SCH), pretectum (PT), PV (paraventicular nucleus), AH (anterior hypothalamus), PEP (posterior entopeduncular area) and TU (tuberal region). The pallium and subpallium of the telencephalon is respectively demonstrated in yellow and green color, while the diencephalon is shown in blue. Modified from Rossant and Tam (2002) and Marin and Rubenstein (2003).



d: dorsal
v: ventral

Following the closure of the neural tube, distinct modes of growth and morphogenesis will be separately initiated along both the A/P and D/V axes in the dorsal and ventral portion of the developing telencephalon, ultimately leading to differential structures between the pallium and subpallium. In the early developing telencephalon, the establishment of different structures in the pallium and supallium as well as diverse functional domains within them are mainly modulated by the combined activities of a group of signaling molecules and transcription factors (Puelles et al., 2000; Rossant and Tam, 2002; Hoch et al, 2009). Specifically, the signaling molecules (also defined as "organizers") generated from the signaling centers at various specific locations in the developing telecephalon first establish and maintain correct spatio-temporal expression of many regional transcription factors by localized expression of themselves or of their activators (Puelles et al., 2000; Rossant and Tam, 2002; Hoch et al, 2009). As such, the expression patterns of these transcription factors are built in a manner that is highly correlated with morphological boundaries in the developing telencephalon (Rallu et al., 2002; Schuurmans and Guillemot, 2002). These transcription factors will then exert their functions in establishing the identities of distinct pallial and subpallial subdomains as well as in producing different types of neurons within these subregions (Rallu et al., 2002; Schuurmans and Guillemot, 2002; Wonders and Anderson, 2006). Indeed, a large number of previous reports have described that different individual subdivisions within the developing mouse subpallium (e.g. LGE, MGE, AEP/POA, and certain parts of the septal and amygdaloid anlages) exhibit unique expression profiles of many transcription factors that strictly resemble their regionalization and are believed to play central roles either individually or in combination in controlling their formation (Bulfone et al., 1993a;

Bulfone et al., 1993b; Guillemont and Joyner, 1993; Porteus et al., 1994; Hsieh-Li et al., 1995; Valerius et al., 1995; Liu et al., 1997; Hallonet et al., 1998; Eisenstat et al., 1999). In addition, during embryonic development, these master regulatory genes are expressed in a time-dependent manner. The expression of some genes, such as *Otx2*, *Six3* and *Vax1*, is detectable in progenitor cells as early as the time of early neural plate formation, whereas others, including *Dlx1, Dlx2, Gsh1, Gsh2, Mash1, Nkx2.1*, and *Islet1*, start their expression at different time points after the completion of neurulation, indicating that the development of various subpallial subdomains may also occur in a stepwise fashion (Bulfone et al., 1993a; Bulfone et al., 1993b; Guillemont and Joyner, 1993; Porteus et al., 1994; Hsieh-Li et al., 1995; Valerius et al., 1995; Liu et al., 1997; Hallonet et al., 1998; Eisenstat et al., 1999). Disrupting function of these genes has been revealed to induce abnormal patterning and specification of the developing subpallial telencephalon. For example, a ventral-to-dorsal transformation has been observed in the basal ganglia (particulary in the LGE and MGE) of *Nkx2.1* mutant mice (Kimura et al., 1996; Sussel et al., 1999), whereas inactivation of *Gsh2* impairs the formation of the LGE and striatum and induces ectopic expression of many dorsal markers in the LGE (Szucsik et al., 1997; Toresson et al., 2000; Yun et al., 2001).

As noted, the expression of these transcription factors is under the control of an overall effect of various signaling proteins originally secreted from different “organizer” centers. To date, a total of three types of signaling cues have been identified to be critically implicated in the patterning and regionalization of the developing subpallium: retinoic acid (RA), Fibroblast growth factors (Fgf) and Sonic hedgehog proteins (Shh), (Puelles et al., 2000; Rossant and Tam, 2002; Hoch et al, 2009). Lateral RA signaling that

is originated from the olfactory placode area may play prominent roles in patterning of progenitor areas at the intermediate level, such as the LGE (LaMantia et al. 1993; Pierani et al. 1999). Indeed, Marklund et al. (2004) cultured the prospective intermediate telencephalon from early chick embryos (~stage 14) and treated them with a RA receptor antagonist (BMS-453) *in vitro*. They found that, following exposure to BMS-453, the production and specification of a large percentage of LGE-derived cells can be significantly blocked (Marklund et al., 2004). In addition, exposure of early-born cells isolated from the dorsal telencephalon of stage 8 chick embryos to RA signaling is sufficient to produce many intermediate features in these cells (Marklund et al., 2004). Another signaling factor is Fgf8, which is originally produced in the regions of the anterior neural ridge/cortical plate and is functionally involved in promoting the patterning and specification of the most ventral subdomains in the subpallium (Rossant and Tam, 2002; Lupo et al., 2006). Although targeted deletion of *Fgf8* gene induces an embryonic lethal phenotype (Sun et al., 1999), aberrant overexpression of *Fgf8*, together with ectopic expression of several ventral markers, have been identified in the rostral areas of the dorsal telencephalon of *Gli3* mutant mice (Kuschel et al., 2003). Ectopic expression of ventral markers has also been observed in the prospective dorsal telencephalic explants dissected from early mouse embryos after exposure to Fgf8 signals (Kuschel et al., 2003). Consistent with these results, the ventral telencephalon of conditional *Fgf8* knockouts is smaller and abnormally organized as compared to that of their wild-type littermates (Storm et al., 2006). The patterning of subpallial structures can be also influenced by Shh signaling that is derived from the telencephalic anlage as well as the rostroventral parts of the telencephalon, such as MGE and POA (Rohr et al., 2001;

Lupo et al., 2006). Impaired Shh signals not only lead to the lack of several subpallial structures but also cause the loss of many key ventral markers, such as *Nkx2.1* and *Dlx2* (Chiang et al., 1996; Muenke and Beachy, 2000). Ectopic expression of Shh signaling in the pallium results in a ventral phenotype, such as ectopic expression of ventral markers (e.g. *Nkx2.1*, *Gsh2* and *Dlx2*) and impaired expression of some dorsal markers (e.g. *Emx1* and *Tbr1*) (Kohtz et al., 1998; Gaiano et al., 1999; Corbin et al., 2000).

1.4. Cell migration in the developing telencephalon

1.4.1. Overview of cell migration in the developing telencephalon

After patterning of the telencephalon and specification of different types of neurons within various regions of the developing telencephalon are accomplished, a process called cell migration can be extensively observed in both the pallium and subpallium. According to their distinct origins, different types of neurons basically utilize two general modes of migration during embryonic development in order to reach and populate their final destinations in the telencephalon as well as in other specific areas in the CNS (Figure 1.2). The first is radial migration, occurring predominantly in the pallium as well as in the mammalian striatum, where migrating glutamatergic and GABAergic projection neurons move along the radial axis through their interaction with fiber tracts built by glial cells. However, the underlying molecular mechanisms may be distinct depending on specific local environment (Nadarajah and Parnavelas, 2002; Marin and Rubenstein, 2003). For instance, a major proportion of striatal GABAergic projection neurons is found to be born in the proliferative VZ within the LGE and then radially migrate (at ~E12) towards the developing striatum in an outward direction that is perpendicular to

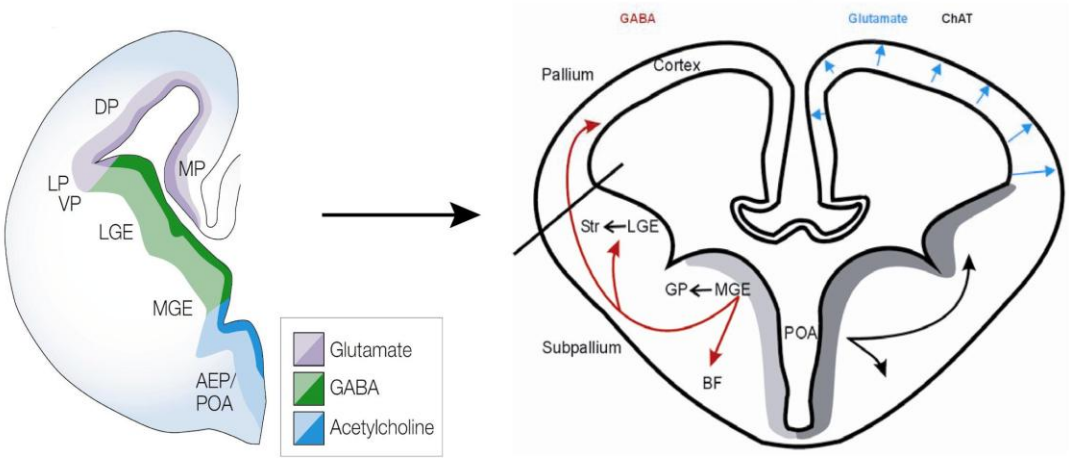
the ventricular surface (Halliday and Cepko, 1992; De Carlos et al., 1996; Hamasaki et al., 2003). The second form of migration is tangential migration, which mainly happens in the progenitor regions (e.g. LGE, MGE and caudal ganglionic eminence, CGE) within the subpallium. Once the processes of proliferation and differentiation are completed, different types of interneurons will tangentially migrate in multiple streams away from the basal telencephalon in a direction that is orthogonal to that of radial migration along both rostrocaudal and mediolateral axes, enter the cortex and eventually occupy their final positions located in different layers of the cortex (radial migration is also involved at this stage) (Nadarajah and Parnavelas, 2002; Marin and Rubenstein, 2003). Considering its closer relevance to my thesis, I will be discussing only tangential migration with some details in the following text.

1.4.2. Tangential migration in the developing telencephalon

As briefly noted above, in the developing mouse forebrain, distinct types of interneurons originate from different progenitor domains within the subpallium and then follow their own specific tangential migratory paths in order to ultimately locate and establish their destined positions in various regions of the forebrain, primarily in the pallium. For example, the progenitor cells that will later differentiate into diverse subtypes of cortical GABAergic interneurons are not generated from the pallium; instead, their origins can be traced back to the proliferative VZ within the LGE, MGE and CGE of the basal telencephalon (Marin and Rubenstein, 2003). After leaving the progenitor regions, the postmitotic immature cells will separate into several superficial or deep migration streams, pass through the developing striatum and intrude into the neocortex in

Figure 1.2. Neuronal migration in the mammalian telencephalon during early embryogenesis

Different types of neurotransmitter are specified in distinct progenitor domains in the developing telencephalon: glutamatergic excitatory neurons are mainly generated and specified within progenitor domains in the pallium, while GABAergic inhibitory neurons as well as cholinergic neurons are derived from progenitor cells within the LGE, MGE and AEP/POA in the subpallium. During early embryogenesis, glutamatergic neurons (blue arrows) migrate radially through different layers of the cortex; in contrast, GABAergic (red arrows) and cholinergic neurons (black arrows on the right) migrate tangentially from the MGE via different routes in order to reach their final destinations in the cortex. Moreover, most GABAergic projection neurons mainly found in the LGE (black arrow on the left) migrate radially from the LGE to the striatum in the developing telencephalon. The black bar marks the boundary between the pallium and subpallium. AEP, anterior entopeduncular area; BF, basal forebrain; DP, dorsal pallium; LGE, lateral ganglionic eminence; LP, lateral pallium; MGE: medial ganglionic eminence; MP, medial pallium; POA, pre-optic area; str, striatum; VP, ventral pallium. Modified from Wullimann (2009).



Progenitor zones

Radial and tangential migration

a tangential manner that is vertical to the orientiation of radial glia fibers (Figure 1.2) (Marin and Rubenstein, 2003). More specifically, three different major streams of neurons near the corticostriatal notch have been revealed by previous cell tracing assays before their invasion into the neocortex (Reviewed in Marin and Rubenstein, 2003; Nadarajah and Parnavelas, 2002; Metin et al., 2006). The first one is found to be derived from the MGE and mainly enter the preplate in the neocortex at ~E12. The cells comprising this stream exhibit unique characteristics representing the typical Cajal–Retzius cells (Lavdas et al 1999). The second tangential migration stream (also the most dominant one) is made up of cells derived from the LGE, MGE and CGE, which mainly travel through the intermediate zone (IZ) in the neocortex at ~E13-E15. At the later stages (~E15 to P0), there exists another migration stream that is composed of cells originating from the LGE and MGE and usually enter the cortex via the lower IZ and SVZ (Anderson et al., 2001). Once arrived in the pallium, these migrating neurons will turn and start moving radially (in waves) towards the pial surface in a fashion highly similar to that of pallial projection neurons (Nadarajah and Parnavelas, 2002). It has been gradually accepted that similar to pallial projection neurons, GABAergic cortical interneurons are also settled in an `inside-out' pattern at various layers of the cortex. Pallial projection neurons and cortical interneurons that are born at the same time points are in general positioned at the same layer (Wichterle et al., 2001; Ang et al., 2003; Valcanis and Tan, 2003; Hevner et al., 2004). Like GABAergic interneurons, progenitor cells of olfactory interneurons are also born in the progenitor areas within the basal ganglia, rather than in the olfactory bulb and, subsequently, migrate tangentially to take up their positions in the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla 1994).

Although the exact origins of olfactory interneurons in the developing subpallium still remain obscure, previous genetic and embryological experiments have provided evidence that a majority of olfactory interneurons may be initially generated in the dorsal subdivision of the LGE (Dellovade et al., 1998; Sussel et al., 1999; Corbin et al., 2000; Wichterle et al., 2001). In addition, the origins of cholinergic interneurons are also considered to be within the MGE as well as within the regions of AEP/POA (Marin et al., 2000; Marin and Rubenstein, 2003). During embryonic development, these neurons can undergo a movement from their origins along a tangentially-oriented trajectory into the LGE (Marin et al., 2000; Marin and Rubenstein, 2003).

The potential molecular mechanisms that are involved in regulating various processes of tangential migration have been well characterized (Marin and Rubenstein, 2003; Nobrega-Pereira and Marin, 2009). A number of molecular cues have been identified, which are capable of potently stimulating the motogenic activity as well as guide the migration of neuronal cells (Marin and Rubenstein, 2003; Nobrega-Pereira and Marin, 2009). Among them, hepatocyte growth factor/scatter factor (HGF/SF), together with its receptor MET, has been reported to participate in driving the migration of GABAergic interneurons, at least in the developing subpallium. Inhibiting normal HGF/SF expression can induce an apparent disorientated migration of cortical interneuron progenitor cells in the ganglionic eminences and hence leads to a significantly reduced number of interneurons in the cortex of newborn mice (Powell et al., 2001). Furthermore, many previous studies have demonstrated that cortical interneuron progenitors express *Neuropilin 1* (*Nrp1*) and *Neuropilin 2* (*Nrp2*) and these two guidance receptors are highly responsive to the chemorepulsive forces that are

exerted by several members of the semaphorin family (mainly Sema 3A and Sema 3F) expressed in the striatal mantle (Marin et al., 2001). As a result, the repulsive activity of semaphorins is believed to act as a key contributing factor for forming a unique exclusion domain in the developing striatum, which can push tangentially migrating neurons into adjacent paths surrounding the striatum (avoiding the striatum) and help to establish different major migration streams when entering the neocortex (Marin et al., 2001; Marin and Rubenstein, 2003).

The guidance of tangentially migrating neurons can also be impacted by proper expression of various transcription factors. For example, the Nkx2.1 transcription factor may be implicated in regulating the sorting of MGE interneuron progenitors through greatly reducing their sensitivity to class 3 semaphorins (Nobrega-Pereira et al., 2008). *Nkx2.1*-positive tangentially migrating cells from the MGE are nonresponsive to chemorepulsive cues produced by the semaphorins and show a markedly decreased level of *Nrp2* expression (Nobrega-Pereira et al., 2008). Intriguingly, in MGE-derived migrating interneurons, the Nkx2.1 protein can directly bind to the regulatory sequences within the *Nrp2* promoter region *in vivo* and modulate the transcription of the *Nrp2* receptor (Nobrega-Pereira et al., 2008). Accumulating evidence has also been shown that tangential migration of MGE-generated interneurons may be influenced by the expression of actin cytoskeletal regulatory proteins or microtubule-associated proteins (e.g. Lis1, Dcx and Dclk), but the precise molecular mechanisms are still unclear at this moment (Kappeler et al., 2006; Friocourt et al., 2007).

1.5. GABAergic interneurons in the telencephalon

Although the neuronal network in the cerebral cortex is fairly complex, it is generally established by two main classes of neurons: excitatory projection neurons and inhibitory local-circuit neurons (also known as interneurons) (Di Cristo, 2007). While projection neurons can send their axons to many different targets either within or farther away from their local environment (e.g. the thalamus, brain stem and spinal cord), inhibitory interneurons mostly set up synaptic connections with nearby projection neurons, inhibit their excitatory activity and maintain the spatio-temporal balance of excitation and inhibition essential for proper telencephalic function (Wonders and Anderson, 2006; Di Cristo, 2007). Projection neurons utilize glutamate as their main neurotransmitter and contribute to ~70-80% of all cortical neurons, whereas around 20-30% of cortical neurons are made up of inhibitory interneurons that basically use GABA as neurotransmitter (Parnavelas et al., 1977; Meinecke and Peters, 1987; De Felipe and Farinas, 1992; Gupta et al., 2002). There also exists a comparatively smaller population of dopaminergic and cholinergic neurons in the cortex than that of glutamatergic and GABAergic cells (Wonders and Anderson, 2006; Di Cristo, 2007). It is worth noting that ~90% of neurons in the striatum of the mammalian telencephalon are projection neurons but they use GABA, rather than glutamate, as their primary neurotransmitter and thus are termed as GABAergic projection neurons (Kawaguchi et al., 1995; Kawaguchi, 1997a; Gerfen, 1992). The remaining ~10% of neurons in the striatum are GABAergic interneurons, which are mainly localized in the patch and matrix compartments of the striatum and produce inhibitory signals to maintain the balance of striatal function (Kawaguchi et al., 1995; Kawaguchi, 1997a; Gerfen, 1992). A very small proportion of

interneurons found in the striatum have also been shown to use dopamine or acetylcholine as their main neurotransmitter (Gall et al., 1987; Kawaguchi et al., 1995).

Unlike projection neurons that have in general similar pyramidal morphology, such as apical and basal dendrites and axons distantly projecting into various sites within the white matter, GABAergic interneurons display a remarkable heterogeneity and different subtypes of GABAergic interneurons are usually classified by their distinct morphology, connectivity patterns, electrophysiological features and biochemical contents (reviewed in Markram et al., 2004; Flames and Marin, 2005; Wonders and Anderson, 2006; Gelman and Marin, 2010). More specifically, GABAergic interneurons can be categorized into the following subgroups according to their morphological shapes, connectivity patterns, electrophysiological properties and their expression of a group of molecular markers reflecting their endogenous biochemical content. Based on the characteristics of axons and dendrites, there are basket cells, bipolar cells, double bouquet cells, bitufted cells, Chandelier cells and Martinotti cells. Based on the location of the branching terminus of their axons, interneurons can be classified as axon-targeting, dendrite-targeting and soma-targeting cells (or any combination of them). Based on their distinct intrinsic firing patterns, GABAergic interneurons can be may further subdivided as fast spiking, burst spiking non-pyramidal, regular spiking non-pyramidal, irregular spiking and late spiking cells. At different stages of either embryonic or adult neurogenesis, GABAergic interneurons are usually classified by detecting their distinct expression patterns of three calcium-binding proteins, calbindin (CB), calretinin (CR) and parvalbumin (PV), and four neuropetides, somatostatin (SOM), neuropeptide Y (NPY), vaso-active intestinal peptide (VIP) and cholecystokinin (CCK) (Markram et al., 2004; Wonders and Anderson,

2006; Gelman and Marin, 2010). It should be emphasized here that under most situations, it is very difficult to precisely distinguish between distinct subtypes of GABAergic interneurons by only checking the expression of one or two of these markers due to the overlaps of their expression profiles. For example, the expression pattern of CB, PV, SOM and CV overlap each other to varying degrees (Kubota et al., 1994; Kawaguchi and Kubota, 1997b). Thereby, using one or more combinations of these molecular markers is required to better define each interneuron subtype, although some previous studies have provided evidence that different interneuron subtypes, such as PV-, CR- and SOM-positive cells, are comprised of largely non-overlapping populations of cells at least in the sensory cortex and suggest that this phenomenon may possibly be applicable to most mammalian species (Kubota et al., 1994; Cauli et al., 1997; Tamamaki et al., 2003).

1.6. Development of the zebrafish telencephalon

1.6.1. Advantages of using zebrafish as a model organism

Teleosts comprise a large and extremely diverse group of fish with bony skeletons, covering approximately 50% of the existing vertebrates (Mueller and Wullimann, 2009). Zebrafish (*Danio rerio*) is a small, freshwater teleost fish that mainly lives in tropical regions, particularly in India and Southeast Asia (Eisen, 1996). In addition to its applicable value in fisheries research, in the past two decades, zebrafish has rapidly emerged as a powerful model organism not only appropriate for developmental and genetic studies, but also for many other research areas, such as carcinogenesis, cardiovascular diseases and biotechnique development (Dodd et al., 2000; Pyati et al., 2007; Ingham, 2009). Compared to mammals, such as the mouse, zebrafish have a

number of unique characteristics that are especially suitable for developmental biology studies. First is their small body size and short generation time. The size of zebrafish embryos is only ~0.7 mm in diameter during fertilization and quickly reaches 3.5 mm within 3 days. Patterning and segmentation of the body plan can be completed within the first day post fertilization (dpf) and the process of primary organogenesis starts at 2 dpf. The vast majority of organs become functional at 3-5 dpf and sexual maturity is accomplished within the first three months of their life. Second, zebrafish have high fecundity. Their embryos are externally fertilized and breeding of one single pair can yield up to hundreds of spawned eggs per day. Third, zebrafish embryos and larvae are optically transparent. By using live-imaging techniques, cellular morphology and movement can be easily and non-invasively observed/traced. Fourth, the genetic tractability of zebrafish, such as high chromosomal integration frequency and good germline transmission when generating transgenic models, allows and facilitates the investigation of a wide variety of developmental events (Westerfield, 1995; Detrich et al., 1999; Kawakami, 2004).

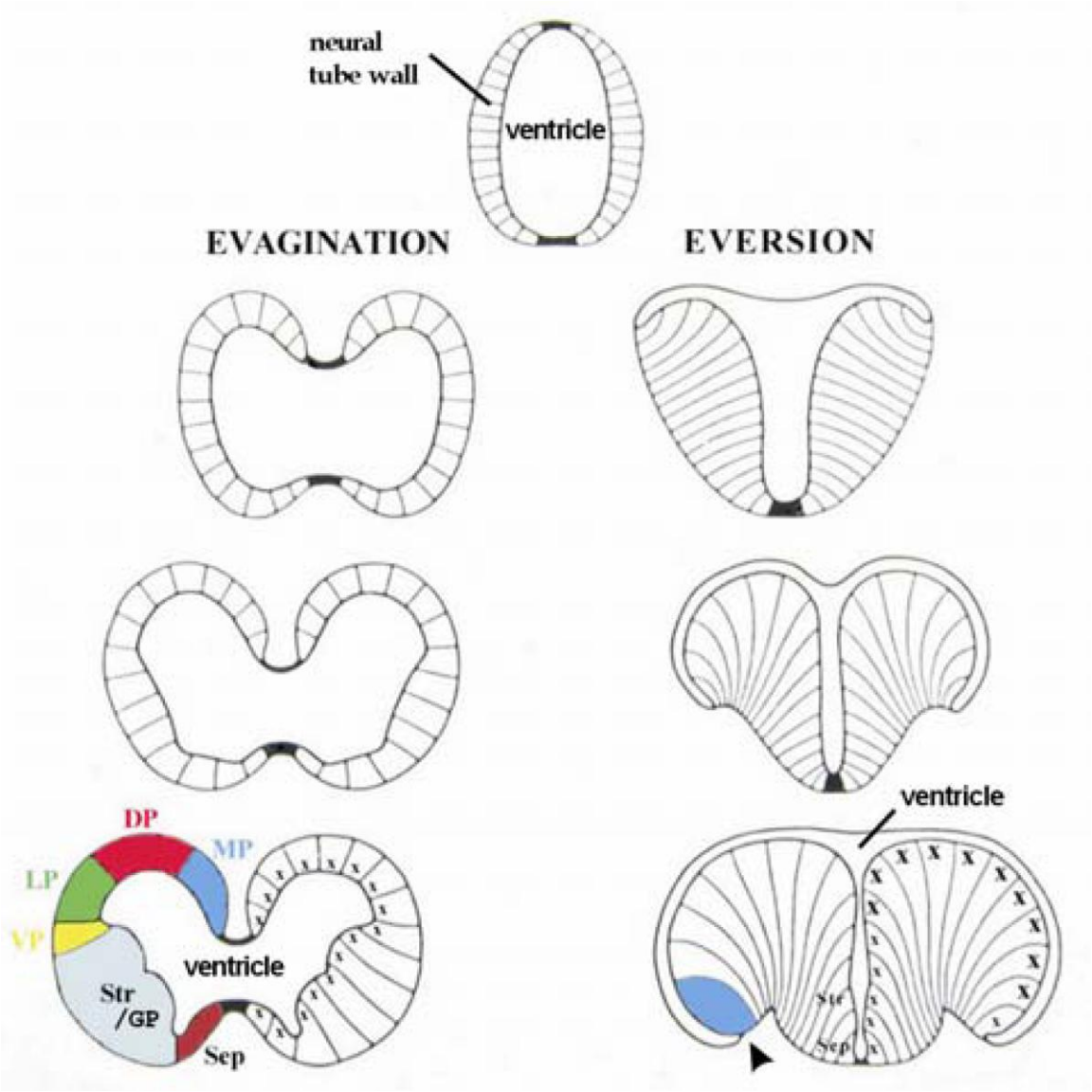
1.6.2. Eversion versus evagination: a different mode of telencephalic development in zebrafish (and other teleost) as compared to most other vertebrate lineages

Similar to that of mammals, the developing zebrafish telencephalon is also made up of four major subdivisions in the pallium (lateral, ventral, medial and dorsal pallium, LP, VP, MP and DP) and a relatively larger ventral subpallium, where increasing numbers of glutamatergic and GABAergic neurons are respectively generated and

differentiatied starting at ~ 2 dpf during early neurogenesis (Mueller and Wullimann, 2009). Despite the fact that the first steps of neural tube formation are in general similar among all vertebrates, the modes of telencephalic morphogenesis differ between teleosts and most other vertebrate groups. Distinct from the evaginated pattern of telencephalic development found in other vertebrates, where the telencephalon is suggested to develop as two hemispheres with a ventricle in the middle, the telencephalon of teleosts (e.g. zebrafish) undergoes an uncommon process of morphogenesis termed as eversion (occuring predominantly in the regions of MP and DP) and leads to a remarkable change of anatomical topography in the telencephalon (Figure 1.3) (Wullimann and Mueller, 2004; Wullimann 2009; Braford, 2009). More specifically, the eversion hypothesis proposes that the pallium at the dorsal midline first rolls out and extends bilaterally, followed by the formation of a non-neuronal epithelial sheet that will subsequently surround and cover the outer surface of the telencephalon. As the consequence of these processes, the proliferative VZ close to the ventricular surface is eventually placed on the outside of the pallium, instead of the inner wall of the two hemispheres as observed in the evaginated telencephalon (Wullimann, 2009). Furthermore, the position of the MP is also significantly altered by lying laterally in the teleost telencephalon, rather than occupying a medial position as seen in the mode of evagination (Wullimann, 2009). Because of these differences, it has been challenging to clearly identify and define the homologies of telencephalic topography between the teleosts and other vertebrates at either developmental or adult stages (Nieuwenhuys, 1999). To date, it still remains largely unknown which molecular mechanisms are responsible for these differences, although Wullimann and Mueller (2004) proposed that the extensive expression of reelin in the

Figure 1.3. Two different modes of telencephalic development from the neural tube in vertebrates

The mode of evagination (left) occurs in a vast majority of vertebrates except for the ray-finned fish. In this process, the telencephalon gradually evolves as two identical hemispheres enclosing a central ventricle. The process of eversion (right) has been proposed to happen duing the telecephalic development of most ray-finned fish, including teleosts. Different from evagination, the pallium in the mode of eversion rolls out bilaterally and eventually makes the proliferative domains that is close to the ventricular surface (labeled with "x") outside of the pallium. DP, dorsal pallium; GP, globus pallidus; LP, lateral pallium; MP, medial pallium; Sep, Septum; Str, striatum; VP, ventral pallium. Note that with respect to the telencephalic development in teleosts, several other hypothetical models have been proposed, such as the partial pallial eversion model (Wullimann and Mueller, 2004) and a new three-dimensional eversion model (Yamamoto et al., 2007). Adapted from Niewenhuys and Meek (1990).


neural
tube wall
ventricle
EVAGINATION
EVERSION
DP
MP
LP
VP
ventricle
Str
/GP
Sep
ventricle
Str
Sep

pallial cells in the telencephalon of teleosts might be one of the potential contributing factors. It is also worthy of mentioning that apart from the simple eversion model, several other hypothetical models, such as a partial eversion model and a more complex three-dimensional eversion model, have been recently proposed (Wullimann and Mueller, 2004; Yamamoto et al., 2007), which definitely will form a good basis for a better understanding of the different telencephalic topography between teleosts and other vertebrates.

1.6.3. Conserved genetic cascades in the developing zebrafish subpallium as compared to the mouse

While the fundamental mode of telencephalic development is divergent between mouse and zebrafish, researchers have made important attempts to compare the embryonic forebrain development between these two distantly related vertebrate species with an aim to identify some homologies between their distinct morphological structures (Mueller et al., 2006; Mueller et al., 2008). By examining the expression patterns of common developmental genes as well as several classic molecular markers for neuronal proliferation and neurogenesis, they discovered a number of anatomical correspondences in both the pallium and subpallium between the mouse and zebrafish forebrain (Wullimann and Mueller, 2004; Wullimann, 2009). Consistent with observations of their mouse orthologs in the developing mouse subpallium, the proneural basic helix-loop-helix (bHLH) gene, *ascl1a* (also known as *zash1a*, an orthologous gene of the mouse *Mash1*) and the regulatory *dlx1a/dlx2a* genes are found to be specifically expressed within restricted domains (close to the ventricular surface) in the zebrafish subpallium

(Figure 1.4A) (Quint et al., 2000; Wullimann and Mueller, 2002; Mueller et al., 2008). Likewise, similar to the expression patterns of their orthologs in the mouse subpallium, the expression domains of LIM-homeobox genes *lhx6* and *lhx7* are predominantly located within in the ventral subpallium (septum, Sv) as well as within the ventral subdivision of the dorsal subpallium (Sdv) in the developing zebrafish telenephalon, but not in the dorsal subdivision of the dorsal subpallium (Sdd) (Figure 1.4B; Mueller et al., 2008). It is worth noting that Sdv and Sdd have been proposed as corresponding homologous structures to the MGE and LGE within the embryonic mouse subpallium (Figure 1.4B; Wullimann, 2009). In addition, *nkx2.1*, whose mouse orthologous gene serves as an essential modulator gene indispensable for proper regional specification within the mouse subpallium, is also found to be expressed in the early developing zebrafish subpallium (Rohr et al., 2001), even though its detailed expression pattern still needs to be further determined. This regional homology between the mouse and zebrafish telencephalon was also seen in the expression pattern of the neurotransmitter GABA (Mueller et al., 2006). In short, these data strongly suggest that despite the difference in the telencephalic development between mouse and zebrafish, the genetic pathways underlying this process may be highly conserved between these two species.

1.7. The *Dlx* gene family

1.7.1. Origin, genomic organization and evolution of the *Dlx* genes

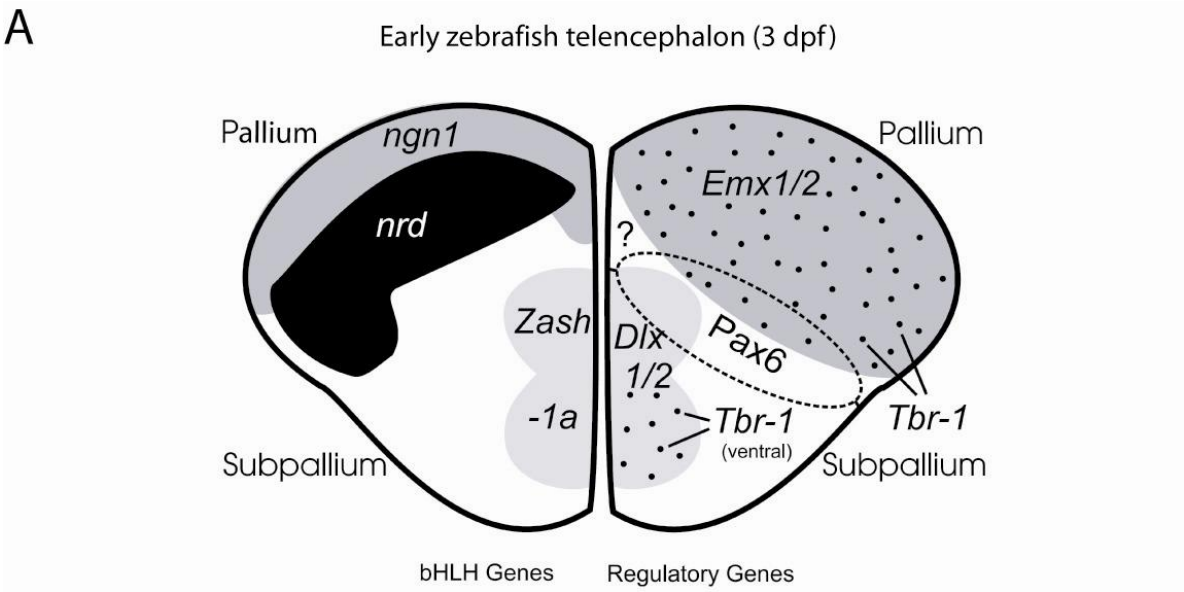
1.7.1.1. The *Drosophila distal-less* (*Dll*) gene

The *Dlx* genes of vertebrates are related in sequence to the *distal-less* (*Dll*) gene that was initially discovered and characterized in *Drosophila* (Panganiban and

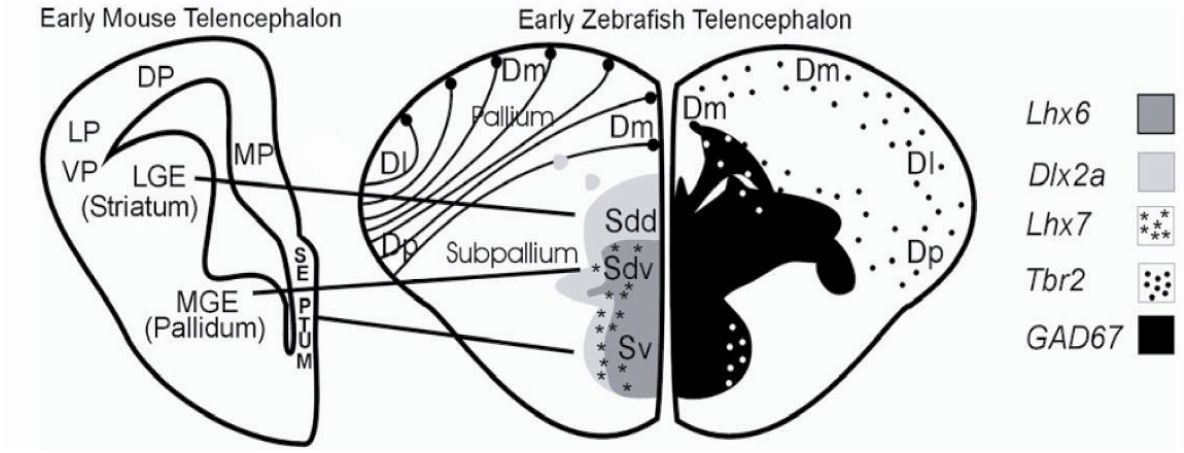
Figure 1.4. Schematic diagrams summarizing expression patterns of various key regulatory genes in the developing zebrafish telencephalon

(**A**) Transverse section of a 3dpf zebrafish telencephalon showing distinct expression domains of a group of important regulatory genes that are commonly used to define different major subdivisions in the developing zebrafish forebrain, such as *zash1a* (*ascl1a*) and *dlx1a/dlx2a* expression in the subpallium. Adapted from Wullimann and Mueller, 2004. (**B**) Structural comparison between the early mouse and zebrafish telencephalon according to the expression patterns of several key regulatory genes (*lhx6*, *dlx2a*, *lhx7*, *tbr2* and *gad67*). The proposed homologous domains between the mouse and zebrafish telencephalon are indicated by solid lines between the corresponding structures. Dl, lateral zones of the dorsal telencephalon; Dm, medial zones of the dorsal telencephalon; Dp, posterior zones of the dorsal telencephalon; DP, dorsal pallium; LP, lateral pallium; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MP, medial pallium; Sdd, dorsal subdivision of dorsal subpallium; Sdv, ventral subdivision of dorsal subpallium; Sv, ventral subpallium; VP, ventral pallium. Adapted from Wullimann (2009).

A


Early zebrafish telencephalon (3 dpf)
Pallium
ngn1
nrd
Zash
-1a
Subpallium
?
Dlx
1/2
Pax6
Tbr-1
(ventral)
Pallium
Emx1/2
Tbr-1
Subpallium
bHLH Genes
Regulatory Genes

B


Early Mouse Telencephalon
DP
LP
VP
LGE
(Striatum)
MP
MGE
(Pallidum)
SEPTUM
Early Zebrafish Telencephalon
Dm
Pallium
Dm
Dl
Dp
Subpallium
Sdd
Sdv
Sv
Dm
Dm
Dl
Dp
Lhx6
Dlx2a
Lhx7
Tbr2
GAD67

Rubenstein, 2002). The *Dll* gene was found to encode a homeodomain-containing transcription factor responsible for controlling proper proximo-distal (P/D) patterning as well as normal specification and organization of several distal appendages, including the limbs, antennae and mouth, during *Drosophila* development. *Dll* null flies die at the early stage of embryogenesis due to the lack of rudimentary larval limbs and antennae, which subsequently serves as the ears and nose of the fly (Cohen et al., 1989; Cohen and Jurgens, 1989; Wu and Cohen, 1999). Consistently, ectopic *Dll* expression can result in abnormal patterning and formation of the limbs and antennae, although the specific phenotypes vary with the specific location where it is expressed (Panganiban and Rubenstein, 2002). In addition to its key role in regulating proper distal appendage development, *Dll* may be also involved in the formation of certain parts of the nervous system in fly. While its specific functional implication in these regions still remains unknown, *Dll* expression has been clearly detected in the two optic lobes of the brain and subsets of glial cells in the ventral nerve cord (Kaphingst and Kunes, 1994; Panganiban and Rubenstein, 2002; Skeath and Panganiban, unpublished observation).

1.7.1.2. Genomic organization of the *Dlx* genes

Vertebrates have multiple *Dlx* genes that are typically organized as convergently transcribed bigene clusters with overlapping expression patterns (reviewed in Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002). For example, a total of six *Dlx* genes have been identified in mouse and human (Price et al., 1991; Robinson et al., 1991; Simeone et al., 1994; Weiss et al., 1994; Nakamura et al., 1996) and they are arranged as three bigene pairs (*Dlx1/Dlx2*, *Dlx3/Dlx4* and *Dlx5/Dlx6*) separated by relatively short

intergenic sequences (<12 kb) (Ghanem et al., 2003). Each of these bigene *Dlx* clusters is also physically linked to a *Hox* gene cluster on distinct chromosomes (Stock et al., 1996). Unlike mammals, some teleost fish including zebrafish contain at least eight *dlx* genes. Six of them are found in the bigene arrangement as *dlx1a/dlx2a*, *dlx3b/dlx4b* and *dlx5a/dlx6a* similar to the six mammalian orthologs (Ekker et al., 1992; Akimenko et al., 1994; Stock et al., 1996; Ellies et al., 1997a). However, the other two additional *dlx* genes (*dlx2b* and *dlx4a*) are not linked to other known *dlx* genes (Stock et al., 1996; Ellies et al., 1997a). All vertebrate *Dlx* genes consist of three exons and two introns and encode 243 to 333-amino acid protein products containing a highly conserved 61 amino acid homeodomain (Liu et al., 1997). According to sequence similarity found in the homeodomain and carboxyl terminus, the six common *Dlx* genes can be generally classified into two subfamilies or clades: *Dlx1/4/6* and *Dlx2/3/5* (Stock et al., 1996; Ellies et al., 1997a). However, the question of whether there is any functional difference between these two groups of *Dlx* genes remains to be answered.

1.7.1.3. Evolution of the vertebrate *Dlx* gene family

Although only one *Dll* gene has been identified in the genome of *Drosophila* and amphioxus, vertebrates have been shown to possess several *Dlx* genes. The tunicate *Ciona intestinalis* has three *Dlx* genes which are organized as one convergently transcribed bigene pair, *Ci-Dll-A/Ci-Dll-B*, and a third unlinked gene, *Ci-Dll-C* (Caracciolo et al., 2000; Irvine et al., 2007). The genome of lampreys contains at least six *Dlx* genes (*LjDlxA-F*), two of which exist as one bigene pair (*LjDlxA*/*LjDlxD*) (Myojin et al., 2001; Neidert et al., 2001; Sumiyama et al., 2003; Kuraku et al., 2009). Similarly,

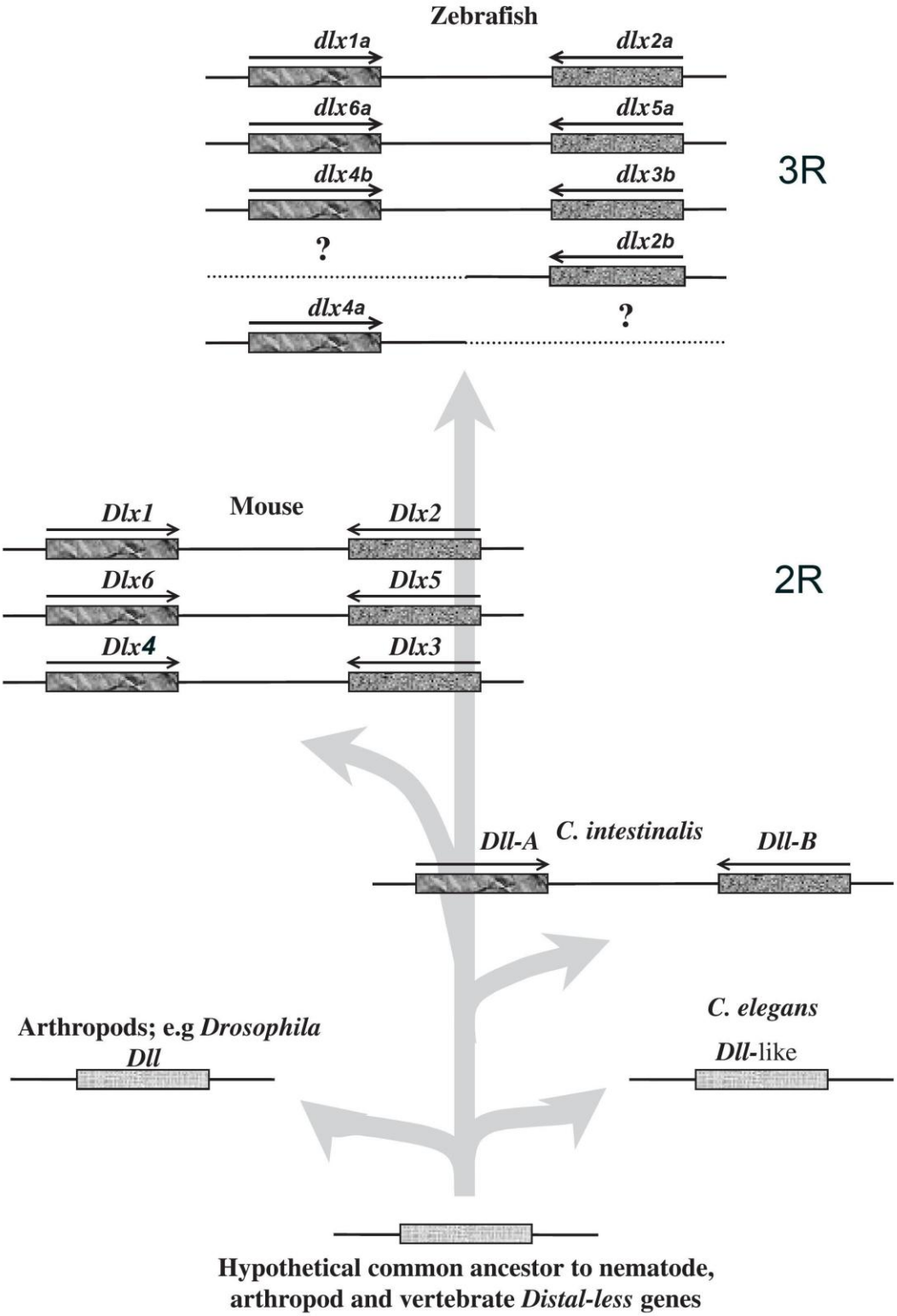
mammals, such as mouse and human, have six *Dlx* genes arranged as three bigene clusters as noted above (Price et al., 1991; Robinson et al., 1991; Simeone et al., 1994; Weiss et al., 1994; Nakamura et al., 1996; Stock et al., 1996). Based on the highly conserved genomic organization of the *Dlx* gene family and the increased number of *Dlx* genes over evolutionary time, a hypothesized model regarding *Dlx* gene evolution has been proposed: an inverted tandem duplication of the single ancestral *Dlx* gene plus two successive rounds of genome duplication events and a subsequent loss of one *Dlx* bigene pair may together contribute to the present organization of six *Dlx* genes as commonly seen in mammals (Figure 1.5) (Zerucha and Ekker, 2000; Sumiyama et al., 2003). With respect to the eight *dlx* genes found in zebrafish or other teleost fish, one explanation would be that an additional genome duplication event specific to certain teleost lineages may likely have occurred that ultimately generates two extra *dlx* genes in the genome. (Figure 1.5) (Stock et al., 1996; Amores et al., 1998; Robinson-Rechavi et al., 2001). Since *Dlx1* shares the highest sequence similarity with the *Drosophila Dll* gene when compared to other vertebrate *Dlx* genes, *Dlx1* has been thought to act as a founding gene during the evolutionary process of the *Dlx* gene family (Holland et al., 1996; Stock et al., 1996).

1.7.2. Expression and function of *Dlx* genes in the forebrain

The *Dlx* genes are expressed in a number of organs and tissues mostly with highly well-organized morphological structures, including the forebrain, branchial arches, sensory organs, limbs/fin buds, bone/cartilage, hematopoeitic and immune systems, and are pivotal for their proper development during embryogenesis and/or later

Figure 1.5. A hypothesized model depicting the evolution of the *Dlx* gene family

Drosophila and *C. elegans* have a single *Dll* gene, while *Ciona intestinalis* has two *Dlx* genes that are organized in a bigene cluster as a result of an initial tandem duplication event. Two rounds of partial or whole genome duplication events (2R) and a subsequent loss of one pair of *Dlx* genes produce the three convergently transcribed *Dlx* bigene clusters which are commonly seen in modern vertebrates, such as mouse. In addition, one more duplication event (3R) specific to certain teleost lineages may likely have occurred, which gives rise to two additional unlinked *dlx* genes as observed in certain fish species, such as zebrafish. The arrow represents the increase of evolutionary time from the theoretical common ancestor to *Dlx* genes in different invertebrate and vertebrate species. Modified from Zerucha and Ekker (2000).



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

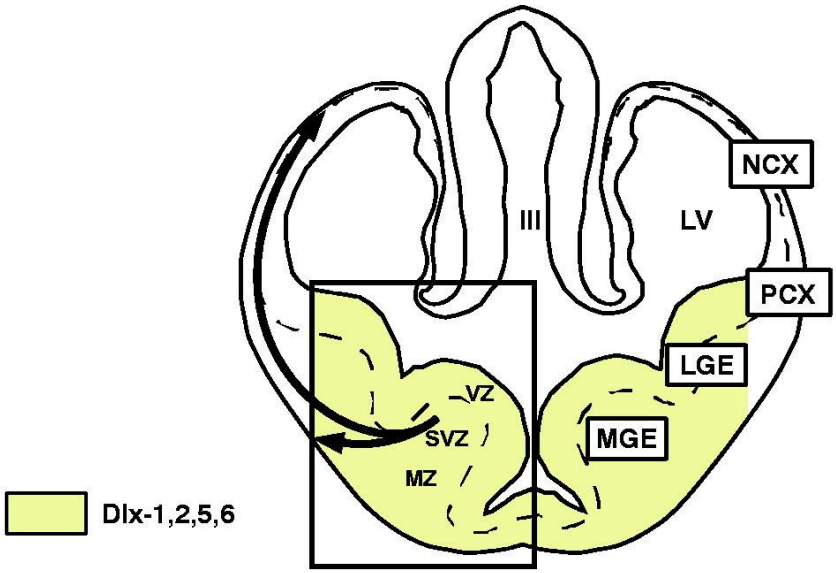
developmental stages (reviewed in Bendall and Abate-Shen, 2000; Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002). In general, there are three major molecular characteristics of *Dlx* expression in these tissues: 1) their expression patterns appear to be evolutionarily conserved among many divergent vertebrate species including human, mouse and zebrafish (Akimenko et al. 1994; Ellies et al. 1997b; Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002); 2) endogenous expression of distinct *Dlx* bigene clusters follows a nested spatial-temportal pattern along the P/D axis, which is particularly apparent in the forebrain and branchial arches (Liu et al., 1997; Qiu et al., 1997; Eisenstat et al., 1999); 3) although subtle differences in the expression of various *Dlx* genes have been observed (and will be further discussed below) that may potentially contribute to their distinct functions, the expression patterns of either paralogous *Dlx* genes from the same bigene pair or those from different clusters are highly overlapping, which make it a very difficult task to distinguish them from one another by simply judging the expression data (Akimenko et al., 1994; Ellies et al., 1997a; Quint et al., 2000). Indeed, a series of loss-of-function studies in mice have revealed that single *Dlx* gene knockouts have relatively milder abnormal phenotypes as compared to that of double *Dlx* null mutants (*Dlx1/2*$^{-/-}$ and *Dlx5/6*$^{-/-}$), which exhibit more severe defects along with some novel malformations in the forebrain and branchial arches (Qiu et al., 1995; Anderson et al., 1997a; Anderson et al., 1997b; Qiu et al., 1997; Acampora et al., 1999; Depew et al., 1999; Cobos et al., 2005a; Cobos et al., 2007; Robledo et al., 2002; Wang et al., 2010). Here, I am only focusing on the expression pattern and functional roles of *Dlx* genes in the forebrain.

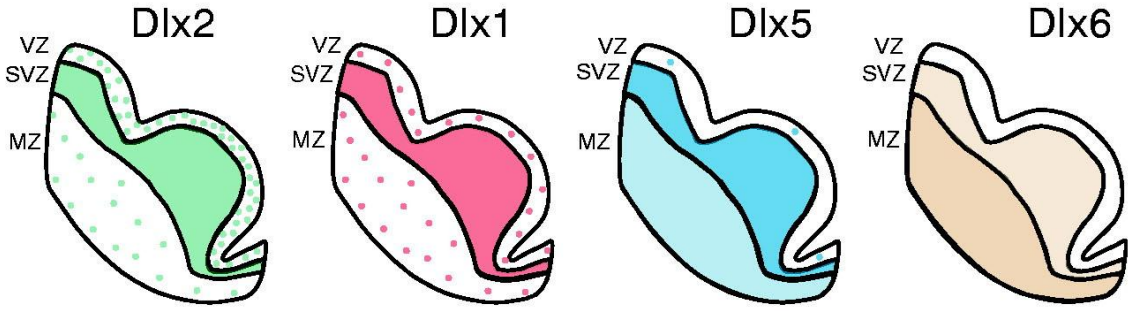
Among the six mammalian *Dlx* genes, four *Dlx* genes (*Dlx2*, *Dlx1*, *Dlx5* and *Dlx6*) are sequentially expressed in the embryonic mouse forebrain (Bulfone et al., 1993b; Liu et al., 1997; Eisenstat et al., 1999; Zerucha et al., 2000). Five out of the eight orthologous *dlx* genes from zebrafish are expressed in the developing zebrafish forebrain: *dlx1a*, *dlx2a*, *dlx5a*, *dlx6a*, and *dlx2b* (Akimenko et al. 1994; Ellies et al. 1997b). *Dlx* gene expression is specifically restricted to two major domains in the developing forebrain: the ventral telencephalon (basal ganglia in the subpallium) and the diencephalon, including both the prethalamus (ventral thalamus) and hypothalamus (Robinson et al., 1991; Bulfone et al., 1993a). These two specific areas have been proposed to be homologous between the mouse and zebrafish forebrain based on the expression data as discussed in previous sections (Mueller and Wullimann, 2009; Wullimann, 2009) and have also been identified in the forebrain of other vertebrates, such as chicks, frogs and lamprey (Fernandez et al., 1998; Puelles et al., 2000; Myojin et al., 2001). In the developing ventral telencephalon of the mouse, various *Dlx* genes display distinct, but highly overlapping, spatial expression patterns, indicative of a possible functional redundancy between each individual *Dlx* gene, at least in certain populations of cells (Figure 1.6): *Dlx1* and *Dlx2* are mainly expressed in the least mature early-born progenitor cells in the VZ and SVZ as well as in a certain number of more differentiated cells scattered in the mantle zone (MZ); *Dlx5* is predominantly expressed in the SVZ and expressed at intermediate levels in the MZ; *Dlx6* is mostly expressed in the most differentiated postmitotic neurons in the MZ and moderate *Dlx6* expression is also found in cells in the SVZ (Bulfone et al., 1993b; Liu et al., 1997; Eisenstat et al., 1999; Zerucha et al., 2000).

In the developing mouse forebrain, *Dlx* genes encode a group of transcription

Figure 1.6. Expression domains of the *Dlx* genes in the mouse ventral telencephalon

Representative schematic of a transverse section of an E12.5 mouse telencephalon showing the combined expression areas of *Dlx1*/*Dlx2* and *Dlx5*/*Dlx6* transcripts (Yellow regions) in the subpallium. The arrows indicate two major tangential migration streams from the subpallium to the neocortex. *Dlx2, Dlx1, Dlx5* and *Dlx6* are sequentially expressed during forebrain development and exhibit distinct spatial but highly overlapping expression patterns in the ventral telencephalon. A majority of progenitor cells within the proliferative regions (VZ and SVZ) of the basal ganglia express one or more *Dlx* genes during the proliferation and/or differentiation process. *Dlx1* and *Dlx2* expression are mainly found in the SVZ as well as some scattered cells in the VZ and MZ. *Dlx5* is mainly expressed in the SVZ with intermediate expression in the MZ. A few scattered *Dlx5*-positive cells can also be identified in the VZ. *Dlx6* is mainly expressed in the MZ and moderately expressed in the SVZ. The number of cells expressing various *Dlx* genes in the VZ, SVZ and MZ is represented by the color (uniform expression: dark color; intermediate expression: light color; scattered expression: dots). Abbreviations: LGE: lateral ganglionic eminence; LV: lateral ventricle; MGE: medial ganglionic eminence; MZ: mantle zone; NCX: neocortex; PCX: palliocortex; SVZ: subventricular zone; VZ: ventricular zone; III: third ventricle. Adapted from Panganiban and Rubenstein (2002).





Model:

Dlx2 → Dlx1 → Dlx5 → Dlx6

factors that play a vital role (amongst others) in manipulating the proliferation, differentiation as well as migration of subpallim-derived GABAergic projection neurons and interneurons that will later populate many different areas in the forebrain, such as the cortex, hippocampus, olfactory bulb and striatum (Anderson 1997a; Bulfone et al., 1998; Marin et al., 2000; Pleasure et al., 2000; Anderson et al., 2001; Stuhmer et al., 2002a). Although several *dlx* genes are also expressed in the zebrafish forebrain, there is presently no systematic study reporting their specific function in this tissue, mainly due to the lack of simple technologies to implement targeted deletions or mutations of *dlx* genes in zebrafish. The function of *Dlx* genes during mouse forebrain development has been primarily assayed in the models of single and double *Dlx* knockout mice (Table 1.1). While *Dlx* single knockouts die very quickly after birth (*Dlx2* and *Dlx5* mutants) or within the first three postnatal weeks (*Dlx1* mutants maintained in a C57BL/6J background) owing to either craniofacial defects or deficiency in the enteric nervous system (Qiu et al., 1995; Qiu et al., 1997; Acampora et al., 1999), forebrain development in these mutant animals seemingly remains largely intact when compared to that of wild-type littermates. However, Cobos et al. (2005) reported that postnatal *Dlx1* mutants (> 1 month old) maintained in a C57BL/6J×CD1 background have a specific loss of several major GABAergic interneurons subtypes (CR-, SOM- and NPY-expressing cells) in the cortex and hippocampus and this phenotype occurs in a time-dependent manner. These mice also exhibit a hyperexcitable and epileptic phenotype possibly as a result of reduced number of GABAergic interneurons and a decreased synaptic inhibition mediated by GABA (Cobos et al., 2005a). Moreover, recent observations in adult *Dlx1*$^{-/-}$ mutant mice revealed that they have significantly increased activities in several behavioral analyses,

such as open field and tail suspension tests, in comparison to wild-type controls (Mao et al., 2009). They also have a reduced response to fear conditioning (Mao et al., 2009). *Dlx2* single mutants have normal morphology and histology of the forebrain during embryogenesis but a reduced number of dopaminergic neurons are found in the olfactory bulb (Qiu et al., 1995). Unfortunately, the potential forebrain phenotypes of *Dlx2*$^{-/-}$ mutants at subsequent postnatal time points cannot be further evaluated because of their death soon after birth (Qiu et al., 1995). Likewise, no apparent abnormalities have been seen in the forebrain of *Dlx5* single mutants except for a decrease of GABAergic neurons in the olfactory bulb (Acampora et al., 1999; Long et al., 2005). To date, the phenotypes of mice lacking the *Dlx6* gene have not yet been documented.

In contrast to *Dlx* single mutants, inactivation of both *Dlx1* and *Dlx2* (although these mice die very shortly after birth) leads to a major block of the production and differentiation of late-born striatal projection neurons (Anderson et al. 1997a; Marin et al. 2000). Most of these neurons remain in the progenitor domains in the ganglionic eminences, rather than radially migrate to their destinations in the striatum (Anderson et al. 1997a; Marin et al. 2000). Similar deficiencies in neurogenesis and neuronal differentiation were also observed in GABAergic, dopaminergic and cholinergic interneuron progenitors born in the LGE and MGE (Anderson et al., 1997a; Bulfone et al., 1998; Marin et al., 2000; Pleasure et al., 2000). These cells accumulate in the basal ganglia and fail to migrate tangentially to the neocortex and hippocampus as well as to the olfactory bulb via the rostral migration stream (Anderson et al., 1997a; Bulfone et al., 1998; Marin et al., 2000; Pleasure et al., 2000). Thus, compared to wild-type mice, *Dlx1/Dlx2* double knockouts display markedly decreased numbers of GABAergic and

Table 1.1. A summary of forebrain phenotypes of single and double *Dlx* knockout mice

Models	Survival	Major phenotypes	References
Dlx1$^{-/-}$	Die within 3 weeks after birth (C57BL/6J) ~50% are viable (C57BL/6J×CD1)	1. Loss of CR-, SOM- and NPY-positive interneurons in the cortex and hippocampus 2. Delayed-onset epilepsy 3. Reduced response to fear conditioning	Qiu et al., 1997; Cobos et al., 2005a; Mao et al., 2009
Dlx2$^{-/-}$	Die shortly after birth	Reduced dopaminergic neurons in the olfactory bulb	Qiu et al., 1995
Dlx5$^{-/-}$	Die shortly after birth	Reduced GABAergic neurons in the olfactory bulb	Acampora et al. 1999; Long et al., 2005
Dlx1$^{-/-}$ */Dlx2*$^{-/-}$	Die shortly after birth	1. Abnormal development of GABAergic, dopaminergic and cholinergic interneurons within the subpallium 2. An almost complete loss of tangential migration from the subpallium to the neocortex	Anderson et al., 1997a; Anderson et al., 1997b; Marin et al., 2000; Pleasure et al., 2000
Dlx5$^{-/-}$ */Dlx6*$^{-/-}$	Viable utill birth	1. Exencephaly in the anterior part of the embryonic brain 2. Impaired tangential migration of GABAergic interneurons to the neocortex 3. Reduced number of PV-positive interneurons	Robledo et al. 2002; Wang et al. 2010

Note that to our best knowledge, *Dlx6* single mutants have not yet been reported thus far.

cholinergic striatal interneurons, olfactory bulb interneurons (>95% are GABAergic and dopaminergic), hippocampal interneurons (>95% are GABAergic) and cortical interneurons (>75% are GABAergic) (Anderson et al., 1997a; Anderson et al., 1997b; Bulfone et al., 1998; Marin et al., 2000; Pleasure et al., 2000; Long et al., 2007). This defective neuronal migration seen in *Dlx1/Dlx2*$^{-/-}$ mutants has been recently shown to be likely due to increased neurite length and reduced branching of migrating cells (Cobos et al., 2007). Furthermore, both *Dlx5* and *Dlx6* expression are almost completely absent in the forebrain of *Dlx1/Dlx2* double knockouts, indicating that *Dlx1* and *Dlx2* are upstream genetic regulators of *Dlx5* and *Dlx6* genes (Anderson et al. 1997a; Zerucha et al. 2000). This interaction between *Dlx1/Dlx2* and *Dlx5/Dlx6* may be mediated by a *cis*-regulatory element (CRE) named I56i that is located in the intergenic sequence between the *Dlx5/Dlx6* bigene cluster (Zerucha et al., 2000, more details on I56i below). While the phenotype in the forebrain of *Dlx5/Dlx6* null mice cannot be assessed in detail because of the exencephaly that also results in the anterior parts of the forebrain, recent laboratory work from Dr. Rubenstein's group showed that simultaneous deletion of *Dlx5* and *Dlx6* genes can significantly slow down the movement of tangentially migrating GABAergic interneurons from the subpallium to the neocortex (Wang et al., 2010; Wang and Rubenstein, personal communication). However, the impact on their migration is not as severe as that seen in *Dlx1/Dlx2*$^{-/-}$ mutants (Anderson et al., 1997a; Bulfone et al., 1998; Marin et al., 2000; Pleasure et al., 2000). *Dlx5/Dlx6*$^{-/-}$ mutants also have a selectively reduced number of PV-expressing cortical interneurons with increased dendrite branching (Wang et al., 2010). Given that *Dlx1* is preferentially involved in the production and maturation of CR-, SOM- and NPY-positive subclasses of cortical

interneurons (but not PV-expressing cells) (Cobos et al., 2005a), it is reasonable to infer that different individual *Dlx* genes may be specifically required for proper development and/or function of distinct subtypes of GABAergic interneurons.

1.7.3. Regulation of *Dlx* gene expression

1.7.3.1. Upstream regulators of *Dlx* genes in the forebrain

To better understand the contributions of *Dlx* genes to the complicated genetic regulatory network during the forebrain development, it is of significant importance to identify and characterize their upstream transcriptional or post-transcriptional modulators. Unfortunately, so far, only a few signaling molecules and transcription factors have been revealed to participate in regulating *Dlx* expression in the developing forebrain. Although the mechanism by which they interact with each other is still unclear, administration of RA to early mouse and zebrafish embryos, before the occurrence of neural crest cell migration, leads to a rapid and marked inhibition of expression of several *Dlx* genes in ectomesenchymal cells (Ellies et al., 1997b; Vieux-Rochas et al., 2007). In addition, Gaiano et al. (1999) reported that ectopic expression of Shh in the pallium of the early developing telencephalon starting at ~E8.5 stimulates and induces ectopic dorsal expression of several ventral markers including *Dlx2* at the sites of ectopic Shh. The level of *Dlx2* expression was found to be significantly declined in the embryonic forebrain of *Shh-/-* mutant mice (Ohkubo and Rubenstein, unpublished data). Other known upstream regulators of *Dlx* genes include the bHLH transcription factor Mash1 and the Fez1 forebrain-specific zinc finger protein (Long et al., 2009a; Yang et al., 2001). Previous *in vitro* assays have shown that when *Mash1* is ectopically expressed in the developing

mouse pallium, ectopic *Dlx1* expression can be induced in the same domains (Fode et al., 2000; Stuhmer et al., 2002b). Further to these data, our laboratory recently found that the Mash1 protein could physically bind to an E-box sequence present in an I12b enhancer element located in the *Dlx1/Dlx2* intergenic region and regulate *Dlx1/Dlx2* expression (Poitras et al., 2007, more information on I12b will be provided below). Forced overexpression of *Fez1* results in ectopic expression of both *dlx2a* and *dlx6a* in the early embryonic zebrafish forebrain, whereas morpholino-based inhibition of *Fez1* expression significantly reduces *dlx2a* expression in the ventral forebrain as compared to that in control embryos (Yang et al., 2001).

1.7.3.2. Downstream targets of *Dlx* genes in the forebrain

Although increasing evidence has shown that Dlx proteins can directly target *Dlx* genes by themselves and regulate their own expression through either cross-regulatory or auto-regulatory mechanisms, up to the present, our general knowledge regarding other direct transcriptional downstream targets of various *Dlx* genes in the forebrain is still very limited. In recent years, a lot of effort has been made to identify additional downstream genes that can be regulated by *Dlx* genes mostly through conducting microarray analyses in *Dlx* knockout mice. Based on gene expression array analysis in *Dlx1/Dlx2* double mutants, Long and coworkers identified a large body of potential downstream candidate genes of *Dlx1* and *Dlx2* in the the subpallial ganglionic eminences, olfactory bulb and striatum (Long et al., 2007; Long et al., 2009a; Long et al., 2009b). Likewise, a microarray assay comparing gene expression profiles between *Dlx1/2*$^{-/-}$ and *Dlx1/2*$^{+/-}$ MGE cells also screened many potential *Dlx* target genes, such as *Pax3*, that are involved

in the regulation of neuronal differentiation and migration (Cobos et al., 2007). More specifically, *Dlx* genes have been demonstrated to act as upstream modulators of *Gad1* and *Gad2* genes, which encode a key rate-limiting enzyme controlling GABA synthesis. Stuhmer et al. (2002b) showed that ectopic *Dlx2* and *Dlx*5 expression *in vitro* are sufficient to induce ectopic expression of both *Gad1* and *Gad2* in many domains of the mouse forebrain. In addition, there have been studies reporting that Dlx1, Dlx2 and Dlx5 proteins can activate the reporter gene expression via acting through an enhancer element identified within the *Gad1* locus *in vitro* (reviewed in Panganiban and Rubenstein, 2002). Consistently, the level of *Gad1* transcript is significantly down-regulated in *Dlx1/Dlx2* null mice (Long et al., 2009a; Long et al., 2009b).

Nrp2 is another well-established direct downstream target of *Dlx* genes. Chromatin immunoprecipitation (ChIP) analysis using E13.5 ganglionic eminences revealed that Dlx1 and Dlx2 proteins can directly bind to a *Nrp2* promoter region 2 (*Nrp2-ii*) *in vivo* (Le et al., 2007). The authors showed that both recombinant Dlx1 and Dlx2 proteins can also bind to this specific region of *Nrp2* promoter *in vitro* and inhibit the transcription of *Nrp2* (Le et al., 2007). These results are further supported by the evidence that disruption of *Dlx1* and *Dlx2* function causes elevated and ectopic *Nrp2* expression in the ventral telencephalon and GABAergic interneurons abnormally overexpressing *Nrp2* accumulate in the LGE and MGE instead of migrating to the neocortex (Marin et al., 2001; Marin and Rubenstein 2003). Moreover, the *Aristaless* (*Arx*) gene, another key player involved in promoting tangential migration of GABAergic interneurons, has been shown to be a direct target of the Dlx2 protein (Colasante et al., 2008). Very interestingly, the

Aristaless-like gene in *Drosophila* is also found to be a downstream target of the *Dll* gene (Depew et al., 2002).

1.7.3.3. *Cis*-regulatory elements (CREs) of *Dlx* genes

The highly conserved genomic organization of multiple *Dlx* bigene clusters among different distant vertebrate species provides us with hints that this genomic arrangement may have important implications and thus have gained selective advantage through the evolutionary process. This viewpoint is further supported by the fact that the expression patterns of orthologous *Dlx* genes in distant vertebrates also appear to be highly conserved in many tissues, including the forebrain, even after a number of genomic duplication events during evolution. One possible explanation for these phenomena would be that the corresponding regulatory mechanisms essential for maintaining *Dlx* expression and function, such as their *cis*- and *trans*-acting elements or certain key signaling pathways, may have been retained by selective pressure during evolution of the *Dlx* gene family. For example, it is likely that sharing of different CREs within the locus of a given *Dlx* bigene cluster (either individually or in combination) may not only help maintain the organization of the two physically-linked *Dlx* genes as a pair through evolution, but also contribute, at least in part, to the overlapping expression pattern and possibly redundant activitiies of paralogous *Dlx* genes.

In the past ten years, our laboratory has employed phylogenetic footprinting to investigate the noncoding sequences surrounding the *Dlx1/Dlx2 and Dlx5/Dlx6* loci and screened several well-conserved CREs across the genomes of five highly divergent vertebrates (mouse, human, zebrafish and two species of pufferfish). Two conserved

CREs, I56i (~440 bp) and I56ii (~310 bp), were identified in the intergenic region of the *Dlx5/Dlx6* bigene cluster of the five different species (I56i: sequence identity > 81%, I56ii: sequence identity > 84%), both of which are able to target expression of reporter transgenes to the basal telencephalon and diencephalon of transgenic mouse and zebrafish in a pattern that highly resembles that of endogenous *Dlx* expression (Figure 1.7A-B) (Zerucha et al., 2000; Ghanem et al., 2003). Transgenic experiments showed that the I56i CRE can also drive reporter gene expression to branchial arches, specifically to the mandibular component of the first branchial arch and the second branchial arch (Figure 1.7B) (Zerucha et al., 2000; Ghanem et al., 2003; Park et al., 2004). In addition, co-transfection assays in cultured cells and DNA-protein binding analyses revealed that Dlx1/Dlx2/Dlx5 protein can activate transcription through binding to putative Dlx binding sites within the I56i sequence, suggesting that I56i CRE may be important for establishing cross-regulatory interactions between various *Dlx* genes (Zerucha et al, 2000; Stuhmer et al., 2002b). Interestingly, similar to a significant reduction of endogenous *Dlx5/Dlx6* expression, the expression of a *lacZ* reporter gene under control of I56i CRE is barely detectable in *Dlx1/Dlx2*$^{-/-}$ mice (Anderson et al., 1997b; Zerucha et al., 2000). These compelling findings suggest that *Dlx1* and *Dlx2* may act as upstream modulators of *Dlx5* and *Dlx6* by functioning via the I56i enhancer (Anderson et al., 1997b; Zerucha et al., 2000).

Data from Kohtz' group also pointed out that *Evf2*, an ultraconserved noncoding RNA that is partially encoded within I56i, is able to recruit Dlx and Mecp2 proteins to I56i by forming a stable complex, impact its transcriptional activity and then regulate *Dlx5*, *Dlx6* and *Gad67* expression (Feng et al., 2006; Bond et al., 2009). *Evf2*$^{TS/TS}$ mutant

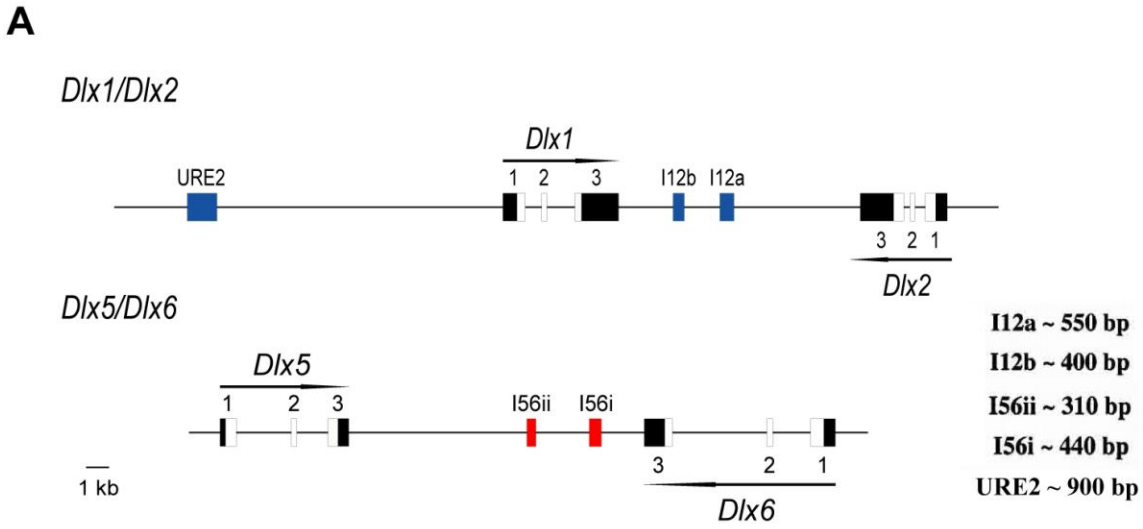
mice (P0) completely lacking the ultraconserved region of *Evf2* have reduced levels of *Gad67* expression and a marked decrease (40-65%) of GABAergic interneurons in the dentate gyrus and CA1 and CA3 regions of the hippocampus (Bond et al., 2009).

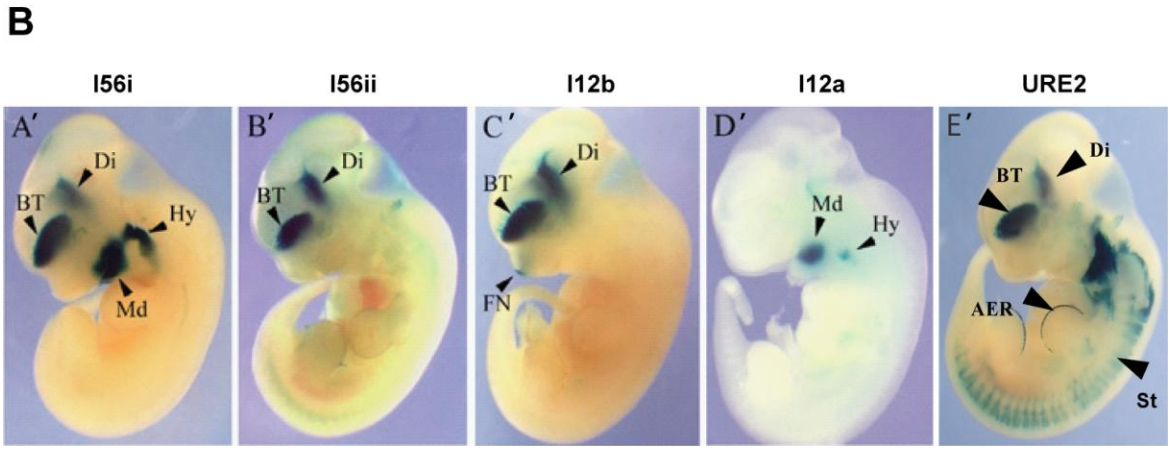
Hamilton et al. (2005) searched sequence variances within *DLX1/DLX2* and *DLX5/DLX6* loci in patients with autism and found a total of 31 single nucleotide polymorphisms (SNPs) and two insertion/deletion polymorphisms. Interestingly, one of them is an adenine to guanine alteration at position 182 within one of the two putative DLX binding sites identified in I56i CRE (Zerucha et al., 2000). By using a transgenic approach in mice, we recently demonstrated that this SNP significantly affects the endogeneous activity of I56i CRE and results in a remarkable reduction in reporter gene expression in the progenitor regions of the early developing telencephalon, in the tangentially migrating streams of GABAergic neurons as well as in various GABAergic interneuron subtypes in the somatosensory cortex of adult animals (Poitras et al., 2010). Our *in vitro* analyses, such as EMSA and co-transfection assays, also showed that this SNP is sufficient enough to impair the physical binding of several Dlx proteins to I56i and significantly decreases the transcriptional activity of the I56i enhancer in response to Dlx2 and Dlx5 proteins (Poitras et al., 2010). These novel findings suggest that disrupted activity of individual *Dlx* CREs due to the presence of a single nucleotide alteration can destroy proper *Dlx* function and trigger abnormal development of GABAergic neurons, which has been found to be related to many human neurological disorders.

Likewise, two conserved CREs, I12a (~550 bp) and I12b (~400 bp), were also discovered in the intergenic region of *Dlx1/Dlx2* bigene cluster with a high sequence identity among human, mouse, zebrafish and two species of pufferfish (I12a: sequence

Fig.1.7. Genomic organization of the mouse *Dlx* genes and the activities of various *Dlx* enhancers

(**A**) Four *Dlx* genes are expressed in the mouse forebrain and are typically organized in convergently transcribed bigene pairs (*Dlx1/Dlx2* and *Dlx5/Dlx6*). The *Dlx1* and *Dlx2* locus contains three highly conserved regulatory elements: upstream regulatory element 2 (URE2), I12b and I12a. I12b and I12b are located in the intergenic sequence between *Dlx1* and *Dlx2*. The intergenic region of *Dlx5* and *Dlx6* genes contains two conserved regulatory elements named I56i and I56ii. Exons are numbered (black, untranslated region; white, coding sequences). Regulatory sequences are shown as blue (URE2, I12b and I12a) and red boxes (I56i and I56ii). **(B)** Enhancer activities of various *Dlx* regulatory elements in E11.5 transgenic mouse embryos. I56i, I56ii, I12b and URE2 regulatory elements target *lacZ* reporter gene expression to the basal telencephalon (BT) and diencephalon (Di) of the mouse forebrain, whereas I12a specifically directs *lacZ* reporter gene expression to the mandibular (Md) component of the first branchial arch and the second branchial arch (Hy). Similar to I12a, strong activity of I56i enhancer is also detected in the Md and Hy. URE2 enhancer is also able to drive *lacZ* reporter gene expression to the apical ectodermal ridge (AER) of the limb buds and somites (St). FN, frontonasal prominence. Modified from Ghanem et al. (2003), Park et al. (2004) and Ghanem et al. (2007).


A
Dlx1/Dlx2
Dlx1
URE2
1 2 3
I12b I12a
3 2 1
Dlx2
Dlx5/Dlx6
Dlx5
1 2 3
I56ii I56i
3 2 1
Dlx6
1 kb
I12a ~ 550 bp
I12b ~ 400 bp
I56ii ~ 310 bp
I56i ~ 440 bp
URE2 ~ 900 bp


B
I56i
I56ii
I12b
I12a
URE2
A′
Di
BT
Hy
Md
B′
Di
BT
C′
Di
BT
FN
D′
Md
Hy
E′
Di
BT
AER
St

identity > 92%, I12b: sequence identity > 77%, Ghanem et al., 2003) (Figure 1.7A). The I12b CRE was found to specifically target reporter gene expression to the basal telencephalon and diencephalon in the mouse forebrain (Figure 1.7B) (Ghanem et al., 2003), whereas I12a targets *lacZ* expresnsion to the mandibular part of the first branchial arch and the second branchial arch, similar to the areas that are targeted by I56i in the branchial arches (Figure 1.7B) (Park et al., 2004). The expression patterns of *I12b-lacZ* and *I12a-lacZ* transgenes faithfully recapitulate the patterns of endogenous *Dlx1/Dlx2* expression in the forebrain and branchial arches. An additional CRE, named URE2 (Upstream Regulatory Element 2, ~900 bp), located at ~12 kb upstream of the *Dlx1* start codon, was identified with > 75% sequence identity between mouse and zebrafish (Ghanem et al., 2007; MacDonald et al., 2010b) (Figure 1.7A). Analyses in transgenic mice showed that URE2 CRE not merely drives reporter gene expression to the basal telencephalon and diencephalon, but also to the apical ectodermal ridge (AER) of the limb buds and to the somites (Figure 1.7B) (Ghanem et al., 2007). Despite the fact that the forebrain expression patterns of the *lacZ* reporter gene targeted by I56i, I56ii, I12b and URE2 appear to be highly similar in whole-mount mouse embryos, further studies on transverse telencephalic sections at various stages revealed some differences between *I56i-lacZ*, *I12b-lacZ* and *URE2-lacZ* transgene expression (Ghanem et al., 2003; 2007). Indeed, a detailed comparison between I56i, I12b and URE2 enhancer activity showed that these three individual CREs target overlapping but distinct populations of GABAergic interneurons in the ganglionic eminences as well as in the cortex of adult animals (Ghanem et al., 2007; Potter et al., 2009). Moreover, a recent study regarding the functional importance of I12b CRE showed that the proneural transcripton factor Mash1

can modulate *Dlx1/Dlx2* expression as a key upstream regulator through directly binding to I12b CRE (Poitras et al., 2007). I12b was also reported to be a direct target of Dlx2 protein and correct *Dlx1* and *Dlx2* expression might be importantly involved in maintaining the proper activity of I12B CRE (Potter et al., 2009).

A total of five conserved CREs with size ranging from ~180 to ~358 bp have been found in the intergenic locus between *Dlx3* and *Dlx4* genes and they all display high sequence identity (over 82%) between mouse and human genome (Sumiyama et al., 2002). I37-2, one of these five CREs, is physically close to *Dlx4* gene and contains two potential homeodomain binding sites as well as two putative Dlx binding sites (Sumiyama and Ruddle, 2003). In transgenic mouse assays, this enhancer can target reporter gene expression to mesenchymal cells in the first and second branchial arches and the expression domains closely mimic that of endogenous *Dlx3/Dlx4* expression in the branchial arches (Sumiyama and Ruddle, 2003). Moreover, at least one of these five CREs was also found in the zebrafish *dlx3b/dlx4b* intergenic sequence (M. Ekker, unpublished observation).

1.7.3.4. Consensus Dlx-binding motifs present in various *Dlx* CREs

So far, several potential binding motifs that can be recognized and bound by Dlx proteins have been identified and characterized. One representative example is an optimal consensus DNA sequence, namely (A/C/G)TAATT(G/A)(C/G), which was originally found as a potential binding module for the *Xenopus* Dlx3 protein and then was proved to be a binding domain for many other Dlx proteins *in vitro* (Feledy et al., 1999). Thus, the authors claim that most of Dlx proteins, if not all, may share similar DNA-binding

activity at least under certain circumstances (Feledy et al., 1999). Although the various *Dlx* CREs identified by our laboratory exhibit some overlapping activities when tested in transgenic mouse and zebrafish, these regulatory elements show only limited sequence similarity (Zerucha et al., 2000; Ghanem et al., 2003; Ghanem et al., 2007), supporting a notion that these individual regulatory elements may be responsive to and modulated by different transcription factors, in addition to Dlx proteins. Previous *in vitro* analyses in culture cells and embryonic brain slices revealed that zebrafish dlx1a/2a/3b/5a/6a proteins as well as mouse Dlx1/2/5 proteins can activate the transcription of reporter genes by acting on the two TAATT Dlx-binding sites found in the I56i CRE (Zerucha et al. 2000; Stuhmer et al. 2002b). Mutagenesis of one or both of these two motifs could significantly disrupt the binding of Dlx proteins to I56i CRE and hence cause a remarkably decreased reporter gene activity in the forebrain of transgenic animals (Zerucha et al., 2000). Additionally, DNA I footprinting assays using nuclear extracts from E13.5 mouse telencephalon and two DNA fragments comprising I12b CRE suggested that I12b contains six protein-protected areas (footprints), each of which indicates a putative protein-DNA binding domains (Poitras et al., 2007). Among them, two putative TAATT Dlx binding sites and a CANNTG E-box binding site (that can be directly bound by the transcriptioin factor Mash1, as mentioned earlier) were shown to be important for correct *lacZ* reporter gene expression in the basal telecephalon and diencephalon of transgenic mice (Poitras et al., 2007; Potter et al., 2008). Mutations of the three binding sites present in I12b result in loss of reporter gene expression, to varying degrees, in many domains of the telencephalon and diencephalon (Poitras et al., 2007). In particular, simultaneous disruption of the two Dlx binding motifs can lead to an

almost entire loss of basal telencephalic and diencephalic expression (Poitras et al., 2007). Aside from the consensus sequence motifs discussed above, researchers also identified a number of TAAT/ATTA core binding motifs at several locations in the sequences of both I56i and I56ii CREs (Zerucha et al., 2000; Ghanem et al., 2003; Zhou et al., 2004), which may play a potential role in recruiting Dlx proteins or other homeodomain-containing transcription factors (e.g. Hox and Islet1 proteins) probably involved in regulating *Dlx* function (Gehring et al., 1994; Pankratova and Polanovsky, 1998).

Statement of Inquiry

During the process of embryogenesis, the CNS including the brain and spinal cord is gradually established and organized by numerous different histological structures, which are originally formed by diverse types of neuronal cells. The correct differentiation of multipotential progenitor cells into specific types of neuronal populations at different developmental stages is under the control of a complicated and precisely-regulated genetic network. The vertebrate *Dlx* genes are typically organized as multiple bigene pairs and are sequentially expressed in a spatial and temporal pattern in the developing forebrain. Specifically, various *Dlx* genes encode a group of homeodomain-containing transcription factors that play essential roles in regulating proper differentiation, migration and survival of GABAergic neurons that will later populate different regions in the telencephalon and diencephalon. Furthermore, the forebrain expression pattern of *Dlx* genes is highly overlapping with that of the two *Gad* genes coding enzymes responsible for synthesizing the inhibitory neurotransmitter GABA and expression of several *Dlx* genes has been shown to be sufficient to induce *Gad* expression both *in vitro* and *in vivo*. Proper *Dlx* forebrain expression is modulated at least in part by several regulatory elements that are originally identified in their surrounding sequences. These regulatory elements appear to be highly conserved among various distantly related vertebrate species, some of which are even ultraconserved, suggesting that they might have undergone a strong evolutionary selection together with the *Dlx* genes over evolutionary time. Using transgenic analysis in mice, our laboratory has found that at least four of those elements, URE2 and I12b in the *Dlx1/Dlx2* loci and I56i and I56ii in the *Dlx5/Dlx6* loci, act as forebrain-specific enhancers by targeting reporter gene expression in a pattern

that strictly resembles endogenous *Dlx* forebrain expression. Moreover, a detailed comparison between URE2, I12b and I56i enhancer activity revealed that these enhancers may regulate *Dlx* expression in distinct but highly overlapping subpopulations of GABAergic interneurons in the developing telencephalon. However, our overall understanding of *Dlx* gene regulation via these various individual enhancers during forebrain development is still very limited in many aspects.

In my PhD project, I have attempted to address several unresolved questions regarding *Dlx* regulation in the developing mouse and zebrafish forebrain: 1) Does another enhancer I56ii possess a similar activity when compared to that of the other three forebrain-specific enhancers (I56i, I12b and URE2; note that I12a was not included for comparison since it is only active in the branchial arches, Park et al., 2004)? 2) Does sequence conservation of these regulatory elements indicate a degree of similar function across the developing forebrain of distantly related vertebrates (e.g. zebrafish), at least during the development of GABAergic neurons? 3) What is the relative contribution of the various forebrain individual enhancers to overall *Dlx* expression? 4) What are the potential impacts of loss of an individual *Dlx* enhancer on the process of forebrain development?

In chapter II of my doctoral thesis, I have specifically answered the first question. In collaboration with Dr. Noël Ghanem, I have conducted a detailed spatio-temporal analysis of I56ii activity in the developing telencephalon between E10.5 and E15.5, and compared its regulatory activity with that of the other three *Dlx* enhancers using *lacZ* reporter genes in transgenic mice. We found that unlike the other three enhancers, I56ii does not label interneuron progenitors in the subpallial telencephalon; instead, I56ii-

positive cells are a subpopulation of GABAergic projection neurons expressing the striatal markers *Meis2* and *Islet1*.

In chapter III, I aimed to address the above-noted second question with a focus on the potential implications of I56i and I56ii enhancers in the *dlx*-mediated regulatory pathways during zebrafish GABAergic neuron development. I have established two independent lines of transgenic zebrafish with reporter constructs under the control of different combinations of I56i and I56ii enhancers and found that these regulatory elements exhibit a fairly conserved activity in the zebrafish forebrain when compared to their corresponding mouse orthologs. We attempted to knock down *dlx1a/dlx2a* and *dlx5a/dlx6a* expression by using morpholino oligonucleotides and found that proper *dlx* expression in the forebrain by acting through the I56i and I56ii enhancers is essential for proper GABAergic neuron production in the diencephalon of zebrafish forebrain but seems less important for those in the telencephalon, a result that is not consistent with that observed in mice. Therefore, we propose that the *dlx*-mediated genetic cascades controlling GABAergic neuron development are evolutionarily conserved in the prethalamus but maybe have undergone divergence in the telencephalon between zebrafish and mammals over evolutionary time. Also, other regulatory programs in addition to the *dlx*-mediated ones may be functionally involved in controlling the establishment of diverse GABAergic interneuron subtypes in the developing zebrafish forebrain.

Finally, in the study reported in chapter IV, I answered partially the third and fourth questions with the purpose of understanding the specific contribution of the I12b enhancer to overall *Dlx* regulation as well as to the process of GABAergic neuron

generation. Working with Dr. Luc Poitras, I have successfully generated a mutant mouse strain with a targeted deletion of the I12b enhancer. My data demonstrated that although mice homozygous for I12b deletion are viable, develop normally, and display no overt morphological abnormalities in the forebrain, targeted disruption of this element leads to a significant decline of *Dlx1* and *Dlx2* levels and seemingly could interfere with normal cell proliferation in the ventral telencephalon, at least in the progenitor domains in the LGE and MGE. These initial phenotype analyses will be of important resource for our future studies and will eventually help us better understand the relative contribution of I12b to *Dlx* forebrain expression as well as to the regulatory cascades associated with this enhancer during vertebrate forebrain development.

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

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Abstract

Regulation of region-specific neuronal differentiation and migration in the embryonic forebrain is a complex mechanism that involves a variety of transcription factors such as the *Dlx* genes. At least four *cis*-acting regulatory elements (CREs) are responsible for the *Dlx* transcriptional regulation in the subcortical telencephalon and the rostral diencephalon. These include I12b and URE2 in the *Dlx1/2* bigene cluster, and, I56i and I56ii in the *Dlx5/6* cluster. We previously reported that URE2, I12b, and I56i, mark different progenitor cell populations in the ganglionic eminences as well as different subtypes of adult cortical interneurons. Here, we carried out a detailed spatial and temporal analysis of the I56ii CRE activity in the developing telencephalon between E10.5 and E15.5, and compared its activity with the other three *Dlx* CREs using *lacZ* reporter genes in transgenic mice. We show that I56ii marks distinct group(s) of neurons located in the superficial mantle of the LGE and MGE between E11.5 and E13.5. The I56ii-positive cells are *Dlx*- and GABA-immunoreactive. However, unlike the other CREs, I56ii does not label interneuron progenitors in the basal ganglia, nor tangentially migrating cells to the cortex at E13.5. Instead, I56ii-positive cells mark a subpopulation(s) of post-mitotic projection neurons that tangentially migrate from the LGE to the deep mantle of the MGE and reside between the subventricular zone and the globus pallidus during midgestation. The majority of these neurons express the striatal markers *Meis2* and *Islet1*. Moreover, both *Meis2* and *Islet1* activate transcription of a reporter gene containing the I56ii sequence in co-transfection assays, indicating that these transcriptional factors may be potential upstream modulators of the *Dlx* genes *in vivo*.

2.1. Introduction

Over the past fifteen years, genetic studies have focused on elucidating the mechanisms underlying the development of the telencephalon, one of the most complex and diverse regions in the central nervous system (CNS). Several models of telencephalic organization and development have emerged implicating a diverse array of signaling molecules and transcription factors that control cell fate specification and proliferation as well as differentiation and migration [for reviews; (Schuurmans and Guillemot, 2002; Zaki et al., 2003; Puelles and Rubenstein, 2003; Guillemot, 2007)]. Hence, programs of regional identity as defined by the expression of distinct transcription factors in different progenitor regions control most aspects of histogenesis within the developing telencephalon. As a result, the embryonic telencephalon is divided into the pallium (primordium of neocortex, hippocampus, piriform cortex and amygdala) and the subpallium (primordium of basal ganglia), which are, in turn, subdivided into distinct progenitor domains (Puelles et al., 1999; Puelles et al., 2000; Marin et al., 2003). Post-mitotic (immature) neurons are generally derived from progenitor cells located nearby in the ventricular zone (VZ) of the neuroepithelium before they translocate to adjacent locations in the mantle through radial migration like during the generation of cortical glutamatergic neurons (Rakic and Lombroso, 1998). However, in some cases, cell types generated in specific progenitor zone(s) migrate orthogonally to the radial axis of the neural tube and intermingle with other neurons in a common final destination, where that cell type apparently is not made, at least in large numbers. This process, known as tangential migration, is widely spread in the developing CNS and contributes to the complexity of the neuronal network found in the vertebrate brain (Corbin et al., 2001;

Marin and Rubenstein, 2001). For instance, the majority of cortical GABAergic interneurons (De Carlos et al., 1996; Anderson et al., 1997a; Tamamaki et al., 1997; Lavdas et al., 1999; Anderson et al., 2001; Cobos et al., 2001; Wichterle et al., 2001; Gorski et al., 2002; He et al., 2001; Nery et al., 2002) and cortical oligodendrocytes (Tekki-Kessaris et al., 2001; Thomas et al., 2000) in mouse and chick are born to ventral progenitors located in the subpallium and reach their final destination by tangential migration.

Several genes including *Lhx6, Arx* and the *Dlx* genes are involved in the regulation of neuronal migration in the ventral telencephalon (Cobos et al., 2007; Colombo et al., 2007; Liodis et al., 2007). The *Dlx* genes are transcription factors that are required for the proper differentiation and migration of most ventrally-derived neurons including striatal projection neurons and cortical interneurons. Thus, *Dlx1/Dlx2* null mice display a major block of cell differentiation in the lateral and medial ganglionic eminences (LGE and MGE), and lack both radial and tangential migration of several types of GABAergic, dopaminergic and cholinergic interneurons derived from the subpallium (Anderson et al., 1997a; Anderson et al., 1997b; Bulfone et al., 1998; Marin et al., 2000; Pleasure et al., 2000; Anderson et al., 2001; Marin and Rubenstein, 2001; Yun et al., 2002; Long et al., 2007). Four *Dlx* genes, *Dlx1, Dlx2, Dlx5 and Dlx6,* are expressed in the forebrain and show highly overlapping expression patterns but with subtle spatio-temporal differences in the telencephalon and diencephalon, suggesting the presence of both redundant and unique functions among these genes (Panganiban and Rubenstein, 2002). Towards elucidating the biochemical mechanisms that control development of ventral progenitors in the telencephalon, our laboratory investigated the regulatory elements and mechanisms

controlling *Dlx* gene expression and showed that shared regulatory mechanisms may underlie the functional redundancy among *Dlx* paralogs (Zerucha et al., 2000; Ghanem et al., 2003). Hence, we identified and characterized four conserved regulatory elements (CREs) acting as forebrain-specific enhancers for the *Dlx* genes in vertebrates: I56i and I56ii in the *Dlx5/Dlx6* bigene cluster (Zerucha et al., 2000), and, URE2 and I12b in the *Dlx1/Dlx2* cluster (Ghanem et al., 2003; Ghanem et al., 2007). Furthermore, we reported that URE2, I12b and I56i, mark different progenitor cell populations in the ganglionic eminences and different subtypes of adult cortical interneurons (Ghanem et al., 2007). These findings suggest that distinct *Dlx* functions could be mediated by different regulatory elements and/or mechanisms.

Here we expand our previous findings by demonstrating the existence of a distinct regulatory role played by I56ii when compared with the three *Dlx* CREs described earlier. We carried out a detailed spatial and temporal analysis of the *lacZ* reporter transgene expression driven by the I56ii CRE between E10.5 and E15.5 and compared its activities with that of three previously described *Dlx* CREs. We report that, unlike the other CREs, I56ii is not active in GABAergic interneuron progenitors in the basal ganglia, nor in tangentially migrating cells to the cortex. Instead, it targets *lacZ* expression specifically to subpopulation(s) of post-mitotic projection neurons that are probably derived from LGE progenitors and have tangentially migrated to the deep mantle of the LGE and MGE between E11.5 and E13.5. In addition, we also identify that the I56ii-positive neurons express two striatal markers, *Meis2* and *Islet1*, during midgestation, both of which can activate transcription via I56ii in co-transfection assays *in vitro*, suggesting these transcriptional factors may be potential upstream regulators of *Dlx* genes *in vivo*.

Together, our data reflect a complex and dynamic regulation of *Dlx* gene expression during the early stages of embryonic development through several regulatory elements with overlapping and distinct function(s).

2.2. Material and methods

2.2.1. Transgenic animals

For transgenic mice, sequences containing the four mouse enhancers (URE2, I12b, I56i and I56ii) were subcloned separately into the p1229/p1230 vectors (Yee and Rigby et al., 1993) that contain a human *β*-globin minimal promoter and the *lacZ* reporter gene. Subclonings were done using a PCR-based approach or using convenient restriction sites and followed by sequencing to verify the integrity of each insert. The p1230-based I12b-alkaline phosphatase (I12b-AP) construct was generated as described earlier (Ghanem et al., 2007). At least two independent transgenic lines that show *lacZ* or AP staining were generated with each construct. Staining results on whole mount embryos and on coronal sections of the forebrain were replicated from independent lines. Transgenic animals were produced and analyzed as previously described (Zerucha et al., 2000).

2.2.2. Histology

E10 to E12.5 mouse embryos were fixed for 45min to 2h in 4% cold paraformaldehyde (PFA) in 1×PBS at 4°C, then, washed and stained for *β*-galactosidase activity overnight (O/N) at 28°C in a solution of 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and 0.02% NP-40 in PBS. The stained brains were dissected and equilibrated in 20% sucrose solution O/N at 4°C prior to sectioning. Brains were

embedded in Tissue-Tek media the following day, cryoprotected and processed for frozen sectioning at 20-50μm using a cryostat (Leica CM3050 S). Sections were mounted with Aquatex (EM Science, VWR). E13.5-P0 mouse brains were fixed for 2h to O/N in 4% cold PFA in PBS at 4°C, dissected and sectioned prior to staining. *LacZ* staining and mounting was performed as described above.

2.2.3. Double immunohistochemistry

Frozen sections of E10.5-E15.5 mouse brain were dried for 1h at room temperature, then, washed for 3×5 min each in 0.1M phosphate buffer (PB) to eliminate residues from tissue protection medium. Immunostaining was performed as described earlier (Ghanem et al., 2007). The following antibodies have been applied in this study: guinea pig anti-*β*-gal (1:1000, a generous gift from Dr. Thomas Sargent), rabbit pan-*Dll* antibody (anti-*Dlx*; 1:100, a kind gift from Drs. Grace Panganiban and J.L.R.R.), rabbit anti-human placental alkaline phosphatase (PLAP, 1:100, Serotec), mouse anti-PCNA (1:300; Vector Laboratories), mouse anti-MAP2 (1:300, Sigma), rabbit anti-active caspase 3 (1:500, BD Biosciences), rabbit anti-GABA (1:4000, Sigma), rabbit anti-calbindin (1:1000, Chemicon), rat anti-somatostatin (1:100, Chemicon), rabbit anti-NPY (1:4000, Immunostar), rabbit anti-TH (1:350, Chemicon), mouse anti-Islet1 (1:100, Developmental Studies Hybridoma Bank), and goat anti-Meis2 (1:100, Santa Cruz Biotechnology). Secondary antibodies were all purchased from Invitrogen: goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 488, goat anti-rat Alexa Fluor 488, donkey anti-goat Alexa Fluor 488, and goat anti-guinea pig Alexa Fluor 594. For PCNA

detection, sodium citrate antigen retrieval, pH 6.0, was performed on sections before incubation with PCNA antibodies.

2.2.4. *In situ* RNA hybridization

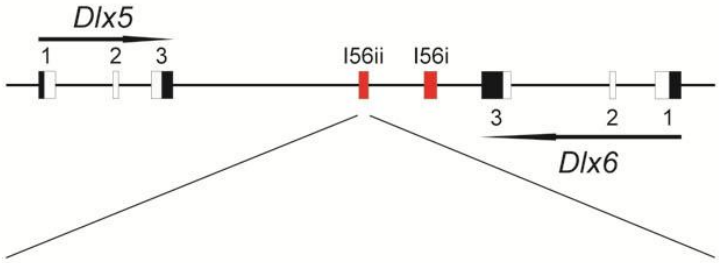
In situ hybridization on frozen tissue sections and digoxigenin RNA probe labeling were performed according to the procedures described in (Wallace and Raff, 1999). Hybridized probes were detected with an AP-conjugated anti-digoxigenin Fab fragment antibody (1:2000, Roche) and visualized with the NBT/BCIP substrate system. Antisense riboprobes for *Dlx5* and *Dlx6* were prepared as previously described (Quint et al., 2000; Cobos et al., 2006; Long et al., 2007).

2.2.5. Co-transfection and chloramphenicol acetyltransferase (CAT) assays

The mouse I56ii CRE was amplified by PCR and inserted upstream of the thymidine kinase (tk) minimal promoter in a pBLCAT2 vector, we called it mI56ii-pBLCAT2. We also created, using the overlapping-PCR method, a modified mI56ii-pBLCAT2 plasmid (mI56ii-mutant-pBLCAT2) containing the mutagenized Meis2 consensus binding site (TGTC to TCTA) in mI56ii sequence (position nt. 262 to nt. 265, Supplementary Figure 2.1). The mouse Islet1 cDNA encompassing the full-length coding sequence (kindly provided by Dr. Steve M Sperber, National Institute of Child Health and Human Development) was subcloned into the EcoRI site of a pCS2+ expression vector, designated as Islet1-pCS2+. The Meis2-pCS2+ expression vector was previously generated in our laboratory (Zerucha et al., 2000). The pSV-β-Gal reporter plasmid encoding β-galactosidase was purchased from Promega and used as an internal control.

Supplementary Figure 2.1. Schematic diagram showing the conserved CREs (I56i and I56ii) within the *Dlx5/6* intergenic region and alignment of I56ii sequences from human, mouse, and zebrafish. Each *Dlx* gene comprises three exons (white box: coding sequences; black box: untranslated regions), which are marked with numbers. Complete sequence identity across all three species is indicated by an asterisk. The putative Meis2 binding sites (TGTC) in I56ii are highlighted in blue, while several ATTA core sequences corresponding to Islet1 recognition sites are indicated in green. The mutagenized Meis2 binding site (TGTC to TCTA, nt. 262-265) is shown in red.

Dlx5/Dlx6




```
          1                                                                                                   60
hI56ii    GTGAGCACATCCAGGTGTGAAATTGTTTGCACACCCCAGCACCTCTTATAT--TGCCAGC
mI56ii    GTGAGCACATCCAGGTGTGAAATTGTTTGCACACCCCAGCACCTCTTATAT--TGCCAGC
zI56ii    GTAAGCAAATCCAGGTGTGAAAGTGTTTGCACACCCCGACACCTCTTATATATTGCCTGC
          ** **** ************** **************  ************  **** **

          61                                                                                                  120
hI56ii    AAAATTAGCTGTTATTACTGTCACTGTTTAGTGATGGTTAGCGTGGTACAAAAAAAAAAA
mI56ii    AAAATTAGCTGTTATTACTGTCACTGTTTAGTGATGGTTAGCGTGATACAAAAAAAAAAA
zI56ii    AAAATTAGCCGTTATTACTGTCACTGTTTAGTGATGGTGAGC------CAGGGAAAAAAC
          ********* ***************************** ***      **   ******

          121                                                                                                 180
hI56ii    AAAAAAAAAAAAAAA---CTGCTGTAATCAAGA-CCTGGCGCATCTTTGCAAATTACAGA
mI56ii    AAAAAAAAAAAAAAAAAGCTGCTGTAATCAAGA-CCTGGCGCATCTTTGCAAATTACAGA
zI56ii    ------------------CTTCTGCAATCAAGAACCAGGCGCATCTTTGCAAATTATAGA
                           ** *** ******** ** ******************** ***

          181                                                                                                 240
hI56ii    TAATTGTAAACGTCCAGATTATGATAATAGCATCCTAATCCAGCCTGCAATATAATTATT
mI56ii    TAATTGTAAACGTCCAGATTATGATAATAGCATCCTAATCCAGCCTGCAATATAATTAT-
zI56ii    TAATTGTAAATGTCCAGATTATGATAATGGAG--CTAATCCTGGTGGAAGTATAATTATT
          ********** ***************** *    ******* *   * * *********

          241                                                                                                 300
hI56ii    ACAGAGTGTTACATCTGAAACTGTCCAGTAGGGCTAATTCAGCCATTATTTAGACCCTAT
mI56ii    ACAGAGTGTTACATCTGAAACTGTCCAGTAGGGCTAATTCAGCCATTATTTAGACCCTAT
zI56ii    GTTGAGTGTTACAGTTGAGGGTGTCCAGTAAAGCTAACT--GTCATTATTTATACGCGGT
             **********  ***    *********  ***** *  * ********* ** *  *
                                TCTA

          301
hI56ii    TT
mI56ii    TT
zI56ii    TT
          **
```

Co-transfection assays were performed as follows: briefly, P19 murine embryonic carcinoma cells were seeded at a density of 3×10^5 in 60-mm-diameter tissue culture plates for 24-36h prior to transfection. Cells were subsequently transfected with 2µg of mI56ii-pBLCAT2, mI56ii-mutant-pBLCAT2, or enhancer-less pBLCAT2, 2µg of each expression vector (Meis2-pCS2+, Islet1-pCS2+, or empty pCS2+), and 2µg of pSV-*β*-Gal using the lipofectamine 2000 reagent (Invitrogen). The CAT activity in cell extracts was analyzed 48h post-transfection on TCL plates by applying the chloroform:methanol solvent system and was quantified by a PhosphorImager as described earlier (Zerucha et al., 2000). After normalizing to the *β*-galactosidase activity, the relative CAT activity for each expression vector was measured and compared to the basal level of activity seen in the control. The statistical analysis of the relative CAT activities among different groups was performed by using the Student *t*-test. All experiments were repeated at least two times in triplicate. *P* values < 0.05 were considered statistically significant.

2.3. Results

2.3.1. Spatio-temporal comparisons of *lacZ* expression in four *Dlx* CRE lines during the development of the telencephalon

Divergence in the sequences of the four *Dlx* forebrain CREs namely, URE2, I12b, I56i and I56ii, suggests that each of them is involved in distinct aspects of *Dlx* regulation either spatially and/or temporally. Previously, we showed that URE2, I12b and I56i, have some distinct and overlapping regulatory properties in the developing telencephalon despite highly overlapping activities (Ghanem et al., 2007). To test whether I56ii promotes expression with a distinct pattern, we conducted a detailed spatial and temporal

analysis of *lacZ* reporter gene expression driven by this CRE in the telencephalon. We then compared the *lacZ* reporter expression of *I56ii-lacZ,* and, *I56i-lacZ, URE2-lacZ* and *I12b-lacZ* on coronal sections between E10.5 and E15.5. All results were confirmed in at least two independent transgenic lines for each CRE.

E10-10.5

The onset of *lacZ* expression conferred by all four CREs is detected around E10 in a small cluster of cells located in the region of the primordia of the prethalamus (previously known as the ventral thalamus) (data not shown). This time point is comparable to the onset of endogenous *Dlx1* and *Dlx2* expression in the prethalamus at E9.0 and E9.5, respectively, as determined by *in situ* RNA hybridization (Bulfone et al., 1993b; Price et al., 1991) and by RNA blot analysis (McGuinness et al., 1996). This is also consistent with the onset of *Dlx5* and *Dlx6* expression which occurs around E9.5 (Simeone et al., 1994).

Shortly after their onset, *lacZ* expression in all four CRE lines becomes visible in the two domains where endogenous *Dlx* genes are expressed in whole mount embryos. Domain I (diencephalon) includes expression in the prethalamus and a *Dlx*+ longitudinal domain in the hypothalamus. Domain II encompasses most of the subpallial telencephalon (Figure 2.1A-D). In *URE2-lacZ*, *I12b-lacZ,* and *I56i-lacZ* transgenics, *β*-galactosidase-positive cells are largely absent from the ventricular zone (VZ) at E10.5 on coronal sections except for scattered cells in the URE2 and I56i lines (Figure 2.1E,G; arrowheads), and are concentrated as a thick subpial layer (Figure 2.1E-G). At this age, the subpial tissue consists of the emerging subventricular zone (SVZ) and mantle zone

(MZ) (Yun et al., 2002). Unlike expression from the other three CREs, *I56ii-lacZ* transgenics produce only a few *lacZ* positive cells that are present in the subpial region of the LGE but not the MGE at this age (Figure 3.1H, arrows).

E11.5

Transgenic animals produced with all four reporter constructs show stronger *lacZ* expression in the subpallial telencephalon at E11.5 compared to E10.5. Transgene expression in the forebrain is restricted to regions where endogenous *Dlx* genes are expressed. Expression patterns of the four transgenes overlap extensively, even though discrete and reproducible differences in enhancer activity are observed, especially between the *I56ii-lacZ* transgene and the other CREs (Figure 2.2A-D and Supplementary Figure 2.2). Therefore, *URE2-lacZ*, *I12b-lacZ*, and *I56i-lacZ* are characterized by a nearly homogeneous expression in the SVZ and MZ in most areas of the subpallium (Figure 2.2A-C and Supplementary Figure 2.2A-C, E-G) with the exception of the VZ in *I12b-lacZ* and *I56i-lacZ* lines (Figure 2.2B, C, and supplementary Figure 2.2B, C, F, G), and, the ventral MGE (vMGE) in *URE2-lacZ* lines (Figure 2.2A and Supplementary Figure 2.2A,E). In contrast, expression of the *I56ii-lacZ* transgene is only detected in a subset of cells in the MZ that are largely limited to parts of the ventral LGE (vLGE), dorsal and medial MGE (dMGE and mMGE), pre-optic area (POA) and caudal ganglionic eminence (CGE) (Figure 2.2D and Supplementary Figure 2.2D,H). Expression of *lacZ* from all four transgenes is sparse in the septum at this age (Supplementary Figure 2.2A-D), as is the case for endogenous *Dlx* expression.

Figure 2.1. Comparative activities of URE2, I12b, I56i, and I56ii CREs in E10.5 transgenic mouse embryos (*A-D*) and coronal sections (*E-H*), as shown by the expression of *lacZ* reporter gene. *A,E*: *URE2-lacZ*; *B,F*: *I12b-lacZ*; *C,G*: *I56i-lacZ*; *D,H*: *I56ii-lacZ*. Lateral views of the mouse embryos (*A-D*) show that all four CREs are able to target *lacZ* gene expression in two main domains in the forebrain, the diencephalon (I) and most of the subpallial telencephalon (II). In *URE2-lacZ*, *I12b-lacZ*, and *I56i-lacZ* transgenic lines, *lacZ* positive cells are largely absent from the VZ except for scattered cells in the URE2 and I56i lines (*E*, *G*; arrowheads), and are concentrated as a thick subpial layer (*E-G*). In contrast, *I56ii-lacZ* transgenics produce only a few *lacZ* positive cells present in the subpial region of the LGE at this age (*H*, arrows). The red arrow in panel E indicates the boundary between the LGE and MGE. 3, third branchial arch; H, hyoid arch (second arch); Hy, hypothalamus; L, limbs; LV, lateral ventricle; Md, mandible; OP, optic eminences; PT, prethalamus (ventral thalamus); ST, subpallial telencephalon; St, somites. Scale bar: (*A-D*), 1mm; (*E-H*), 12.5 μm.

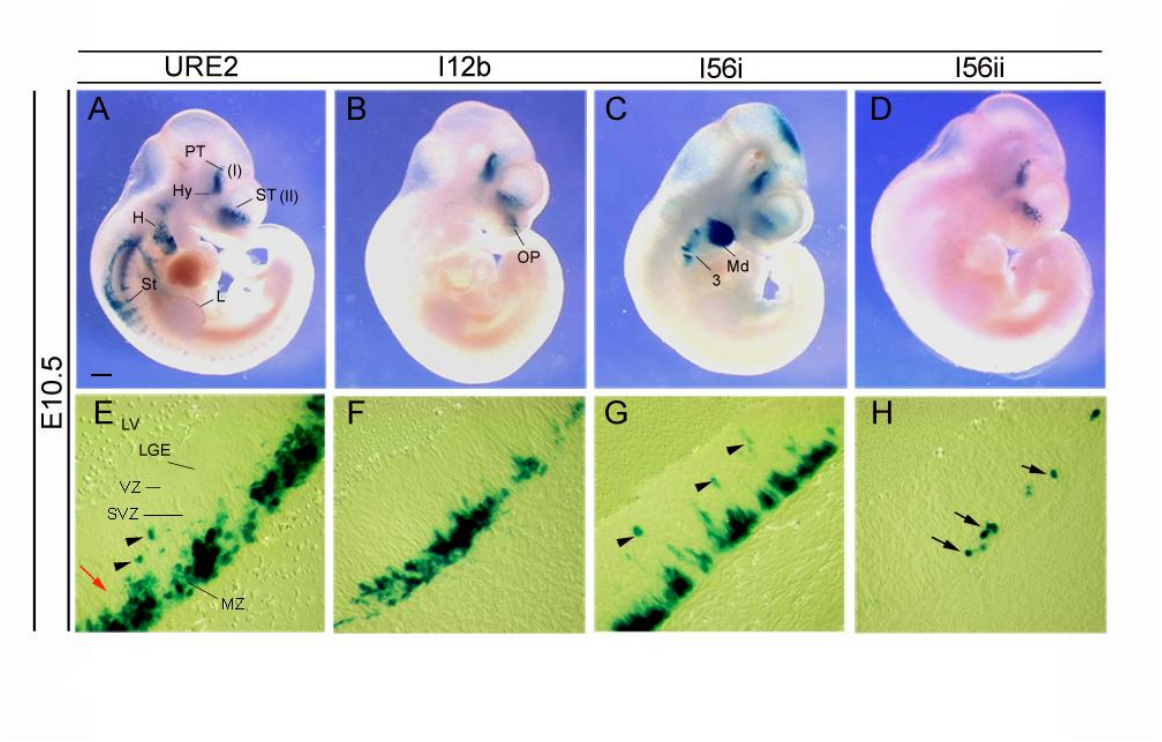
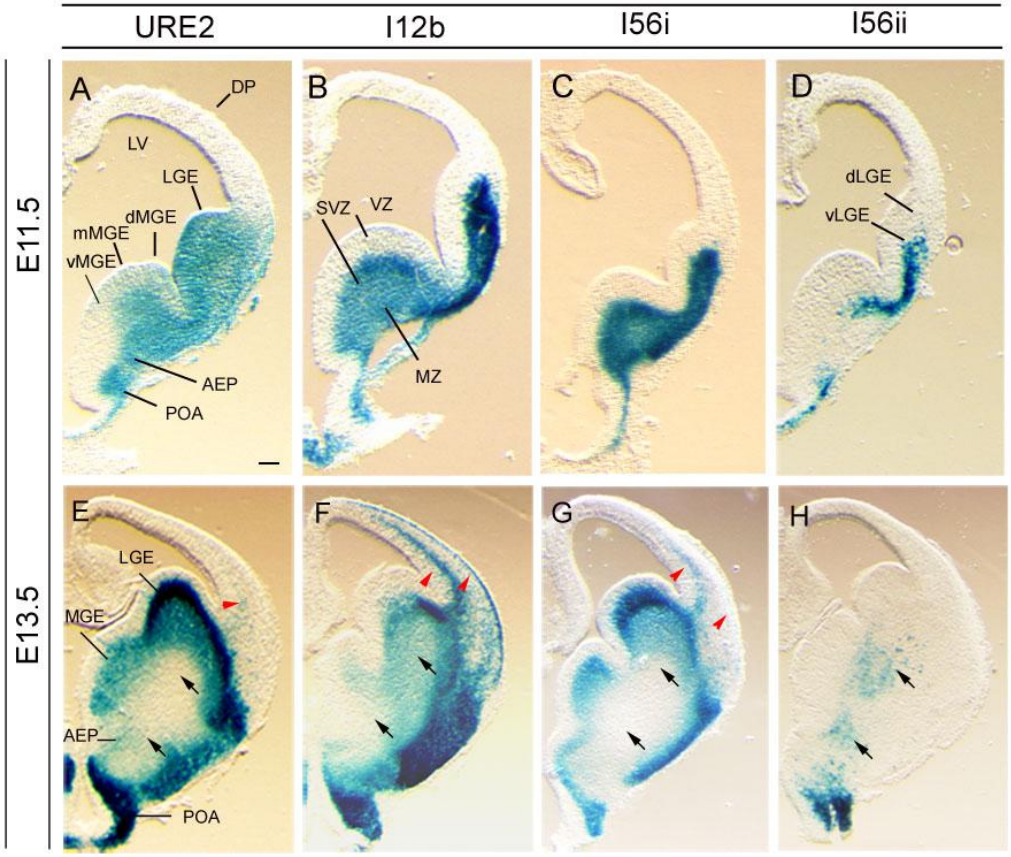
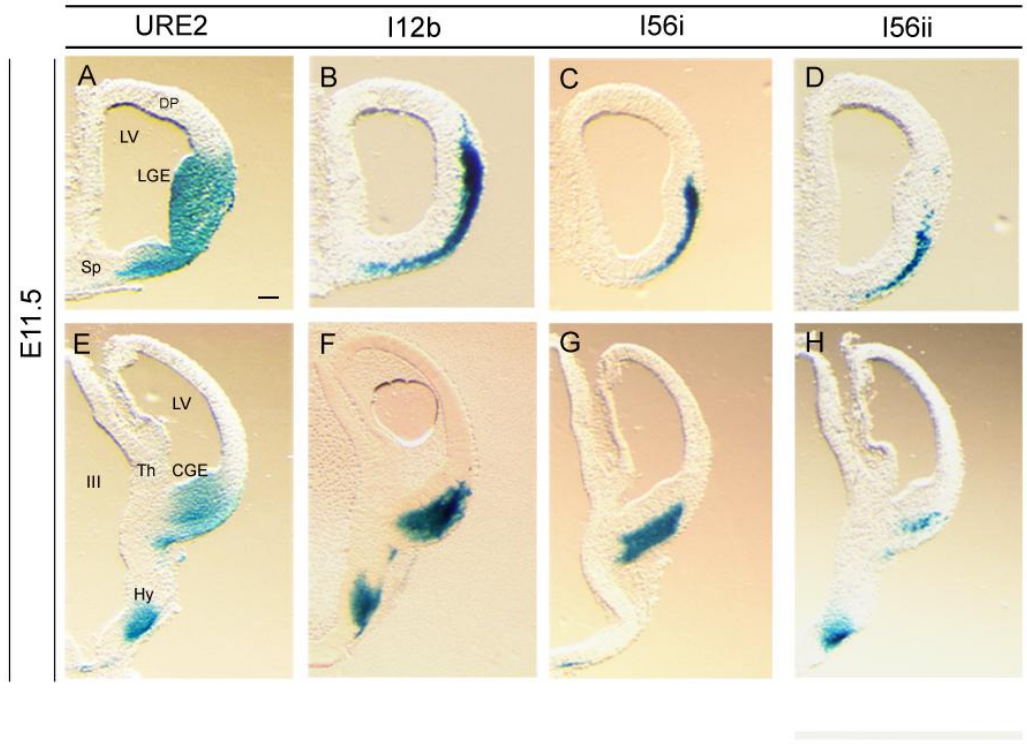

URE2
I12b
I56i
I56ii
E10.5
A
PT
(I)
Hy
ST (II)
H
St
L
B
OP
C
Md
3
D
E
LV
LGE
VZ
SVZ
MZ
F
G
H

Figure 2.2. Different enhancer activities of URE2, I12b, I56i, and I56ii in the subpallial telencephalon of transgenic mice. Coronal hemisections showing *lacZ* expression under the control of each enhancer at E11.5 (*A-D*) and E13.5 (*E-H*) in medial levels. *A,E*: *URE2-lacZ*; *B,F*: *I12b-lacZ*; *C,G*: *I56i-lacZ*; *D,H*: *I56ii-lacZ*. At both E11.5 and E13.5, *URE2-lacZ, I12b-lacZ-*, and *I56i-lacZ-* positive cells display a nearly homogeneous pattern of expression in most areas of the subpallium (*A-C, E-G*). In contrast, expression of the *I56ii-lacZ* transgene is mainly restricted to a subset of cells in the superficial mantle of the vLGE, MGE, AEP and POA (*D, H*, arrows), the regions where the other three CRE lines produce weak or no *lacZ* expression (*E-G*, arrows).Unlike in the *URE-lacZ, I12b-lacZ*, and *I56i-lacZ* lines, *I56ii-lacZ* does not label cells migrating tangentially to the cortex at E13.5 (*H* versus *E-G*, arrowheads). DP, dorsal telencephalon; LV, lateral ventricle. Scale bar: (*A-H*), 250 μm.


URE2
I12b
I56i
I56ii
E11.5
E13.5
A
B
C
D
E
F
G
H
DP
LV
LGE
dMGE
mMGE
vMGE
AEP
POA
SVZ
VZ
MZ
dLGE
vLGE
MGE

Supplementary Figure 2.2. Activities of four different *Dlx* CREs in the rostral (*A-D*) and caudal levels (*E-H*) of subpallial telencephalon at E11.5. *A,E*: *URE2-lacZ*; *B,F*: *I12b-lacZ*; *C,G*: *I56i-lacZ*; *D,H*: *I56ii-lacZ*. As described in Figure 2.2, although expression patterns of the four transgenes overlap extensively, discrete and reproducible differences in enhancer activity can be identified at this age, especially between the *I56ii-lacZ* transgene and the other CREs (URE2, I12b, and I56i). III, Third ventricle; DP, dorsal telencephalon; Hy, hypothalamus; LV, lateral ventricle; Sp, septum; Th, thalamus. Scale bar: (*A-H*), 250 μm.


URE2
I12b
I56i
I56ii
E11.5
A
DP
LV
LGE
Sp
B
C
D
E
LV
III
Th
CGE
Hy
F
G
H

E12.5-birth

Regional differences in the activity of I56ii and the other three CREs become more pronounced between E12.5 and E13.5, particularly in the mantle of the ventral telencephalon. Consequently, *I56ii-lacZ* expression is still restricted to a subgroup of cells located in the deep mantle of the LGE and septum at rostral level (Supplementary Figure 2.3D and 2.4D). It is also found along the superficial mantle of the vLGE, MGE, anterior entopeduncular area (AEP) and POA at medial level (Figure 2.2H, arrows, and Supplementary Figure 2.2H), and in the deep MZ of the CGE more caudally (Supplementary Figure 2.3L and 2.4H, arrow). In contrast, all of the above regions display weak or no *lacZ* expression in the other CRE lines at E12.5 (Supplementary Figure 2.3E-G, I-K) and E13.5 (Figure 2.2E-G and supplementary Figure 2.4E-G, arrows), suggesting that I56ii may be labeling a distinct group(s) of cells at these stages. Furthermore, unlike in the *URE2-lacZ*, *I12b-lacZ*, and *I56i-lacZ* lines, *I56ii-lacZ* does not label tangentially migrating cells to the cortex at E13.5 (compare Figure 2.2H, Supplementary Figure 2.4D,H with Figure 2.2E-G, supplementary Figure 2.4A-C, E-G, respectively; arrowheads) and E15.5 (compare Supplementary Figure 2.5D, H, and L with Supplementary Figure 2.5A-C, E-G, and I-K, respectively; arrowheads). Starting at E14.5, I56ii activity is greatly reduced in the ventral telencephalon (data not shown) and is only maintained in a group of hypothalamic cells after E15.5 (Supplementary Figure 2.5D, H, L). In contrast, the expression of *URE2-lacZ* and *I56i-lacZ* is still strong in the ganglionic eminences at this time (Supplementary Figure 2.5A, C, E, G, I, K and data not shown). *I12b-lacZ* expression remains also visible in most subdivisions of the basal ganglia but with weaker intensity (Supplementary Figure 2.5B, F, J).

In summary, our data suggest that I56ii CRE displays a different activity compared with URE2, I12b and I56i, that is confined to group(s) of cells lining the mantle of the ganglionic eminences between E11.5 and E13.5.

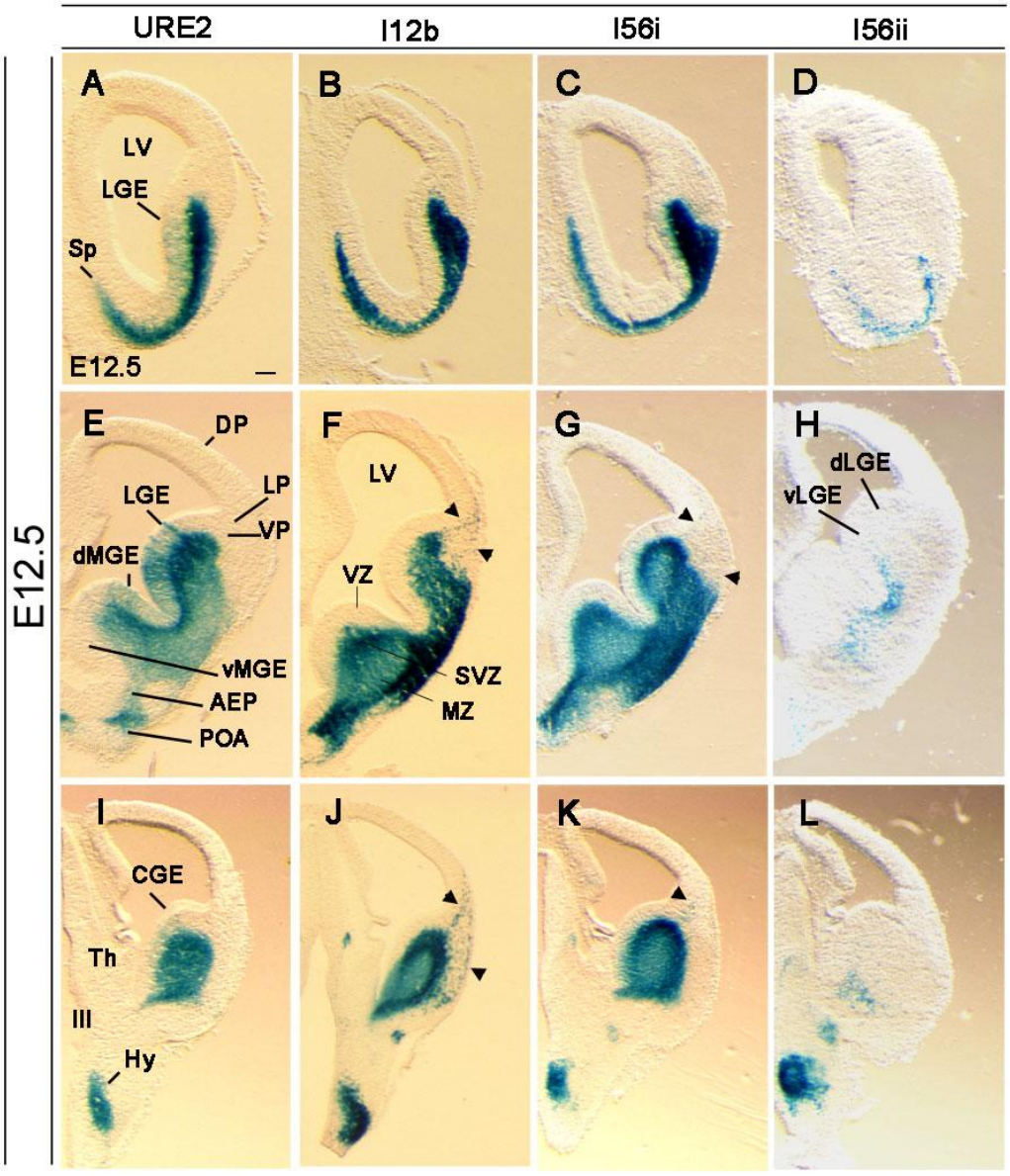
2.3.2. I56ii marks a subgroup of striatal projection neurons expressing *Meis2* and *Islet1*

We sought to characterize the identity of the *I56ii-lacZ* positive cells. First, we checked that *I56ii-lacZ*-expressing cells are *Dlx*-positive between E11.5 and E13.5 comparing sections stained with X-gal with consecutive sections hybridized to *Dlx5* or *Dlx6* cRNA probes (Figure 2.3 and data not shown). We also applied double-labeling with a pan-*Dll* antibody and the *β*-galactosidase antibody (Figure 2.4A-D and data not shown). Interestingly, we found that a stripe of cells that extends from the ventral MGE towards the LGE and expresses the reporter transgene also express relatively high levels of *Dlx6* transcripts (Figure 2.3C,E; arrowheads).

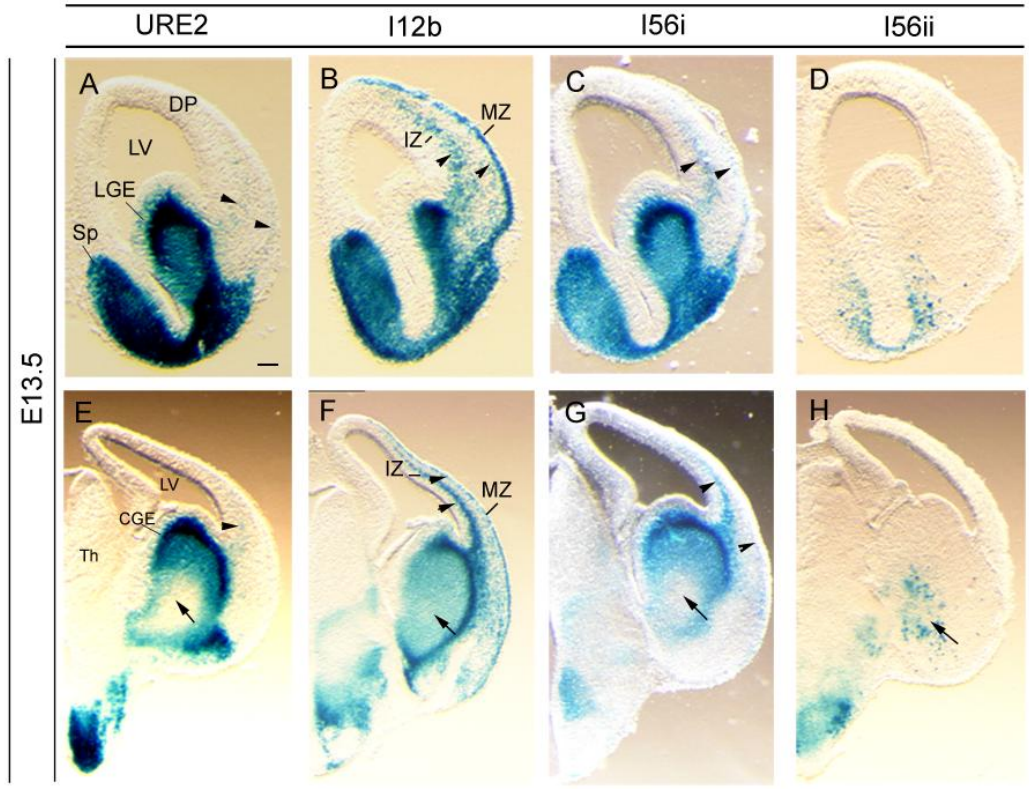
Then, in order to test whether I56ii and I12b/I56i are active in the same or distinct group(s) of cells, we crossed the *I56ii-lacZ* line with an alkaline phosphatase (AP) reporter line under the control of I12b (*I12b-AP*). We then performed double immunohistochemistry on brain sections from double hemizygote embryos (*AP+/lacZ+*) at E12.5 and E13.5 using *β*-gal and AP antibodies. As a result, we found that *I56ii-lacZ* positive cells almost never overlap with cells expressing the *I12b-AP* transgene at all levels and ages examined (Figure 2.4E-H and data not shown).

Next, we investigated whether *I56ii-lacZ* positive cells proliferate and whether they undergo apoptosis after E13.5. We found that between E11.5 and E13.5, most *I56ii-lacZ*

Supplementary Figure 2.3. Comparisons of various activities of URE2, I12b, I56i, and I56ii CREs in rostral (*A-D*), medial (*E-H*) and caudal levels (*I-L*) of the subpallial telencephalon at E12.5. *A, E, I*: *URE2-lacZ*; *B, F, J*: *I12b-lacZ*; *C, G, K*: *I56i-lacZ*; *D, H, L*: *I56ii-lacZ*. In contrast to *lacZ* expression patterns in the other three *Dlx* CREs lines, *I56ii-lacZ* expression is only limited to a subgroup of cells located in the deep MZ of the LGE and septum at rostral level (*D*), the superficial mantle of the vLGE, MGE, AEP and POA at medial level (*H*), as well as the MZ of the CGE at caudal level (*L*). Unlike *I12b-lacZ* and *I56i-lacZ* positive cells, *I56ii-lacZ* does not target tangentially migrating cells to the cortex at E12.5 (*H, L* versus *F, G, J, K*, arrowheads). Note that tangential migration of *URE2-lacZ* positive cells was not observed at this stage. Symbols are the same as in Figure 2.2 and supplementary Figure 2.2; VP, ventral pallium. Scale bar: (*A-L*), 250 μm.


URE2
I12b
I56i
I56ii
E12.5
A
LV
LGE
Sp
E12.5
B
C
D
E
DP
LP
LGE
VP
dMGE
vMGE
AEP
POA
F
LV
VZ
SVZ
MZ
G
H
dLGE
vLGE
I
CGE
Th
III
Hy
J
K
L

Supplementary Figure 2.4. Enhancer activities of URE2, I12b, I56i, and I56ii in rostral (*A-D*) and caudal levels (*E-H*) of the subpallial telencephalon at E13.5. *A,E*: *URE2-lacZ*; *B,F*: *I12b-lacZ*; *C,G*: *I56i-lacZ*; *D,H*: *I56ii-lacZ*. Similar to results obtained at E12.5 (see Supplementary Figure 2.3), *I56ii-lacZ* activity is continuously maintained in the subpopulation of cells located in the deep MZ of the LGE and septum at rostral level (*D*) and in the deep MZ of the CGE more caudally (*H*, arrow) at E13.5. *I56ii-lacZ* does not label tangentially migrating cells to the cortex at E13.5 (see also Figure 2.2). The cells migrating to cortex are indicated with arrowheads. Symbols are the same as in Figure 2.2 and Supplementary Figure 2.2. Scale bar: (*A-H*), 250 μm.


URE2
I12b
I56i
I56ii
E13.5
A
DP
LV
LGE
Sp
B
MZ
IZ
C
D
E
LV
CGE
Th
F
IZ
MZ
G
H

Supplementary Figure 2.5. Activities of four *Dlx* CREs in the subpallial telencephalon, as shown by coronal hemisections at E15.5. *A, E, I*: *URE2-lacZ*; *B, F, J*: *I12b-lacZ*; *C, G, K*: *I56i-lacZ*; *D, H, L*: *I56ii-lacZ*. The expression of *URE2-lacZ* (*A, E, I*) and *I56i-lacZ* (*C, G, K*) are strong in the ganglionic eminences, whereas *I12b-lacZ* expression persists in most subdivisions of the basal ganglia but with weaker intensity (*B, F, J*). In contrast, I56ii activity is greatly reduced in the ventral telencephalon (*D, H*) and is only maintained in a group of hypothalamic cells after E15.5 (*L*). Arrowheads indicate the streams of migrating cells to cortex. Symbols are the same as in Figure 2.2 and Supplementary Figure 2.2. Scale bar: (*A-L*), 250 μm.

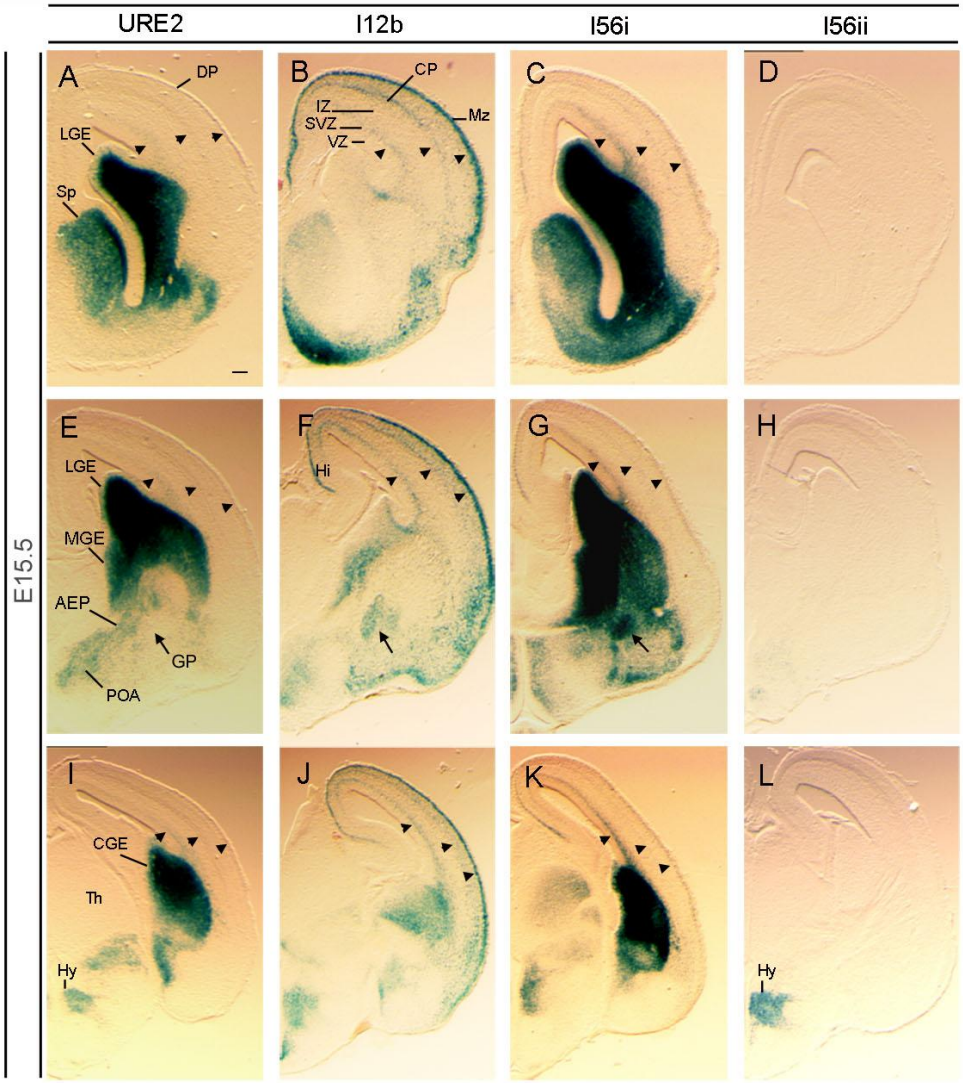
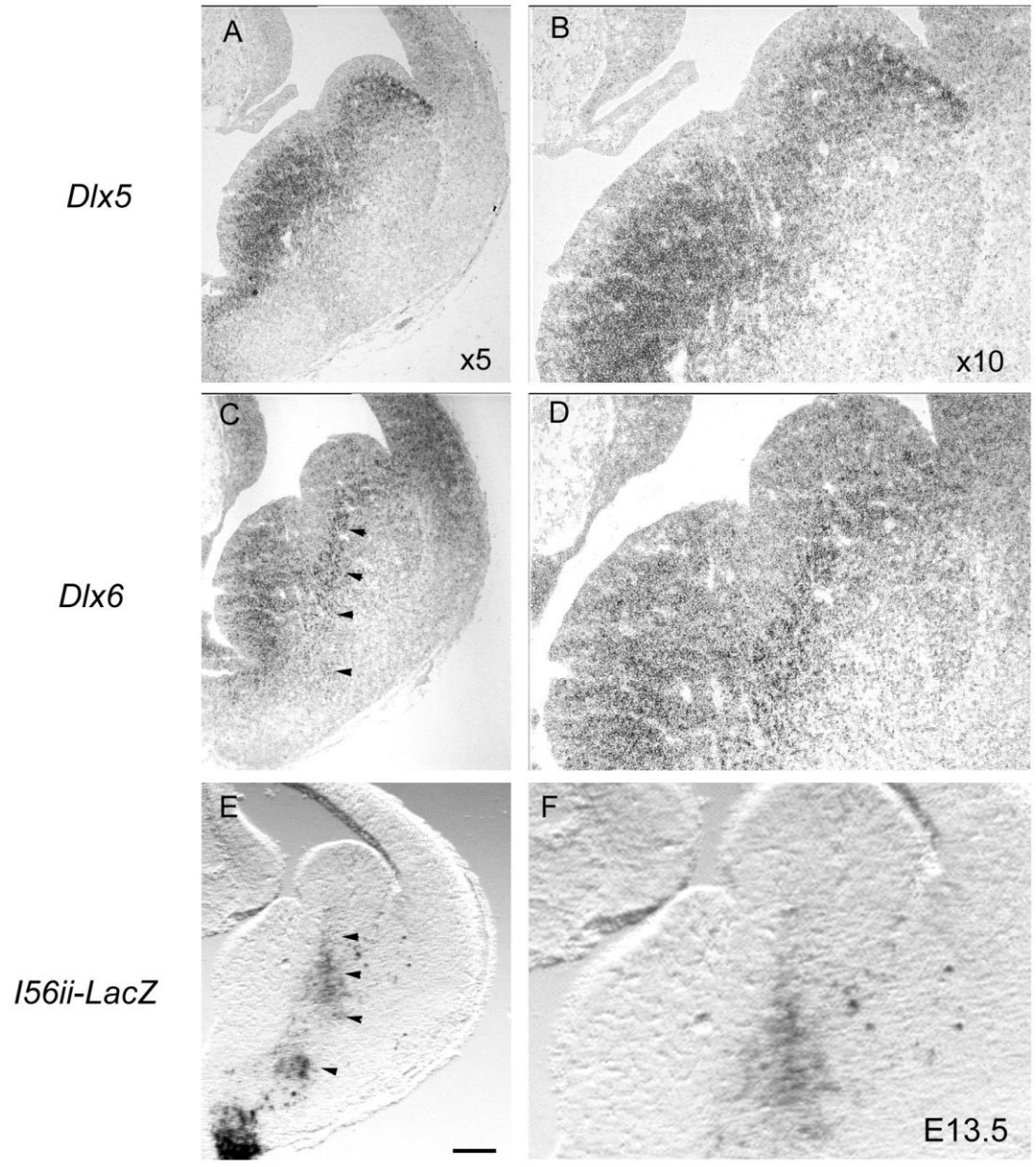

URE2
I12b
I56i
I56ii
E15.5
A
DP
LGE
Sp
B
CP
IZ
SVZ
VZ
Mz
C
D
E
LGE
MGE
AEP
GP
POA
F
Hi
G
H
I
CGE
Th
Hy
J
K
L
Hy

Figure 2.3. The I56ii CRE is active in a number of *Dlx5*- and *Dlx6*-expressing cells in the telencephalon. Distribution of *Dlx5* (*A, B*) and *Dlx6* (*C, D*) transcripts was compared to the expression of the *I56ii-lacZ* transgene (*E, F*) in the telelencephalon at E13.5. Note that a stripe of cells, where the I56ii CRE is particularly active, express relatively high levels of *Dlx6* transcripts (*C, E*; arrowheads). Scale bar: (*A, C, E*), 200 μm; (*B, D, F*), 100 μm.


A
B
x5
x10
C
D
E
F
E13.5
Dlx5
Dlx6
I56ii-LacZ

positive cells have already exited the cell cycle since they do not express the proliferating cell nuclear antigen (PCNA), which marks all proliferating cells (Figure 2.4I-L and data not shown). In contrast, the vast majority of these cells express microtubule associated protein-2 (MAP2), a marker of post-mitotic neurons (Figure 2.4M-P and data not shown). Furthermore, the *I56ii-lacZ*-expressing cells do not appear to be in the process of undergoing apoptosis at E13.5 as they did not co-express the cell death marker, active-caspase 3 (Figure 2.4Q-T).

Since *Dlx* genes are expressed in striatal projection neurons as well as GABAergic interneurons both of which are derived form subpallial progenitors, we tested whether I56ii labels neurons belonging to either of these two groups. First, we found that *I56ii-lacZ*-expressing cells express GABA between E11.5 and E13.5 (Figure 2.5A-D and data not shown). However, they do not express any of the interneuron subtype-specific molecular markers expressed at E13.5 including somatostatin and neuropeptide Y (NPY) or the dopaminergic marker — tyrosine hydroxylase (TH), suggesting that they are not immature interneurons (Figure 2.5E-P and data not shown). This is true except for a few *I56ii-lacZ*-expressing cells that are calbindin-positive cells at E11.5 and E13.5 (Figure 2.5H, arrowheads and data not shown). As comparisons, the other three CREs label one or more of these interneuron subtypes at E13.5 (Ghanem et al., 2007).

Some *I56ii-lacZ* positive cells express calbindin, a marker of both striatal projection neurons and cortical interneurons; thus we investigated whether I56ii-positive cells are striatal projection neurons. We co-labeled these cells with two markers of striatal projection neurons, *Meis2* and *Islet1*, whose expression largely resembles that of *I56ii-lacZ* in the MZ of the ganglionic eminences. The majority of *I56ii-lacZ* cells are located

in a deep mantle layer that is intercalated between the SVZ and more superficial parts of the mantle of the ventral LGE (striatum) and the MGE (globus pallidus) (note: superficial, as in the cortex, refers to being close to pia). This region also expresses the *Islet1* (Figure 2.6A-D) and *Meis2* (Figure 2.6I-L) transcription factors at E13.5. In contrast, we did not detect *I56i-lacZ*- nor *I12b-lacZ*-expressing cells that were co-labeled with anti-Islet1 or anti-Meis2 antibodies at this age (Figure 2.6E-H, M-P, and Supplementary Figure 2.6E-H, M-P). Furthermore, we found that only a few *URE2-lacZ* positive cells express either of these two transcription factors in the mantle of the MGE (Supplementary Figure 2.6C, D, K, L). Note that, some *URE2-lacZ* positive cells located in the mantle of the dorsal LGE also express either *Islet1* or *Meis2*; however, I56ii is not active in this region (Supplementary Figure 2.6C, D, K, L).

Taken together, our results lead to the conclusion that *I56ii-lacZ*-expressing cells in the subpallial telencephalon are a subpopulation(s) of post-mitotic projection neurons that may be derived from ventral LGE progenitor cells, as suggested by their expression of two striatal markers, *Meis2* and *Islet1*. They constitute a distinct population of *Dlx*-expressing cells compared to those where the other CREs, URE2, I12b, and I56i, are active.

2.3.3. Meis2 and Islet1 proteins can bind and activate reporter gene transcription via the I56ii enhancer sequence

To further clarify the existence of a potential regulation of the *Dlx* genes by Meis2 and Islet1 proteins, we searched the I56ii sequence using the genomatix software and identified two putative binding sites (TGTC) for Meis homeodomain proteins as well as

Figure 2.4. Characterization of the I56ii CRE activity in the LGE and MGE at E13.5. *A-D*: Double-immunohistochemistry showing that *I56ii-lacZ*-expressing cells are *Dlx*-positive by co-labeling using a pan-*Dll* antibody and a *β*-galactosidase specific antibody; *E-H*: *I56ii-lacZ* positive cells do not overlap with *I12b-AP*-expressing cells, indicating that I56ii and I12b/I56i are active in distinct subpopulation(s) of cells at this time point; *I-L*: At E13.5, most *I56ii-lacZ* positive cells have already exited the cell cycle because they do not express the PCNA, which marks all the proliferating cells; *M-P*: *I56ii-lacZ* positive cells are post-mitotic cells since they are co-localized with the molecular marker MAP2; *Q-T*: These cells are not apoptotic as they do not co-express the cell death marker, active-caspase 3. Cells expressing *I56ii-lacZ* are shown in red; while cells expressing *Dlx*, AP, PCNA, MAP2 or active-caspase 3 are shown in green, respectively. *D, H, L, P, T* are high-magnification pictures of boxes shown in *C, G, K, O, S*. Scale bars: (*A-C, E-G, I-K, M-O, Q-S*), 12.5 μm; (*D, H, L, P, T*), 8.7 μm.

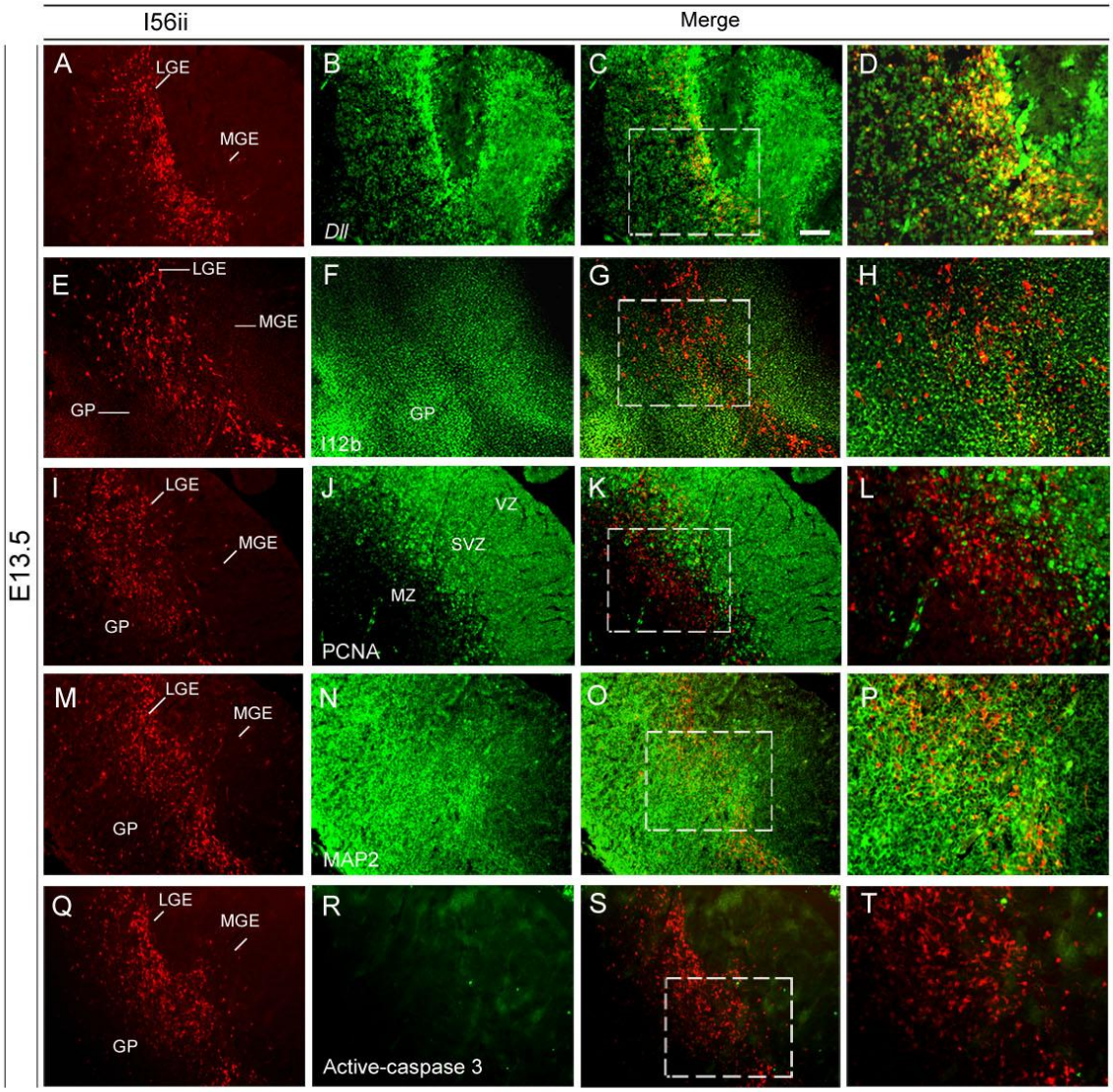

I56ii
Merge
E13.5
A
LGE
MGE
B
Dll
C
D
E
LGE
MGE
GP
F
GP
I12b
G
H
I
LGE
MGE
GP
J
VZ
SVZ
MZ
PCNA
K
L
M
LGE
MGE
GP
N
MAP2
O
P
Q
LGE
MGE
GP
R
Active-caspase 3
S
T

Figure 2.5. *I56ii-lacZ* positive cells are immunoreactive for GABA (*A-D*) at E13.5. In contrast to the other *Dlx* CREs (URE2, I12b, and I56i, Ghanem et al., 2007), I56ii is not active in interneuron progenitor cells (calbindin: *E-H*; somatostatin: *I-L*; NPY: *M-P*) in the basal ganglia or tangentially migrating cells to the cortex at E13.5, except for a few *I56ii-lacZ*-expressing cells that are calbindin-positive (*H*, arrowheads). Cells expressing *I56ii-lacZ* are shown in red; whereas cells expressing GABA, calbindin, somatostatin or NPY are shown in green, respectively. *D*, *H*, *L*, *P* are high-magnification pictures of boxes shown in *C*, *G*, *K*, *O*. Scale bars: (*A-C*, *E-G*, *I-K*, *M-O*), 12.5 μm; (*D*, *H*, *L*, *P*), 8.7 μm.

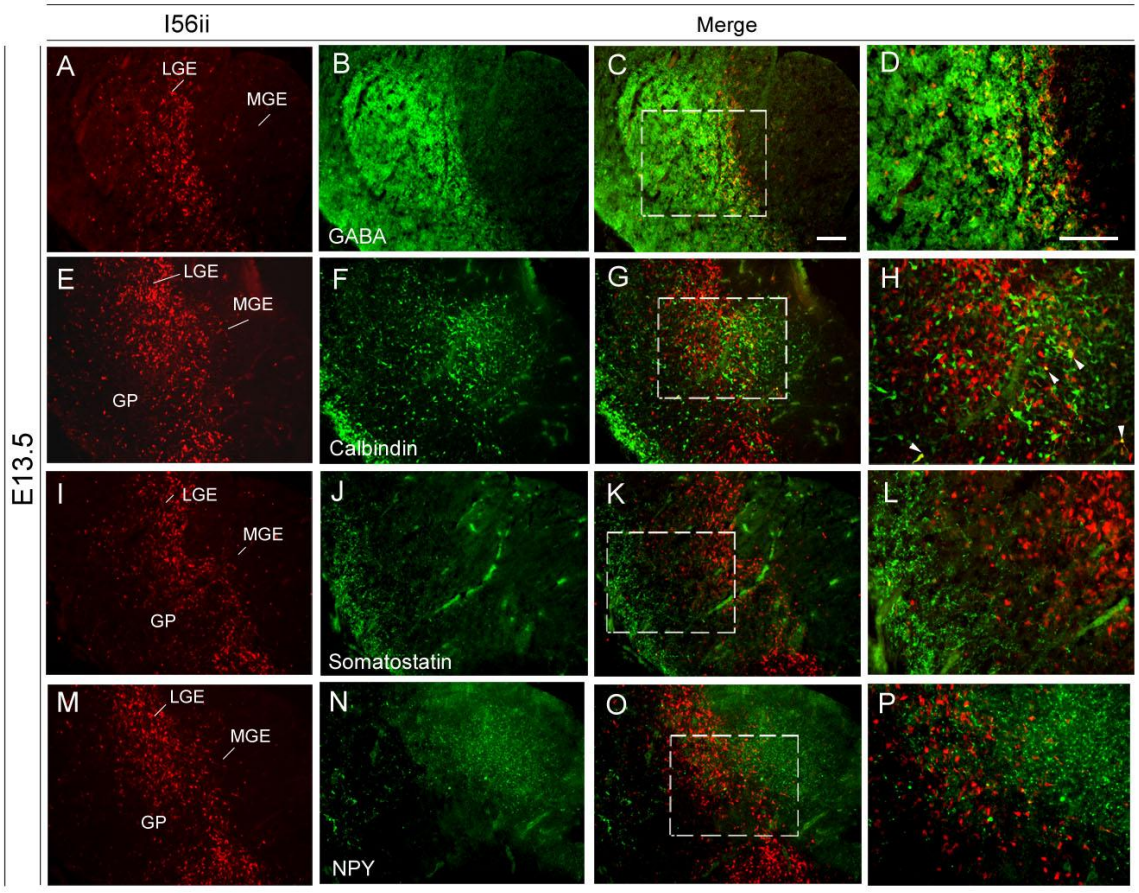
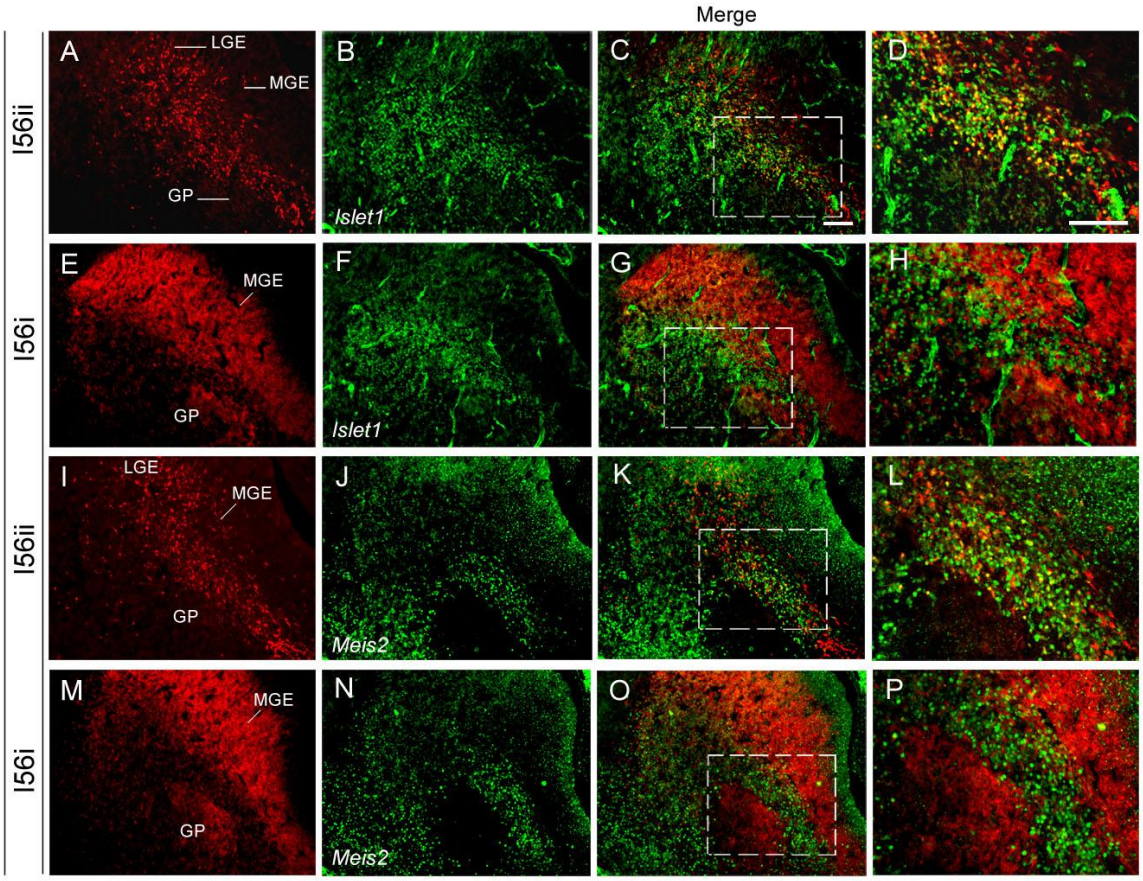
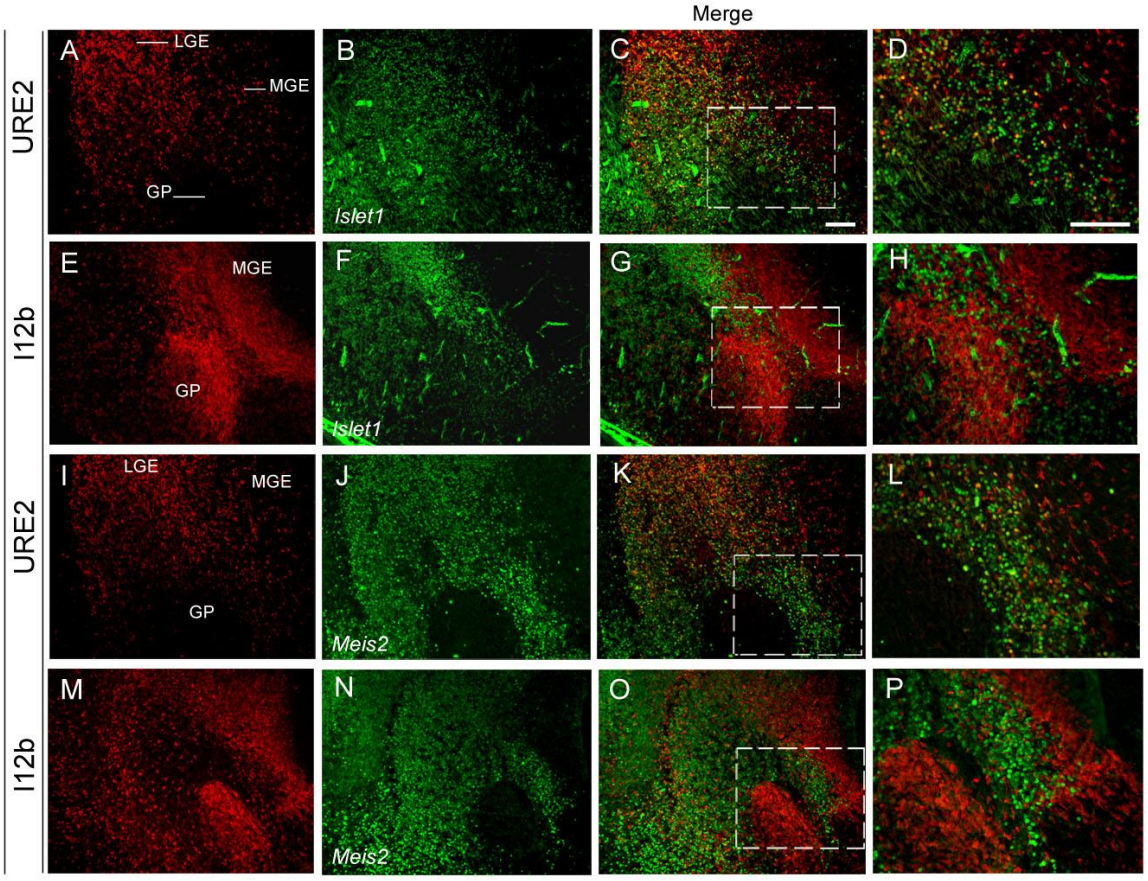

I56ii
Merge
E13.5
A
LGE
MGE
B
GABA
C
D
E
LGE
MGE
GP
F
Calbindin
G
H
I
LGE
MGE
GP
J
Somatostatin
K
L
M
LGE
MGE
GP
N
NPY
O
P

Figure 2.6. The I56ii CRE is active in a subset of cells expressing *Islet1* and *Meis2*. The vast majority of *I56ii-lacZ*-expressing cells located in a deep mantle layer that is intercalated between the SVZ and more superficial parts of the mantle of the ventral LGE (striatum) and the MGE (globus pallidus) express two markers of striatal projection neurons, *Islet1* (*A-D*) and *Meis2* (*I-L*) at E13.5. No such co-localization is observed for the *I56i-lacZ* positive cells at this stage (*Islet1*: *E-H*; *Meis2*: *M-P*). Cell expressing *I56ii-lacZ* or *I56i-lacZ* are shown in red; Islet1- or Meis2- positive cells are shown in green. *D*, *H*, *L*, *P*: high magnifications of boxes shown in *C*, *G*, *K*, *O*, respectively. Scale bars: (*A-C*, *E-G*, *I-K*, *M-O*), 12.5 μm; (*D*, *H*, *L*, *P*), 8.7 μm.


Merge
I56ii
I56i
I56ii
I56i
A
LGE
MGE
GP
B
Islet1
C
D
E
MGE
GP
F
Islet1
G
H
I
LGE
MGE
GP
J
Meis2
K
L
M
MGE
GP
N
Meis2
O
P

Supplementary Figure 2.6. Unlike the activity of the I56ii CRE, only a few *URE2-lacZ* positive cells express either *Islet1* (*A-D*) or *Meis2* (*I-L*) in the mantle of the MGE at E13.5. Note: many *URE2-lacZ* positive cells located in the deep mantle of the LGE also express *Islet1* (*C, D*) or *Meis2* (*K, L*), but I56ii is not active in this region (see Fig. 2.6A-D, I-L). In addition, none of the *I12b-lacZ*-expressing cells is co-labeled with anti-Islet1 (*E-H*) or Meis2 (*M-P*) antibodies at this stage. Cell expressing *URE2-lacZ* or *I12b-lacZ* are shown in red; Islet1- or Meis2- positive cells are shown in green. *D*, *H*, *L*, *P*: high magnifications of boxes shown in *C*, *G*, *K*, *O*, respectively. Scale bars: (*A-C*, *E-G*, *I-K*, *M-O*), 12.5 μm; (*D*, *H*, *L*, *P*), 8.7 μm.


Merge
URE2
I12b
URE2
I12b
A
LGE
MGE
GP
B
Islet1
C
D
E
MGE
GP
F
Islet1
G
H
I
LGE
MGE
GP
J
Meis2
K
L
M
N
Meis2
O
P

several potential core binding sites (ATTA) for Islet proteins (Supplementary Figure 2.1). It is noteworthy that these ATTA-core motifs are also shared by a large number of other homeodomain proteins, such as Hox and Oct proteins (Gehring et al., 1994; Pankratova and Polanovsky, 1998). To explore whether Meis2 or Islet1 binds and activates transcription via these binding sites, we conducted transient co-transfection reporter assays. As noted above, P19 embryonic carcinoma cells were transfected with the mI56ii-pBLCAT reporter construct, together with Meis2-pCS2+ or Islet1-pCS2+ expression vectors (see material and methods for experimental details). We then quantified the relative CAT activity in the presence of Meis2 or Islet1, and found that it was significantly increased by about 4.50- and 3.53-fold, respectively, when compared to controls and after normalization with the β-galactosidase expression, even though the increase in both cases was not statistically significant ($P = 0.052$ and $P = 0.081$; Figure 2.7). Co-transfection of a modified mI56ii-pBLCAT vector containing the mutant Meis2 binding site (mI56ii-mutant-pBLCAT2) significantly decreased the transcriptional activation of the reporter gene in the same assay and virtually no activation by Meis2 was obtained with an enhancer-less vector (empty pBLCAT2) (Figure 2.7). These data suggest that both Meis2 and Islet1 proteins are potential upstream regulators of *Dlx* gene expression *in vivo* and this regulation could be mediated by the I56ii CRE.

2.4. Discussion

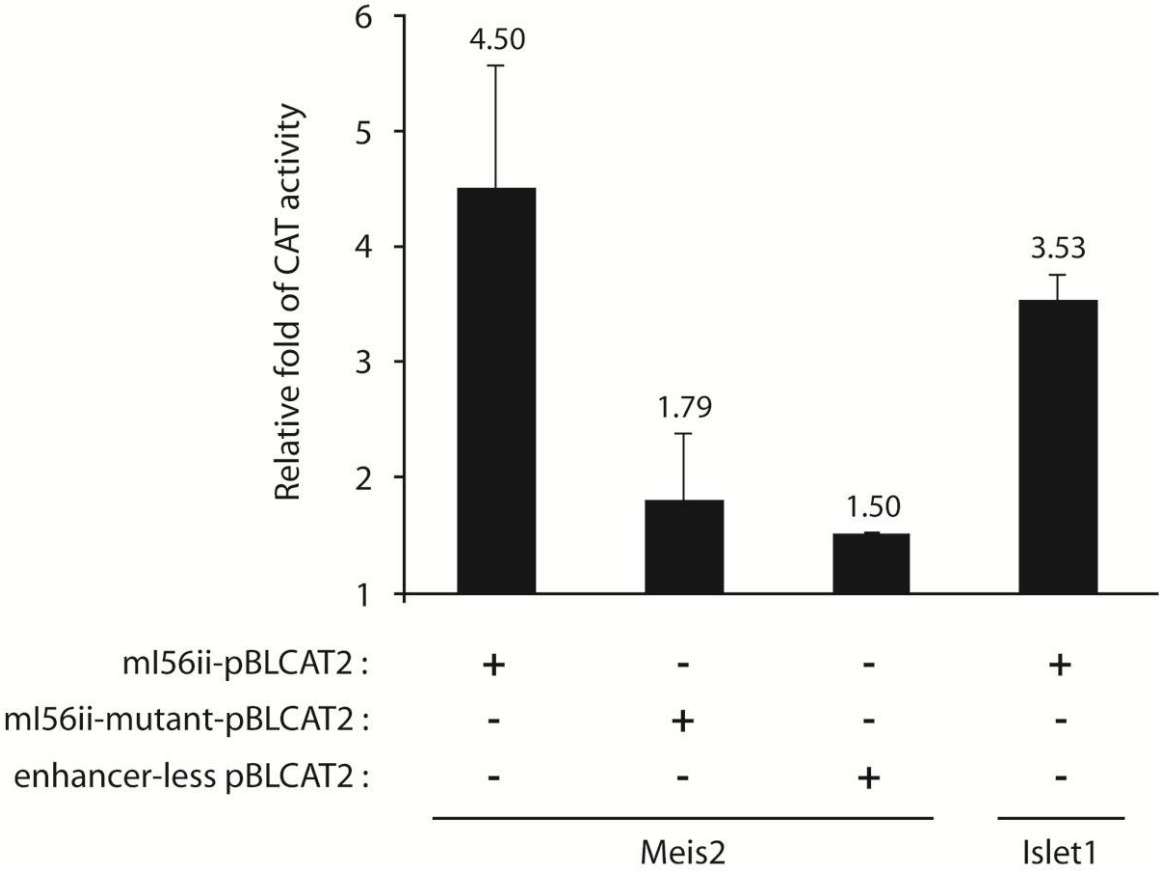
In the present report, we analyzed the regulatory role of the I56ii CRE in the mouse telencephalon using *lacZ* reporter transgenes. We report that the I56ii CRE shows a strikingly different activity during midgestation when compared with the other three *Dlx*

CREs that are also active in the forebrain: I56i from the *Dlx5/6* bigene cluster and, URE2 and I12b, from the *Dlx1/2* cluster. Thus, the *I56ii-lacZ*-expressing cells display the following properties: 1) They express the transgene more transiently, between E11.5 and E13.5. Their expression is restricted to a deep mantle layer that is intercalated between the subpallial SVZ and more superficial parts of the mantle of the ventral LGE (striatum) and the MGE (globus pallidus). The other three *Dlx* CREs are weakly or not active in these cells (Figure 2.2, arrows); 2) Unlike the other three *Dlx* CREs, the *I56ii-lacZ*-expressing cells do not express markers of interneurons including somatostatin and NPY (Figure 2.5 and data not shown) and do not tangentially migrate to the cortex. Rather, the *I56ii-lacZ*-expressing cells are a subpopulation of post-mitotic GABAergic projection neurons expressing two striatal markers, *Meis2* and *Islet1* (Figures 2.4-2.6); and, 3) Both Meis2 and Islet1 proteins can induce transcription *in vitro* via one or more putative binding sites found in the I56ii sequence (Figure 2.7).

2.4.1. Comparison of the *lacZ* reporter gene expression driven by the four *Dlx* CREs with endogenous *Dlx* gene expression

The onset of expression of the *lacZ* transgene in all four CRE lines is detected in a group of prethalamic cells around ~E10 and, therefore, slightly contrasts with the onset of the endogenous *Dlx* gene expression which occurs between E9-9.5. This difference could be due to low levels of the *β*-galactosidase protein that are undetectable before E10 and/or to the existence of other regulatory sequence(s) that are necessary to refine the temporal expression of the *Dlx* genes. In all four transgenic lines, the *β*-galactosidase-positive cells are *Dlx*-positive as suggested by their co-labeling with a pan-*Dll* antibody

Figure 2.7. The Meis2 and Islet1 proteins can activate reporter gene transcription via the I56ii CRE *in vitro*. In a transient co-transfection assay, the activity of the CAT reporter construct containing the I56ii sequence (mI56ii-pBLCAT2) was increased by the transcription factors whereas almost no change was obtained when an enhancer-less construct was co-transfected with Meis2. When one of the two potential Meis2 binding sites in I56ii was mutagenized, a much lower fold activation was obtained in the presence of Meis2. Data are presented as the mean of six values obtained from two independent experiments in triplicate ± standard error on the mean (SEM).


Relative fold of CAT activity
6
5
4
3
2
1
4.50
1.79
1.50
3.53
mI56ii-pBLCAT2 :
mI56ii-mutant-pBLCAT2 :
enhancer-less pBLCAT2 :
+ - - +
- + - -
- - + -
Meis2
Islet1

(Figure 2.4A- D and data not shown; Ghanem et al., 2007). In addition, the patterns of enhancer activity between E10.5 and E15.5 overlap with the endogenous patterns of *Dlx* expression (Figure 2.3 and data not shown). However, the transgene expression in all lines examined lack one feature of telencephalic *Dlx* expression at E10.5: previous studies showed that the dorsal LGE (dLGE) has a high concentration of *Dlx2*+ cells in the VZ (Eisenstat et al., 1999; Yun et al., 2002). As none of the transgenic lines studied here show this property, it suggests that this expression is conferred by an unknown enhancer element. Furthermore, the *β*-galactosidase-positive neurons are still found in the mouse neocortex at birth as well as one month after birth in all CRE lines (Ghanem et al., 2007) except in the I56ii lines where *lacZ* expression is maintained only in a group of hypothalamic cells after E14.5 (Supplementary Figure 2.5D, H, L and data not shown).

2.4.2. I56ii CRE displays a differential activity in comparison with the I56i, I12b and URE2 CREs

Using double labeling, we showed that *I56ii-lacZ*- and *I12b-AP*-expressing cells do not overlap between at E12.5 and E13.5 in the ganglionic eminences (Figure 2.4E-H and data not shown). We previously showed that I12b and I56i are co-active in more than 80% of the progenitor population(s) found in the proliferative zones between E11.5 and E13.5 (Ghanem et al., 2007). Therefore, we can infer that I56ii and I56i are likely to be active in distinct group(s) of cells at these time points. Several lines of additional evidence support our conclusion: 1) We found that I56ii-positive cells are *Dlx*+/GABA+. They also specifically express *Meis2* and *Islet1*, two markers of striatal projection neurons that are primarily derived from the LGE. In contrast, none of the other three

lacZ-CRE transgenes co-expresses *Meis2* or *Islet1* in the superficial mantle of the ganglionic eminences, except for a few cells weakly expressing *URE2-lacZ* (Figure 2.6 and Supplementary Figure 2.6); 2) We showed that URE2, I12b, and I56i are active in distinct subpopulations of immature cortical and hippocampal interneurons that tangentially migrate from subpallium, including populations expressing calbindin, somatostatin and NPY at E13.5 (Ghanem et al., 2007 and this study, data not shown). In addition, we showed that expression from the elements continue to mark different subtypes of adult interneurons (Ghanem et al., 2007). In contrast, the I56ii CRE is inactive in cells migrating tangentially to the cortex and its activity is not detectable past E14.5 in the basal ganglia as discussed earlier.

2.4.3. I56ii marks a subgroup of striatal projection neurons that are probably derived from the ventral LGE that tangentially migrate into the pallium

Based on their profile and pattern of expression as well as the orientation of their migratory processes, we suggest that the *I56ii-lacZ*-expressing cells are GABAergic projection neurons that may derive from progenitors found in the ventral LGE and then migrate tangentially following a dorsal-to-ventral route before they finally settle down between the SVZ and the globus pallidus in the deep mantle of the MGE (Figures 2.2 and 2.6).

There is recent evidence of a novel dorsal-to-ventral tangential migration of neural stem cells in the perinatal forebrain (Willaime-Morawek et al., 2006). Furthermore, tangentially migrating cells can translocate between subdivisions within the subpallium itself including the LGE and MGE as well as the AEP and POA. For instance, progenitor

cells found in the AEP/POA region migrate dorsally into the striatum to become cholinergic interneurons (Marin et al., 2000; Zhao et al., 2003). Also, it was shown recently that a subgroup of projection neurons, namely "corridor cells", tangentially migrate from the LGE to the MGE around E13.5 to establish a permissive route that is essential for thalamocortical axon guidance in mouse (Lopez-Bendito et al., 2006). Furthermore, the so-called "corridor cells" are GABAergic neurons that, like the *I56ii-lacZ*-expressing cells described here, specifically express LGE markers such as *Islet1*, *Ebf1* and *Meis2* (Lopez-Bendito et al., 2006). It will be interesting to determine in the future whether I56ii is a marker of these "corridor cells" during midgestation.

2.4.4. *Meis2* and *Islet1* are potential upstream regulators of *Dlx* genes

Our data suggest that the *Dlx* genes may be potentially regulated by *Meis2* and *Islet1* to possibly maintain their activities via the I56ii sequence, at least between E11.5 to E13.5. This observation is supported by the ability of both proteins to activate the transcription (by about 3.5-4.5 fold) of a reporter gene via I56ii in transient co-transfection assays *in vitro* (Figure 2.7). There are two potential binding sites for Meis2 in I56ii (Supplementary Figure 3.1) and when one of the two sites is mutated in the above sequence, activation by Meis2 is almost totally abolished. Future experiments such as chromatin immunoprecipitation (ChIP) could determine whether this interaction also takes place *in vivo*. As for Islet1, it was not possible to identify a precise binding site in I56ii as the binding preferences of this transcriptional activator are poorly defined as the ATTA sequence which is found in several locations in I56ii (Supplementary Figure 2.1) and which is shared by numerous homeodomain transcription factors.

In conclusion, we showed in the present study that *I56ii-lacZ*-expressing cells in the ventral forebrain are a subpopulation(s) of post-mitotic projection neurons that may be derived from progenitor cells located in the LGE as suggested by their expression of two striatal markers, *Meis2* and *Islet1*. They constitute a distinct population of *Dlx*-expressing cells compared to those where the other CREs, URE2, I12b and I56i, are active. Therefore, the distinct activities of the four *Dlx* CREs, we have identified thus far, reflect a complex and dynamic spatio-temporal regulation of *Dlx* gene expression during the early stages of embryonic development. Moreover, it highlights that the *Dlx* genes may have distinct functions in distinct progenitor populations born and derived from the basal ganglia.

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3. Roles of the *cis*-regulatory elements, I56i and I56ii, from the *dlx5a/dlx6a* intergenic region, during zebrafish GABAergic neuron development and their cross-regulatory interaction

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Abstract

During vertebrate forebrain formation, *Dlx* homeobox genes play essential roles in the differentiation, migration and survival of subpallium-derived progenitors that will later give rise to diverse subtypes of γ-aminobutyric acid (GABA)-expressing neurons, including inhibitory cortical interneurons. They also participate in the regulation of the two *Gad* genes that encode the enzymes necessary for GABA synthesis. In mice, at least four *cis*-regulatory elements (CREs) control *Dlx* expression in the telencephalon and diencephalon: URE2 and I12b in the *Dlx1*/*Dlx2* bigene cluster, and, I56i and I56ii in the *Dlx5*/*Dlx6* bigene cluster. However, little is known so far with respect to the function of orthologous *dlx* genes and their regulatory elements during zebrafish GABAergic neuron development. To investigate whether similar *dlx*-mediated pathways exist in the early developing zebrafish forebrain, we characterized two independent lines of transgenic zebrafish carrying GFP reporter genes: one under the control of a 1.4kb *dlx5a/dlx6a* intergenic sequence (encompassing I56i and I56ii) and one with a 1.1kb fragment containing only I56i CRE, respectively. Our comparative analysis showed that GFP expression patterns of these two transgenic lines are largely overlapping throughout the basal forebrain. Intriguingly, compared to the *1.4kbdlx5a/dlx6a:GFP* fish, populations of GFP-positive cells detected in the mesencephalic tectum are almost completely unlabeled in the *1.1kbI56i:GFP* embryos, probably due to the lack of I56ii CRE activity. Despite that difference, almost all GFP-expressing cells are GABA-positive. Co-expression of the GFP transgene with various GABAergic interneuron markers was only observed in certain domains in the ventral portion of the telencephalon and diencephalon as well as in the midbrain tectum. Simultaneous inactivation of *dlx1a/dlx2a* or *dlx5a/dlx6a* function

led to a marked loss of *dlx* CRE activity specifically restricted to the diencephalon and midbrain tectum, but a comparatively milder extent of reduction in the telencephalon, suggesting the cross-regulation of *dlx5a/dlx6a* by *dlx1a* and *dlx2a* is similar, at least in part, to that previously reported for their mouse orthologs. Together, our data suggest that *dlx* genes and their CREs are involved in a conserved genetic pathway necessary for proper GABAergic neuron production and *dlx* expression in the developing zebrafish forebrain, particularly in the diencephalon.

3.1. Introduction

The *Dlx* gene family encodes homeodomain-containing transcription factors that are implicated in the development of the mammalian forebrain (Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002). In the developing mouse forebrain, four *Dlx* genes (*Dlx1*, *Dlx2*, *Dlx5* and then *Dlx6*) are sequentially expressed starting at around embryonic day 9.5 (E9.5) and exhibit highly overlapping but distinct expression patterns in the ganglionic eminences of the ventral telencephalon as well as in the ventral diencephalon (prethalamus and hypothalamus) (Liu et al., 1997; Yang et al., 1998; Eisenstat et al., 1999). Specifically, *Dlx1* and *Dlx2* are predominantly expressed in the least mature early-born progenitor cells within the ventricular and subventricular zones (VZ and SVZ), whereas *Dlx5* and *Dlx6* genes are mainly expressed in the more differentiated postmitotic cells within the SVZ and mantle zone (MZ) (Eisenstat et al., 1999; Panganiban and Rubenstein, 2002). The forebrain expression patterns of *Dlx* genes largely overlap with that of the two *glutamic acid decarboxylase* (*Gad*) genes, *Gad1* and *Gad2*, which encode the key enzymes required for the production of the neurotransmitter

γ-amino butyric acid (GABA) (Yun et al. 2002; Long et al., 2009a). Ectopic expression of Dlx proteins *in vitro* is able to induce ectopic *Gad* expression in many domains of the developing mouse forebrain (Anderson et al., 1999; Stuhmer et al., 2002b; Yun et al., 2002). Previous loss-of-function studies have shown that *Dlx* genes are particularly responsible for the proper proliferation, differentiation, and survival of GABAergic interneuron progenitor cells and those cells will later migrate and populate various areas of the forebrain (Panganiban and Rubenstein, 2002; Wonders and Anderson, 2006). For example, inactivation of both *Dlx1* and *Dlx2* genes leads to a major deficiency in the generation and differentiation of GABA-expressing striatal projection neurons and GABAergic interneuron progenitors born in the ganglionic eminences (Anderson et al., 1997a; Anderson et al., 1997b; Marin et al., 2000; Pleasure et al., 2000; Long et al., 2007). These cells accumulate in the basal ganglia and fail to migrate to the neocortex, olfactory bulb and hippocampus through different migratory routes (Anderson et al., 1997a; Anderson et al., 1997b; Marin et al., 2000; Pleasure et al., 2000; Long et al., 2007). Likewise, simultaneous inactivation of *Dlx5* and *Dlx6* function results in exencephaly of the anterior portion of the embryonic forebrain and the mutants have a decreased number of PV-expressing cortical interneurons with increased dendrite branching (Wang et al., 2010).

Vertebrates have multiple *Dlx* genes that are generally arranged as convergently transcribed bigene clusters on distinct chromosomes (Zerucha and Ekker, 2000). The two genes of a bigene pair are separated by a short intergenic sequence (~3.5 to 16 kb) (Zerucha and Ekker, 2000). Mammals have six *Dlx* genes organized as three bigene pairs (*Dlx1/Dlx2*, *Dlx3/Dlx4* and *Dlx5/Dlx6*), whereas some teleost fish including zebrafish

have eight *dlx* genes, among which six (*dlx1a/dlx2a*, *dlx3b/dlx4b* and *dlx5a/dlx6a*) are organized in a manner similar to their mammalian counterparts (Stock et al., 1996; Amores et al., 1998; Quint et al., 2000). The other two additional *dlx* genes (*dlx2b* and *dlx4a*) are not linked with other *dlx* genes and are considered as the respective duplicates of ancestral *Dlx2* and *Dlx4* genes, following the occurrence of a teleost-specific whole or partial genome duplication event (Amores et al., 1998). Consistent with mouse *Dlx* forebrain expression, five zebrafish *dlx* genes (*dlx1a/dlx2a*, *dlx2b* and *dlx5a/dlx6a*) are expressed in a highly similar pattern in the developing zebrafish telencephalon and diencephalon (Akimenko et al. 1994; Ellies et al. 1997; MacDonald et al., 2010a). In addition, *dlx1a* and *dlx2a* expression has been mainly found in the cell populations that are located more close to the ventricle as compared to that of *dlx5a* and *dlx6a*, a situation that resembles the spatio-temporal forebrain expression of mouse *Dlx* genes (Zerucha et al., 2000; MacDonald et al., 2010a). Proper *Dlx* expression in the developing mouse and zebrafish forebrain is transcriptionally regulated, at least in part, by a number of highly conserved *cis*-regulatory elements (CREs, enhancers) present in their surrounding genome regions (Zerucha et al., 2000; Ghanem et al., 2003; Ghanem et al., 2007): URE2 located upstream of *Dlx1* (*dlx1a*), I12a and I12b within the *Dlx1/Dlx2* (*dlx1a/dlx2a*) intergenic region, I56i and I56ii within the *Dlx5/Dlx6* (*dlx5a/dlx6a*) intergenic region. Our previous studies using transgenic mice have shown that URE2, I12b and I56i CREs target GABA-expressing progenitors in the ganglionic eminences in the subpallial telecephalon as well as diverse subtypes of GABAergic interneurons in the adult cortex (Ghanem et al., 2007). In contrast, I56ii activity is mainly restricted to a subpopulation of

GABAergic striatal projection neurons at E11.5-E13.5 during early embryogenesis (Ghanem et al., 2008).

To date, the roles played by *dlx* genes and their various individual CREs in the genetic cascades involved in GABAergic neuron formation are not well understood, especially in non-mammalian vertebrates. Using triple fluorescence *in situ* hybridization techniques, our laboratory has shown that *dlx* genes are expressed in highly overlapping domains with that of the *gad* genes in the developing zebrafish subpallium (MacDonald et al., 2010a). The spatial-temporal forebrain expression of *dlx* and *gad1* genes and the subpallial-to-pallial migration of *dlx/gad1*-expressing neurons are consistent with previous studies done in the mouse, indicating that these genes may be involved in a conserved genetic pathway mediating GABAergic neuron development (Panganiban and Rubenstein, 2002; Marin and Rubenstein, 2003; Mueller et al., 2008; MacDonald et al., 2010a). To further understand the regulatory roles of the *dlx* CREs during zebrafish GABAergic neuron generation, we characterized two stable lines of transgenic zebrafish: one in which GFP is under the control of a 1.4kb *dlx5a/dlx6a* intergenic sequence (encompassing I56i and I56ii; Ghanem et al., 2003) and a newly-produced line containing a 1.1kb fragment encompassing only I56i CRE. In addition to a comparative analysis conducted between the two transgenic lines, we also sought to determine whether any potential association exists between GFP expression and several GABAergic interneuron markers using double immunohistochemical staining in an effort to evaluate the functional importance of these two enhancers in the differentiation of zebrafish GABAergic neurons. The possible auto- and/or cross-regulation of *dlx* genes during

zebrafish GABAergic neuron development was also investigated in our transgenic models by employing morpholino-mediated knockdown assays.

3.2. Materials and methods

3.2.1. Animal maintenance

Adult wild-type and transgenic zebrafish were maintained in circulating system water at 28.5°C under a constant photoperiod (a light/dark cycle of 14/10h) according to standard methods (Westerfield, 2000). Fish were fed once or twice daily with No.1 crumble (Zeigler^{TM}, Aquatic Habitats). Embryo-collection traps were placed inside each 9L acrylic tank containing 20-30 fish in the evening and embryos were harvested the next morning. Embryos were raised in an incubator at 28.5°C, staged in days post fertilization (dpf) according to specific criteria outlined by Kimmel et al. (1995) and euthanized with an overdose of tricainemesylate (ethyl 3-amino-benzoate methanesulfonate, Sigma) when needed. All experimental procedures in this study were carried out in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) and were approved by the University of Ottawa Animal Care Committee.

3.2.2. Construction of *dlx* CRE transgene vectors

The *1.4kbdlx5a/dlx6a:GFP* transgene construct has been previously generated in the laboratory as described in (Zerucha et al., 2000; Ghanem et al., 2003). In brief, a ~1.4kb *dlx5a/dlx6a* intergenic region containing both I56i and I56ii CREs was subcloned into a modified SP72-pEGFP-N1 vector using *Kpn*I/*Cla*I restriction sites and placed downstream of a ~3.5kb fragment from the immediate 5' flanking region of zebrafish

dlx6a, including part of the 5'UTR. This *dlx6a* 5' flanking fragment *per se* does not generate tissue-specific expression pattern in transgenic zebrafish (Ghanem et al., 2003).

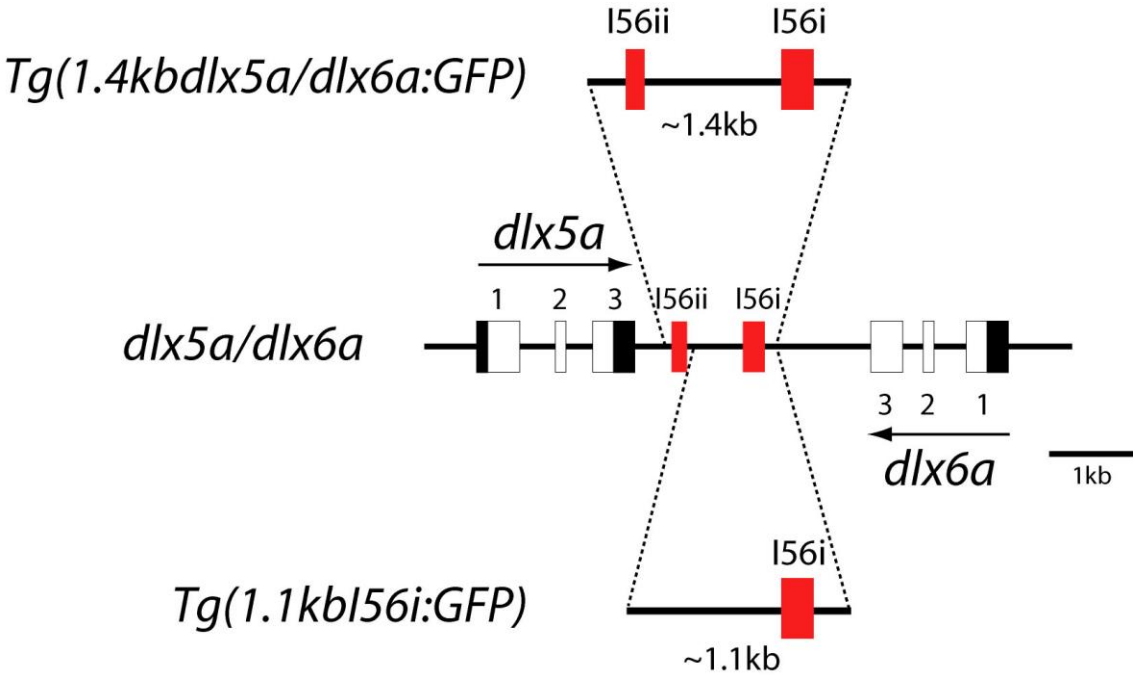
The *1.1kbI56i:GFP* transgene construct was made up of a ~1.1kb region from the *dlx5a/dlx6a* intergenic locus containing only I56i CRE and its flanking sequences. This fragment shares the same sequence with the 1.4kb *dlx5a/dlx6a* intergenic segment with the only difference being the lack of the ~300 bp I56ii CRE (Figure 3.1). Specifically, a ~1.1kb *Sal*I-*Sal*I I56i fragment was first PCR-amplified from the *1.4kbdlx5a/dlx6a:GFP* transgene plasmid using the following primer pair (forward: 5'-GTC GAC GCT CAA TTA TTA AGG TAT TGA CAA-3'; reverse: 5'-GTC GAC ACA AGC GGT TAC AAC GCT ATC-3') and was cloned into the pDrive vector (Qiagen). After a *Sal*I digestion, the linearized I56i fragment was separated in 1% agarose gel, recovered and purified using a gel extraction kit (Qiagen). This fragment was then ligated into the unique *Sal*I site immediately upstream of a β-globin minimal promoter linked to the coding sequence of a GFP reporter cassette in a Tol2 transposon plasmid that was modified from a SP72 vector (Promega) (Kawakami, 2004; MacDonald et al., 2010a). The resulting I56i-β-globin-GFP sequence was eventually flanked at both ends by Tol2 transposable element recognition sites. The *300bpI56ii:GFP* transgene vector that is under the control of a ~300 bp zebrafish I56ii sequence was generated in the same way by PCR amplification and subcloning into the Tol2 transposon plasmid.

3.2.3. Generation and visualization of transgenic zebrafish

The *tg(1.4kbdlx5a/dlx6a:GFP*) transgenic line has been previously established and expresses GFP in the domains of *dlx* expression in the telencephalon and diencephalon

Figure 3.1. A schematic drawing of GFP transgene constructs used to establish stable transgenic zebrafish lines

Similar to mammalian *Dlx* genes, zebrafish *dlx5a/dlx6a* genes are physically linked in a bigene arrangement and contain two highly conserved intergenic CREs named I56i and I56ii. The *tg(1.4kbdlx5a/dlx6a:GFP)* vector is controlled by a 1.4kb *dlx5a/dlx6a* intergenic sequence encompassing both I56i and I56ii CREs, while the *tg(1.1kbI56i:GFP)* construct is under the regulation of a ~1.1kb fragment containing all elements of the 1.4kb sequence, except for the I56ii CRE. Exons are numbered (black, untranslated region; white, coding sequences). I56i and I56ii regulatory elements are shown as red boxes.


I56ii
I56i
Tg(1.4kbdlx5a/dlx6a:GFP)
~1.4kb
dlx5a
1
2
3
I56ii
I56i
dlx5a/dlx6a
3
2
1
dlx6a
1kb
I56i
Tg(1.1kbI56i:GFP)
~1.1kb

(Zerucha et al., 2000; Ghanem et al., 2003). The *tg(1.1kbI56i:GFP)* line was generated by means of Tol2-mediated transgenesis. For the production of transgenic zebrafish, Tol2 transposase mRNA was first synthesized and purified with the mMessage SP6 Kit (Ambion) from the pCS2-TP vector after digestion with *Not*1 according to the manufacturer's instructions. The *1.1kbI56i:GFP* construct was then co-injected at approximately 50 ng/µl in standard DNA microinjection buffer (0.2 mM KCl, 0.1% phenol red), along with 50 ng/µl of *in vitro* transcribed Tol2 transposase mRNA, into fertilized one-cell stage embryos following procedures described previously (Fisher et al., 2006). Injected embryos were screened and those with strong GFP expression were raised to sexual maturity. Founder transgenic zebrafish (F0) were interbred with each other or outcrossed in pairs with wild-type fish. At least 100 embryos for each pair were screened by GFP fluorescence starting at 1-3 dpf. F1 progeny expressing strong GFP were selected and allowed to grow up to adulthood for establishing stable transgenic lines. Four independent transgenic lines (#11, #15, #17 and #21) were obtained and all gave identical and consistent GFP expression patterns in the developing forebrain, albeit with varying GFP signal intensity. The *300bpI56ii:GFP* transgene construct was injected into one-cell stage embryos in the same manner as the *1.4kbdlx5a/dlx6a:GFP* vector and expression of the *1.4kbdlx5a/dlx6a:GFP* construct was examined in primary transgenic embryos. Screening for GFP-positive offspring was performed on a Nikon NBZ 1500 dissecting fluorescence microscope and image acquisition was done with a Nikon DXM 1200C digital camera.

3.2.4. Immunohistochemistry

For immunostaining assays, zebrafish embryos were collected at different time points (3 and 5 dpf) and fixed in 4% paraformaldehyde (PFA)/1×PBS O/N at 4°C. Samples were then equilibrated in 30% sucrose/1×PBS O/N at 4°C prior to cryosectioning. The next day, fixed embryos were oriented and embedded in Tissue Freezing Medium (Triangle Biomedical Sciences) and transverse sections of 10-12 µm thickness were put onto Superfrost Plus slides (Fisher) using a Leica CM1850 standard cryostat (Leica Microsystems) at -20°C. After washing 3×10 min with 1×PBS to remove cryoprotectant, tissue sections were preincubated with a blocking solution containing 1% bovine serum albumin (BSA), 10% newborn calf serum and 0.03% Tween 20 for 2h at RT. The solution was subsequently replaced with a fresh aliquot containing the primary antibody (for dual-labeling, two primary antibodies were added simultaneously) and incubation was carried out in a humid chamber O/N at 4°C. Following 3×15 min washes in 1×PBS, sections were incubated in the dark with the appropriate secondary fluorescence-coupled antibody for 2h at RT and the slides were cover-slipped with a Vectashield mounting medium (Vector Labs). Histological sections were analyzed with a Zeiss Axiophot fluorescence microscope by using the appropriate filter sets and photographed with a Zeiss Axiocam digital camera.

The following primary antibodies were used in this study: rabbit polyclonal anti-GFP (1:500, Invitrogen), mouse monoclonal anti-GFP (1:500, Invitrogen), rabbit polyclonal anti-calretinin (CR, 1:1000, Swant), rabbit polyclonal anti-calbindin (CB, 1:1000, Swant), mouse monoclonal anti-parvalbumin (PV, 1:500, Sigma). The following secondary antibodies were applied: Alexa Fluor 488® goat anti-rabbit IgG (H+L) (1:300 Invitrogen), Alexa Fluor 488® goat anti-mouse IgG (H+L) (1:300, Invitrogen),

AlexaFluor 594® goat anti-rabbit IgG (H+L) (1:300, Invitrogen), Alexa Fluor 594® goat anti-mouse IgG (H+L) (1:300, Invitrogen).

3.2.5. Morpholino knockdown of *dlx* mRNA expression

Antisense morpholino oligonucleotides (MOs) complementary to the translation initiation sites of zebrafish *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* mRNA as well as a standard control morpholino were purchased from Gene Tools (Philomath, OR) and were diluted with nuclease-free H_2O. Serial concentrations of MOs (1, 2 and 4 ng/nl) were then prepared in 1×Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES pH 7.6] and phenol red was added to the MO injection solution at a final concentration of 0.05%. Approximately 1 nl of MO was microinjected into one cell-stage transgenic zebrafish embryos using a Narishige IM300 microinjector. Injected embryos were incubated in 30 ml Petri dishes with E3 embryo medium containing 0.03% methylene blue at 28.5°C. The morphology of morphants and their GFP expression patterns were examined under a Nikon NBZ1500 dissecting microscope and pictures were taken using a Nikon DXM 1200C digital camera. For double MO knockdown experiments, two MOs (*dlx1a+dlx2a* or *dlx5a+dlx6a*) were always co-injected in equal amounts. Standard control MOs were injected at the same concentrations simultaneously. The following translation blocking MOs were used: *dlx1a* (Sperber et al., 2008), *dlx2a* (Sperber et al., 2008), *dlx5a* (Walker et al., 2007), *dlx6a* (5'-TGG TCA TCATCA AAT TTT CTG CTT T-3'). The sequence for the standard control MO is 5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3'.

3.2.6. Neuroanatomical terminology

Neuroanatomical designations for different structures in the developing zebrafish brain are referenced from the atlas of early zebrafish brain development (Mueller and Wullimann, 2003). Gene symbols were used according to the ZFIN database (http:// zfin.org).

3.3. Results

3.3.1. Characterization and comparison of the activity of *dlx5a/dlx6a* intergenic CREs in the zebrafish forebrain

To better understand the roles of *dlx* genes in the process of zebrafish GABAergic neuron generation, it is necessary to characterize the underlying mechanisms responsible for their spatio-temporal expression and/or for their response to different regulatory signals. Multiple evolutionarily conserved *Dlx* CREs, such as I56i and I56ii, have been shown to exert strong forebrain-specific activity and target populations of GABAergic neurons in transgenic mouse studies (Ghanem et al., 2003; Ghanem et al. 2007; Ghanem et al. 2008). To test if orthologous regions from the zebrafish genome retain similar regulatory function, we characterized a previously established stable transgenic line, *tg(1.4kbdlx5a/dlx6a:GFP)*, which reflects the regulatory activity of a 1.4kb *dlx5a/dlx6a* intergenic sequence containing both I56i and I56ii enhancers (Figure 3.1). GFP transgene expression in live embryos was mainly analyzed from 1 to 5 dpf, a time during which the zebrafish forebrain is beginning to undergo secondary neurogenesis (Mueller and Wullimann, 2003) and during which GABA is expressed throughout the forebrain (Martin et al., 1998; Mueller et al., 2006). We found that the 1.4kb I56i/I56ii fragment

specifically drives GFP expression in the telencephalon starting at approximately 1 dpf (Figure 3.2A,B), and in certain domains of the ventral diencephalon (prethalamus and hypothalamus) starting at 2 dpf (Figure 3.2C,D). GFP expression in the prethalamus and hypothalamus becomes more apparent from 3 dpf onwards (Figure 3.2E-H). Scattered populations of GFP-positive cells were also clearly seen in the mesencephalic tectum (also known as optic tectum, Mueller and Wullimann, 2003) starting at 2 dpf and persisted until at least 5 dpf (Figure 3.2C,E,G, arrows). Our immunohistochemistry analysis on *1.4kbdlx5a/dlx6a:GFP* brain sections revealed that overall GFP expression patterns in the telencephalon and ventral diencephalon (Figure 3.3A,E,I,M) closely mimic the endogenous *dlx5a/dlx6a* expression (Mione et al., 2008; MacDonald et al., 2010a).

To confirm whether the zebrafish I56i and I56ii CREs target *dlx* expression to cells that are GABAergic neurons, we co-labeled 3 dpf transverse sections with antibodies against GFP and GABA and observed an extensive co-expression between GFP and GABA at different planes throughout the forebrain (Figure 3.3). It should be noted that a small number of GFP-positive cells located close to the ventral midline of the telencephalon and diencephalon, an area known to be a proliferative region (Mueller and Wullimann, 2003), remain free of GABA staining (Figure 3.3D,H,L,P, arrows), indicating those cells are probably still in the cell cycle and have not yet acquired the GABAergic phenotype. These results demonstrate that similar to their corresponding mouse orthologs, zebrafish *dlx5a/dlx6a* intergenic CREs are also active in GABAergic neurons during zebrafish forebrain development. In addition to their highly conserved sequences, zebrafish and mammalian *Dlx* CREs may possibly share at least a certain

Figure 3.2. Comparison of reporter gene expression patterns in the *1.4kbdlx5a/dlx6a:GFP* (A-H) and *1.1kbI56i:GFP* live embryos (I-P) at various developmental stages (1, 2, 3 and 5 dpf)

In the *1.4kbdlx5a/dlx6a:GFP* fish, robust GFP expression is first found in the telencephalon starting at 1 dpf (**A**,**B**), while GFP expression in the ventral diencephalon (prethalamus and hypothalamus) appears at ~2 dpf (**C**,**D**) and becomes more apparent after 3 dpf (**E-H**). Scattered populations of GFP-positive cells are also clearly identified in the midbrain tectum starting at 2 dpf and are present until 5 dpf (**C**,**E**,**G**, arrows). Although GFP expression patterns found throughout the ventral telencephalon and diencephalon in the *1.1kbI56i:GFP* fish (**I-P**) largely resembles those detected in the *tg(1.4kbdlx5a/dlx6a:GFP)* line at the same time points, *1.1kbI56i:GFP* larvae completely lack GFP expression in the tectum (**K**,**M**,**O**) and seem to have fewer GFP-positive cells in the telencephalon and diencephalon (**L**,**N**,**P**). Note that all injected embryos were treated with 0.003% 1-phenyl-2-thiourea (PTU) to inhibit pigmentation. (**A**,**C**,**E**,**G**,**I**,**K**,**M**,**O**): lateral views, anterior to the left, dorsal up; (**B**,**J**): dorsal views, anterior down; (**D**,**L**): ventral views, anterior to the left; (**F**,**H**,**N**,**P**): dorsal views, anterior to the left. Scale bars: 50 μm (**A**,**B**,**I**,**J**) and 100 μm (**C**,**D**,**K**,**L**; **E-H**, **M-P**). Abbreviations: hb, hindbrain; pTh, prethalamus; hy: hypothalamus; tec, tectum; tel, telencephalon.

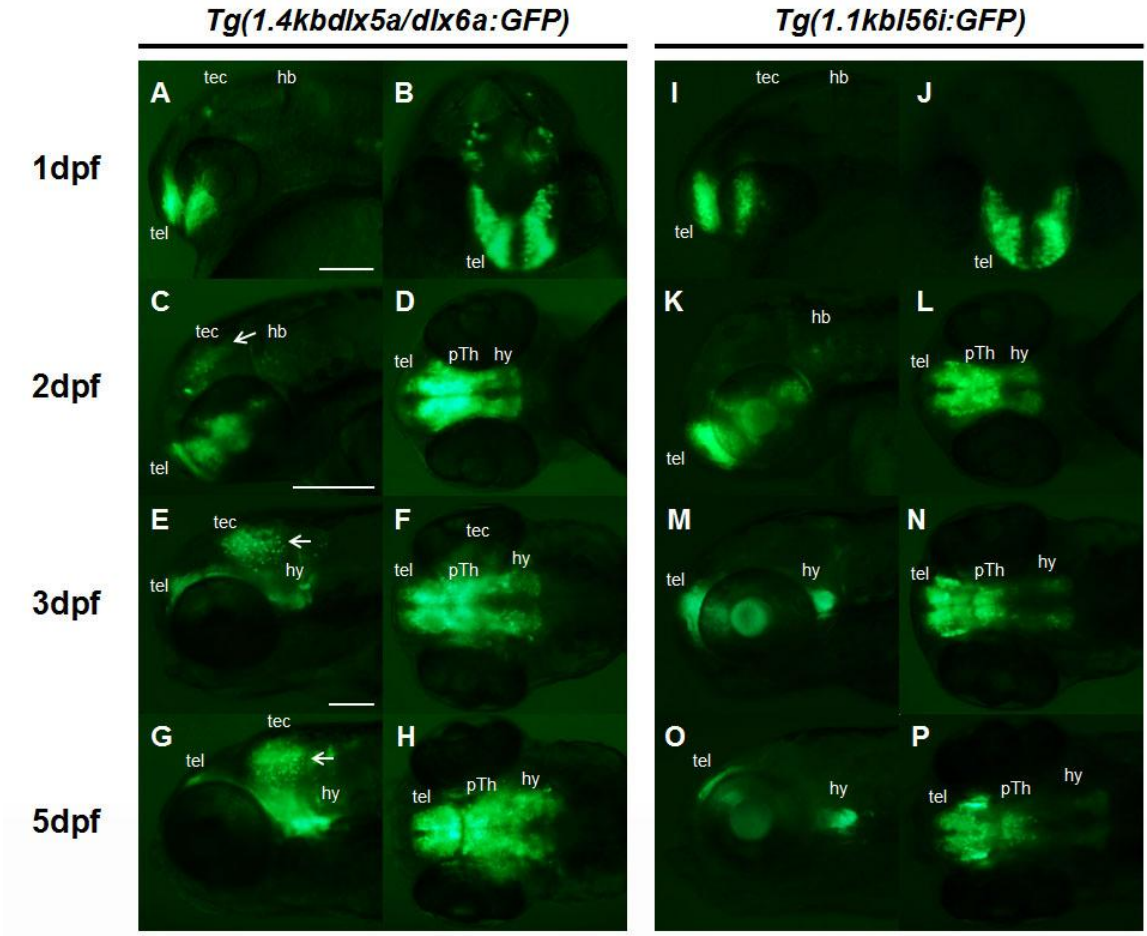
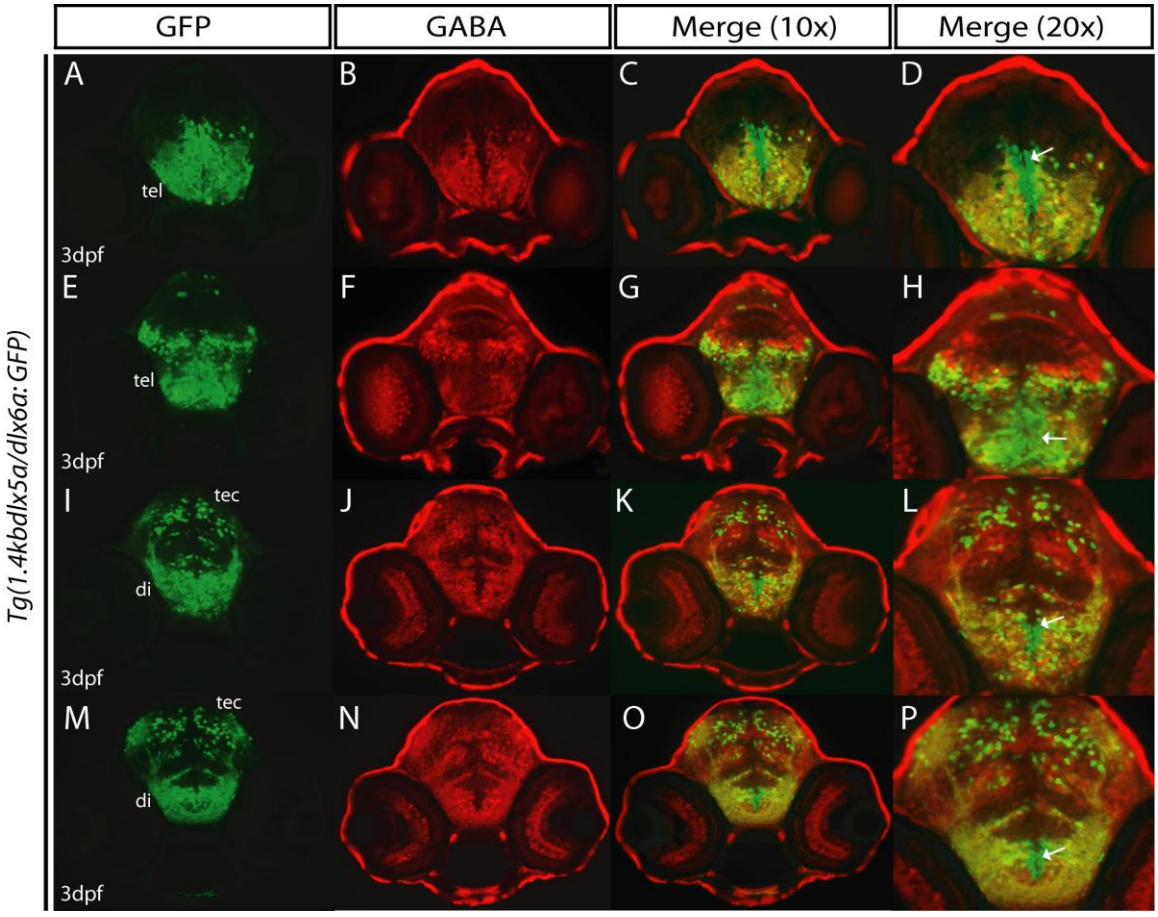

Tg(1.4kbdlx5a/dlx6a:GFP)
Tg(1.1kbI56i:GFP)
1dpf
2dpf
3dpf
5dpf
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
tec
hb
tel
pTh
hy

Figure 3.3. Double immunostaining on transverse sections showing colocalization of GFP and GABA in the *1.4kbdlx5a/dlx6a:GFP* transgenic embryos at 3 dpf

A vast majority of GFP-positive cells (**A**,**E**,**I**,**M**) in the ventral telencephalon and diencephalon as well as in the midbrain tectum co-express GABA (**B**,**F**,**J**,**N**), indicating *dlx5a/dlx6a* regulatory elements are specifically active in GABAergic neurons. Note that a small number of GFP-positive cells mainly located close to the ventricle in the ventral telencephalon and diencephalon are free of GABA labeling (**D**,**H**,**L**,**P,** arrows), consistent with the presence of large proliferative zones. Dorsal is upward in all the panels. **C**,**G**,**K**,**O** are merged images of (**A**,**B**), (**E**,**F**), (**I**,**J**) and (**M**,**N**), respectively. **D**, **H**, **L** and **P** are higher magnification pictures of **C**, **G**, **K** and **O** showing double-labelling of GFP and GABA. Abbreviations: di, diencephalon; tec, midbrain tectum; tel, telencephalon.


GFP
GABA
Merge (10x)
Merge (20x)
Tg(1.4kbdlx5a/dlx6a:GFP)
A
B
C
D
tel
3dpf
E
F
G
H
tel
3dpf
I
J
K
L
tec
di
3dpf
M
N
O
P
tec
di
3dpf

degree of functional conservation.

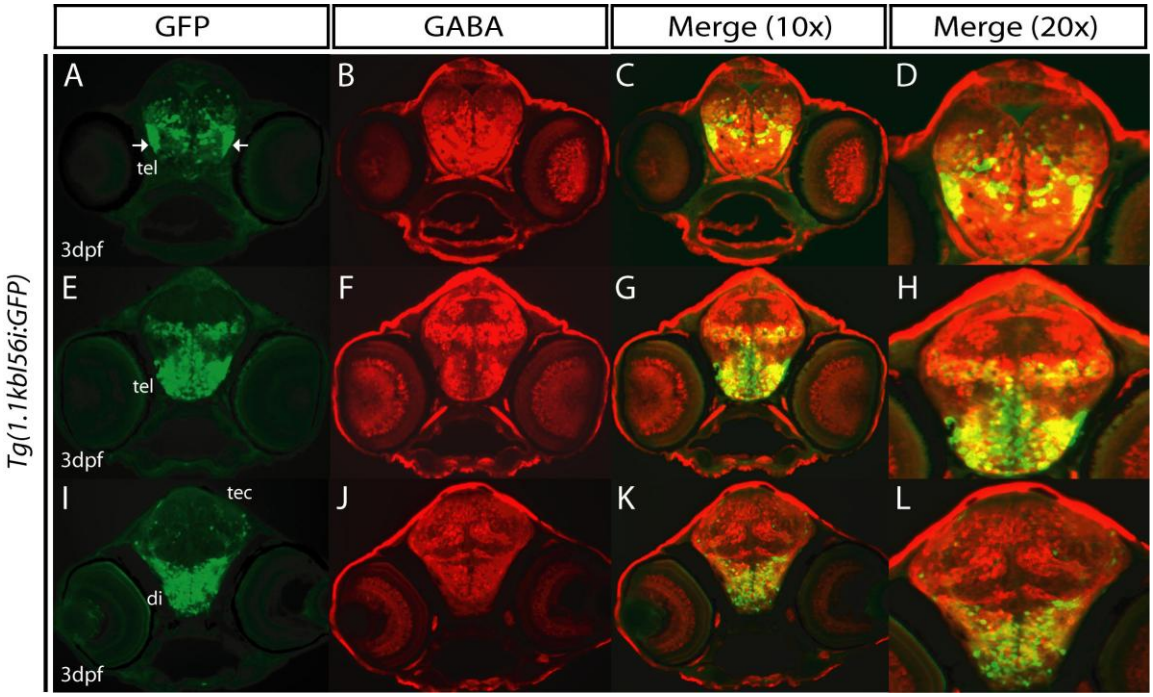
Although our analyses showed that *dlx5a/dlx6a* intergenic CREs are able to target zebrafish GABAergic neurons and reconstitute most, if not all, of the endogenous pattern of *dlx* forebrain expression, the specific contributions of each individual CRE has yet to be determined. For this purpose, we first generated a transgene construct named *300bpI56ii:GFP* that is under the control of a ~300 bp I56ii CRE cloned from the *dlx5a/dlx6a* intergenic region and tested its potential activity. The zebrafish I56ii enhancer, alone, failed to display a detectable specific forebrain activity in primary transgenic fish (n > 200), precluding the generation of a stable line (data not shown). This result suggests that contrary to its mouse counterpart, zebrafish I56ii sequence cannot by itself target gene expression in the forebrain. However, it may collaborate with other CREs in regulating *dlx* expression and necessary for their proper activity. To test this possibility, a ~1.1kb fragment containing all of the previously used 1.4kb fragment, except for the I56ii CRE, was inserted into a GFP reporter construct (Figure 3.1) and an independent transgenic line, named *tg(1.1kbI56i:GFP)*, was successfully created. We found that the 1.1kb I56i sequence, when tested individually, is sufficient to direct specific GFP expression to the telencephalon as well as the ventral diencephalon including both prethalamus and hypothalamus areas (Figure 3.2I-P), which faithfully recapitulates the GFP expression observed in the *1.4kbdlx5a/dlx6a:GFP* transgenic fish at the same time points (Figure 3.2A-H). However, compared to the *1.4kbdlx5a/dlx6a:GFP* fish, the *1.1kbI56i:GFP* embryos showed a complete lack of GFP reporter expression in the midbrain tectum starting at 2 dpf (Figure 3.2K,M,O) and fewer GFP-positive cells were seen in the telencephalon and diencephalon (Figure 3.2L,N,P),

most likely due to the loss of the regulatory function of the I56ii CRE. These observations are supported by immunostaining assays on tissue sections showing that GFP expression in the midbrain tectum is almost entirely absent in the *1.1kbI56i:GFP* embryos (Figure 3.4E,I), whereas expression patterns in the ventral telencephalon and diencephalon are essentially identical between the two constructs (Figure 3.3A,E,I,M and Figure 3.4A,E,I). Furthermore, the *1.1kbI56i:GFP* fish appear to have a markedly reduced number of GFP-positive cells in the ventral telencephalon at the rostral level (Figure 3.4A) as well as a loss of a small subpopulation of GFP-expressing cells in the ventral diencephalon (prethalamus, Figure 3.4I) as compared to the corresponding regions in *1.4kbdlx5a/dlx6a:GFP* fish (Figure 3.3A and Figure 3.3I). In the *1.1kbI56i:GFP* fish, robust GFP expression is also found in the forebrain bundles assembled bilaterally in the basal forebrain (Figure 3.4A, arrows). As expected, the 1.1kbI56i-directed GFP expression within domains of the ventral telencephalon and diencephalon is highly co-localized with that of GABA-positive neurons (Figure 3.4), consistent with the observations made in the *1.4kbdlx5a/dlx6a:GFP* fish (Figure 3.3). These findings support a notion that whereas the overall activity of intergenic regulatory elements associated with the *dlx5a/dlx6a* locus is primarily restricted to GABAergic neurons, the different individual enhancers, I56i and I56ii, seem to perform distinct, non-redundant roles in modulating the development of different subpopulation of GABA-expressing cells, with I56ii necessary for expression in the tectum.

3.3.2. *dlx5a/dlx6a* intergenic CREs do not extensively mark diverse GABAergic interneuron subtypes

Figure 3.4. Double immunohistochemistry analysis of GFP and GABA on transverse sections of the 3 dpf *1.1kbI56i:GFP* forebrain

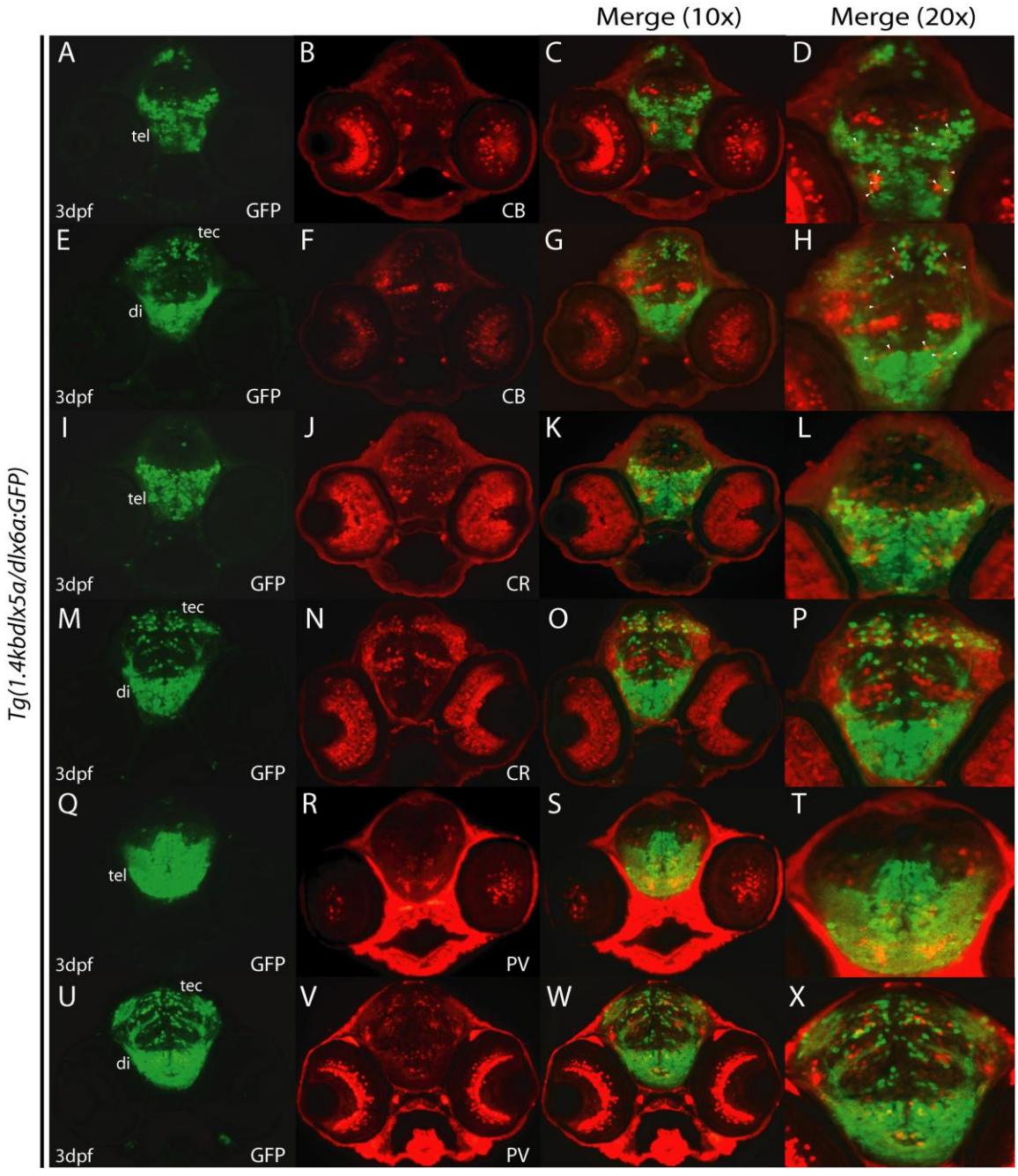
The overall distribution of 1.1kbI56i-directed GFP-positive cells (**A**,**E**,**I**) in the ventral telencephalon and diencephalon at 3 dpf recapitulates the patterns seen in the *1.4kbdlx5a/dlx6a:GFP* fish (Figure 3**A**,**E**,**I**,**M**). In addition, GFP expression within domains of the ventral telencephalon and diencephalon is highly colocalized with that of GABA-positive neurons (**B**,**F**,**J**), consistent with the observations made in the *1.4kbdlx5a/dlx6a:GFP* fish. Dorsal is upward in all the panels. **C**,**G**,**K** are merged images of (**A**,**B**), (**E**,**F**)and (**I**,**J**), respectively. **D**, **H** and **L** are higher magnification pictures of **C**, **G** and **K** showing the presence of both GFP and GABA. Arrows in **A**: lateral forebrain bundles. Abbreviations are as in figure 3.3.


GFP
GABA
Merge (10x)
Merge (20x)
Tg(1.1kbl56i:GFP)
A
B
C
D
tel
3dpf
E
F
G
H
tel
3dpf
I
J
K
L
tec
di
3dpf

In mice, *Dlx* enhancers have been shown to be highly active in various major subtypes of GABAergic interneurons (Ghanem et al., 2007), suggesting that proper function of these CREs might be important to target *Dlx* gene expression to specific populations of differentiating GABAergic neurons. To further examine this possibility in the zebrafish forebrain, we thereby utilized a double-immunohistochemistry analysis in the *1.4kbdlx5a/dlx6a:GFP* transgenic fish in order to evaluate whether *dlx5a/dlx6a* CREs can target transgene expression to groups of cells that express particular GABAergic neuron subtypes. Surprisingly, comparison of GFP expression with commonly used interneuron markers such as calbindin (CB), calretinin (CR) and parvalbumin (PV), revealed that a vast majority of GFP-positive cells within the telencephalon and diencephalon of 3 dpf embryos are not co-labeled by the three interneuron markers that we tested (Figure 3.5). Nonetheless, we did identify some co-localization between GFP and these molecular markers in certain populations of cells. For instance, many GFP-expressing cells appear to coexpress CR in the ventral diencephalon (prethalamus, Figure 3.5L) as well as in the optic tectum (Figure 3.5P). A number of GFP-expressing cells in the ventral telencephalon (Figure 3.5T) and in the rostral hypothalamic region (Figure 3.5X) were found to PV-positive. In the case of GFP and CB coexpression, a few scattered cells in the basal forebrain and in the midbrain tectum were found to be double-positive (Figure 3.5D,H, arrowheads). Similar results were also observed at 5 dpf and later developmental stages (data not shown). These data are, therefore, at least in part contrasting with our earlier studies in mice (Ghanem et al., 2007).

Figure 3.5. Immunolocalization of GFP and various GABAergic interneuron markers in the *1.4kbdlx5a/dlx6a:GFP* transgenic embryos at 3 dpf

The majority of GFP-positive cells (**A**,**E**,**I**,**M**,**Q**,**U**) within the telencephalon and diencephalon are not extensively overlapped with three common interneuron markers, calbindin (**B**,**F**), calretinin (**J**,**N**) and parvalbumin (**R**,**V**). However, coexpression of GFP and CR/PV could be seen in certain populations of cells located in the ventral telecephalon (**T**), the prethalamus (**L**), the rostral hypothalamus (**X**) as well as in the midbrain tectum (**P**). In addition, in the case of GFP and CB double labeling, a few scattered cells in the basal forebrain and in the tectum are double positive (pointed by arrowheads in **D**,**H**). Dorsal is up in all the panels. **C**,**G**,**K**,**O**,**S**,**W** are merged pictures of (**A**,**B**), (**E**,**F**), (**I**,**J**), (**M**,**N**), (**Q**,**R**) and (**U**,**V**), respectively. **D**,**H**,**L**,**P**,**T,X** are magnified photographs of **C**,**G**,**K**,**O**,**S**,**W**. Abbreviations are as in figure 3.3.


Merge (10x)
Merge (20x)
Tg(1.4kbdlx5a/dlx6a:GFP)
A
tel
3dpf
GFP
B
CB
C
D
E
tec
di
3dpf
GFP
F
CB
G
H
I
tel
3dpf
GFP
J
CR
K
L
M
tec
di
3dpf
GFP
N
CR
O
P
Q
tel
3dpf
GFP
R
PV
S
T
U
tec
di
3dpf
GFP
V
PV
W
X

3.3.3. Loss of *dlx1a/dlx2a* or of *dlx5a/dlx6a* function dramatically affects enhancer activity in the zebrafish diencephalon

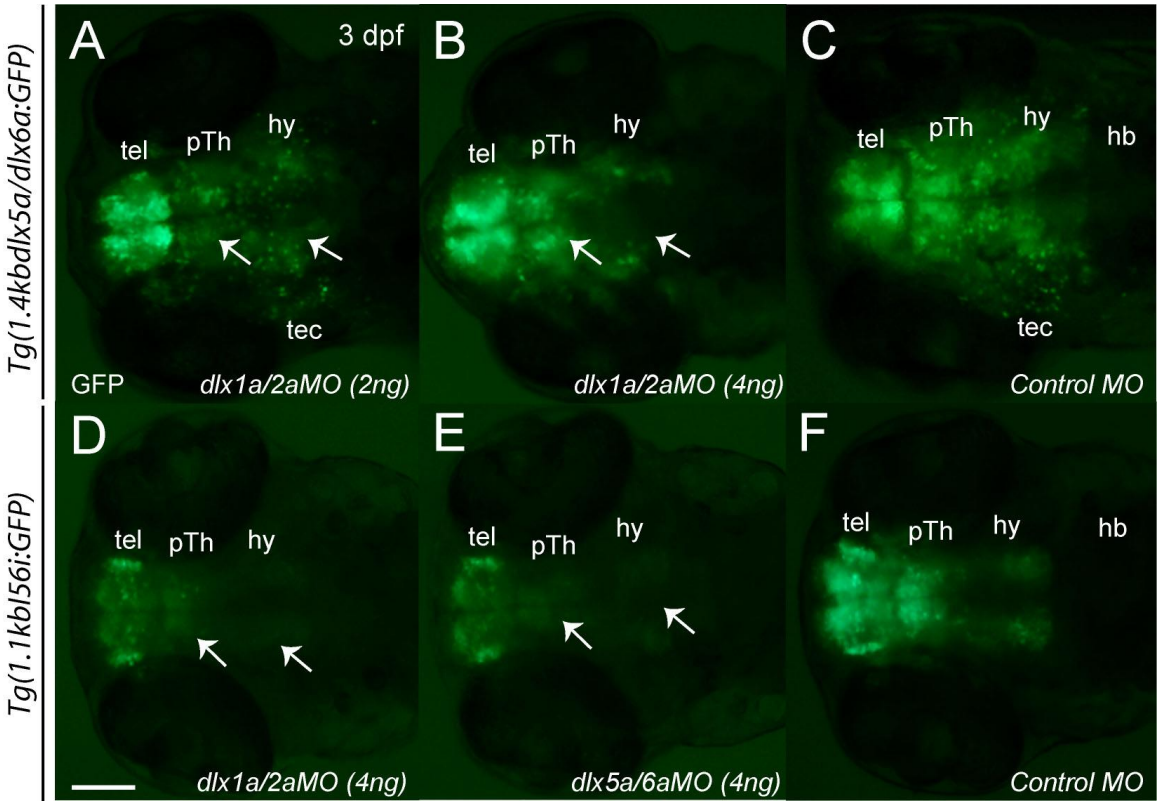
In mice, *Dlx* genes have been revealed to regulate their own expression or expression of their paralogous genes through acting on the CREs within their loci or those from other *Dlx* bigene clusters (Zerucha et al., 2000; Zhou et al., 2004; Poitras et al., 2007; Potter et al., 2009). To explore whether auto- or cross-regulatory loops between distinct *dlx* bigene pairs exist in zebrafish as is the case in mice, different combinations of translation blocking MOs against *dlx* mRNA transcripts were injected into fertilized one-cell stage embryos from the *1.4kbdlx5a/dlx6a:GFP* and *1.1kbI56i:GFP* transgenic fish and the effects on the reporter activity were assessed (Table 3.1). MO-treated embryos were examined at 3 dpf, a stage when all *dlx* genes are highly expressed and various distinct regions of the developing forebrain become identifiable. We found that inhibition of single *dlx* gene (*dlx1a*, *dlx2a*, *dlx5a* or *dlx6a*) did not result in a marked loss or visible alteration of GFP expression (data not shown), indicating a potential functional redundancy among *dlx* genes. In contrast, the *1.4kbdlx5a/dlx6a:GFP* embryos that received the *dlx1a+dlx2a* MOs displayed an extensive depletion of GFP expression in the entire diencephalon and in the midbrain tectum (Figure 3.6A,B, arrows). However, telencephalic GFP expression in a majority of morphants remained largely intact (Figure 3.6A,B). Likewise, compared with the embryos injected with control MO, the simultaneous loss of *dlx1a* and *dlx2a* function in the *1.1kbI56i:GFP* embryos caused a marked decrease in reporter transgene expression in the diencephalon (Figure 3.6D, arrows) and a reduction of GFP expression was also noticed in the telencephalon of some fish (Figure 3.6D).

Table 3.1. A summary of forebrain phenotypes after knockdown of *dlx1a/dlx2a* and *dlx5a/dlx6a* genes in the 3dpf *1.4kbdlx5a/dlx6a:GFP* and *1.1kbI56i:GFP* embryos

Model	MO Treatment	Number of embryos assessed	Reduced telencephalic expression (%)	Reduced diencephalic expression (%)
1.4kb line	*dlx1a/dlx2a*MO (2ng each)	69	11/69 (16.0%)	45/69 (65.2%)
	*dlx1a/dlx2a*MO (4ng each)	60	16/60 (26.7%)	44/60 (73.3%)
	control MO (4ng)	42	2/42 (4.8%)	4/42 (9.5%)
1.1kb line	*dlx1a/dlx2a*MO (4ng each)	57	22/57 (38.6%)	46/57 (80.7%)
	*dlx5a/dlx6a*MO (4ng each)	46	13/46 (28.3%)	25/46 (54.3%)
	control MO (4ng)	38	4/38 (10.5%)	5/38 (13.2%)

Figure 3.6. Double knockdown of *dlx1a/dlx2a* and *dlx5a/dlx6a* markedly reduces GFP reporter expression in the diencephalon and the midbrain tectum in the *1.4kbdlx5a/dlx6a:GFP* and *1.1kbI56i:GFP* morphants at 3 dpf

Simultaneous injection of combined MOs against *dlx1a+dlx2a* (2 ng/nl for each MO in **A** and 4 ng/nl for each MO in **B**) significantly disrupted GFP expression in the diencephalon (prethalamus and hypothalamus) and the midbrain tectum in the *1.4kbdlx5a/dlx6a:GFP* embryos (**A**,**B**), as compared to that seen in 3 dpf embryos injected with the standard control MO (**C**), although telencephalic GFP expression in a majority of morphants remained seemingly unaffected (**A**,**B**). Similarly, double knockdown of *dlx1a+dlx2a* and *dlx5a+dlx6a* (4 ng/nl for each MO) in the *tg(1.1kbI56i:GFP)* line also led to a marked loss of diencephalic and midbrain GFP expression (**D**,**E**), in comparison with the corresponding controls (**F**), similar to the loss observed in the *1.4kbdlx5a/dlx6a:GFP dlx1a+dlx2a* morphants (**A**,**B**). In addition, there was also an noticeable reduction in the number of 1.1kbI56i-targeted GFP-positive cells in the telencephalon of some fish (**D**,**E**). The loss of GFP expression in the prethalamus and hypothalamus in the morphants are indicated by arrows in **A**,**B**,**D**,**E**. Note that the effects of double knockdown of *dlx5a* and *dlx6a* were not examined in the *1.4kbdlx5a/dlx6a:GFP* embryos due to the promoter sequence used in the original transgene construct. In all the panels, embryos are oriented anterior to the left, dorsal views. Scale bar: 100 μm. Abbreviations are as in figure 3.1.


Tg(1.4kbdlx5a/dlx6a:GFP)
Tg(1.1kbl56i:GFP)
A
3 dpf
tel
pTh
hy
tec
GFP
dlx1a/2aMO (2ng)
B
dlx1a/2aMO (4ng)
C
hb
Control MO
D
dlx1a/2aMO (4ng)
E
dlx5a/6aMO (4ng)
F
Control MO

We also knocked down the function of both *dlx5a* and *dlx6a* and examined reporter gene expression in the *1.1kbI56i:GFP* embryos. As expected, an obvious loss of GFP expression was seen in the diencephalon (Figure 3.6E, arrows) as well as in the telecephalon of the *dlx5a+dlx6a* morphants (Figure 3.6E), resembling the changes seen after systemic knockdown of both *dlx1a* and *dlx2a*. Our data support a viewpoint that self- and cross-regulation of different *dlx* genes may be conserved in the developing zebrafish forebrain.

3.4. Discussion

3.4.1. Conserved activities of *dlx* regulatory elements between zebrafish and mouse

Teleost fish are one of the most diverse groups of vertebrates and are composed of more than 23000 different species including zebrafish (Powers, 1991). Teleost fish have been proposed to undergo divergence from mammals about 430 millions years ago (Powers, 1991). Although the basic mode of telencephalic development varies greatly between teleosts (e.g. zebrafish) and mammals (e.g. mouse) (Nieuwenhuys and Meek, 1990), comparative studies of the expression patterns of a group of developmental genes including *Dlx* genes as well as neurotransmitters have revealed many regional homologies between the developing mouse and zebrafish forebrain (Wullimann and Rink, 2002; Mueller et al., 2006; Mueller et al., 2008; Moreno et al., 2009; Wullimann, 2009). *Dlx* forebrain expression patterns of mouse and zebrafish appear to be well conserved, suggesting that the potential mechanisms underlying *Dlx* regulation might be also conserved over evolutionary time (Akimenko et al., 1994; Ellies et al., 1997;

Panganiban and Rubenstein, 2002; MacDonald et al., 2010a). One potential factor contributing to this conservation may be the conservation of various *Dlx* CREs within with their respective *Dlx* loci. Indeed, orthologous *Dlx* CREs exhibit a high degree of sequence similarity among distantly related vertebrate species (Ghanem et al., 2003). The I56i and I12a enhancers also belong to a group of 481 ultraconserved DNA fragments, whose sequences are above 200 bp and are completely identical between the human, mouse and rat genomes (Bejerano et al., 2004). While there exists a debate concerning whether high sequence conservation of noncoding regulatory elements among distinct vertebrates does faithfully reflect their conserved activities (Elgar and Vavouri, 2008; McEwen et al., 2009), our present study showed that the 1.4kb *dlx5a/dlx6a* intergenic region and a 1.1kb zebrafish I56i fragment both specifically drive GFP expression to GABAergic neurons in the ventral telencephalon and diencephalon, mimicking the activities of their mouse orthologs when tested in transgenic mice (Ghanem et al., 2007; Ghanem et al., 2008). Consistent with these results, other *dlx* CREs, such as URE2 and I12b, also showed activities similar to that of their mouse orthologous sequences as reflected by GFP reporter gene expression in the developing zebrafish forebrain and pharyngeal arches (Ghanem et al., 2003; Ghanem et al., 2007; MacDonald et al., 2010a; MacDonald et al., 2010b). Furthermore, heterologous transgenic assays showed that zebrafish I56i/I56ii and URE2 CREs are able to activate reporter gene expression in the mouse subpallial telencephalon in a pattern resembling that of endogenous *Dlx* forebrain expression (Zerucha et al., 2000; Stuhmer et al. 2002a; MacDonald et al., 2010b). The genetic pathway involving *Mash1* and *Dlx* that is required for proper GABAergic neuron development is also likely to be conserved between mouse and zebrafish. The *Mash1*

ortholog, *ascl1a*, is not only co-expressed with *dlx1a*, *dlx5a* and *gad1* in the early zebrafish forebrain, but also acts as a upstream regulator mediating *dlx* expression (MacDonald et al., unpublished data), which is consistent with previous observations made in mice (Yun et al., 2002; Poitras et al., 2007). These data suggest that highly conserved sequences and activities of various *Dlx* CREs may be crucial for maintaining proper *Dlx* functioning and the overall genetic cascade modulating GABAergic neuron development through vertebrate evolution.

3.4.2. Differential activity and contribution of zebrafish I56i and I56ii CREs

Modified gene expressions and gene-gene interactions due to altered regulatory elements and/or changes in binding of *trans*-acting factors to these elements have been long hypothesized as key molecular events that can induce novel morphological structures and genetic networks over evolutionary time (Tvrdik and Capecchi, 2006; Papatsenko and Levine, 2007; Kleinjan et al., 2008). Expression patterns of the *1.4kbdlx5a/dlx6a:GFP* and *1.1kbI56i:GFP* transgenes overlap extensively in most areas of the ventral forebrain. Nevertheless, differences were observed in the mesencephalic tectum most probably as a result of the absence of I56ii activity in the *tg(1.1kbI56i:GFP)* construct. Despite the unknown roles of *dlx* genes in this area, this finding indicates that zebrafish I56i and I56ii CREs may exert differential regulatory roles, at least in certain domains in the developing zebrafish brain.

The apparent absence of specific activity for I56ii, when tested individually in zebrafish, contrasts markedly with what we previously observed in mice. In mice, I56ii is unique in its ability to specifically target expression to a distinct subpopulation of

postmitotic striatal projection neurons, termed “corridor cells”, which express two homeoproteins Islet1 and Meis2 and is essential for the growth and guidance of thalamocortical projections during midgestation (Lopez-Bendito et al., 2006; Ghanem et al., 2007; Ghanem et al., 2008). One potential implication of this inter-species difference would be the absence of zebrafish forebrain cells corresponding to the mouse corridor cells. Moreover, our former results in transgenic mice revealed that zebrafish I56ii is much less efficient at targeting reporter gene expression to the forebrain (0 of 2 transgenic lines; 1 of 10 primary transgenic embryos) compared to the full-length *dlx5a/dlx6a* intergenic region as well as the individual zebrafish I56i enhancer (Zerucha et al., 2000). Nevertheless, I56ii sequence conservation between mammals and teleosts is hard to reconcile with its apparent inability to drive reporter transgene expression, at least spatially. We cannot at present rule out a specific role for I56ii in driving expression in later stage larvae or in adults. An essential cooperative effect between I56i and I56ii in the zebrafish forebrain could also explain the conservation in sequence. In order to function properly and efficiently, the zebrafish I56ii may require the presence of additional regulatory sequences, either from I56i or from the promoter of one or both flanking *dlx* genes. Similarly, I56ii could improve the efficiency of additional *dlx5a/dlx6a* CREs, such as I56i. The I56i and I56ii enhancer differ markedly in sequence. We have demonstrated that, compared to the mouse I56i CRE, the putative Dlx binding sites and the surrounding nucleotides appear to be less conserved in I56ii (Zerucha et al., 2000; Ghanem et al., 2003). Furthermore, the I56ii enhancer is less responsive to the activation of Dlx proteins in *in vitro* transfection assays (Zerucha et al., 2000; Ghanem et al., 2003). Finally, both Dlx1 and Dlx2 proteins can directly bind to I56i CRE *in vivo* but

not to I56ii as revealed by ChIP analysis (Zhou et al., 2004). In light of this evidence, we propose that zebrafish I56ii CRE may be only bound by a particular set of transcription factors, which are probably distinct from those acting through the I56i enhancer. As such, I56ii may be necessary for the proper development of a particular subpopulation of GABAergic neurons, for instance, those located in the midbrain tectum.

It is important to emphasize here that the two transgenic lines used in this study were generated in very different ways: the 1.1kb line carries a β-globin minimal promoter, whereas the 1.4kb line is flanked by a *dlx6a* 5'-flanking region. This difference may potentially impact our results. Therefore, in order to provide more direct comparisons, we are in the process of generating a novel transgenic line that contains the 1.4kb intergenic fragment as well as the same β-globin minimal promoter as the 1.1kb line. The establishment and analysis of this stable transgenic line will help us understand whether the *dlx6a* 5'-flanking region contribute to some extent the differential GFP expression patterns seen in the two transgenic lines shown in the present study.

3.4.3. Functional divergence of *dlx* forebrain CREs between zebrafish and mouse

Spatio-temporal expression of a given gene in specific tissue types is usually an overall effect of multiple transcription factors which can exert their regulatory functions in a correct combination by acting on one or more regulatory elements (Strahle and Rastegar, 2008; Panne, 2008). On the other hand, if such a combination cannot be accomplished under certain cellular microenvironments or if any of these transcription factors are prevented from binding to their targeting CREs due to certain reasons, the

CREs will stay in an inactive status (Smale and Kadonga, 2003; Arnosti and Kulkarni, 2005). Although the sample size of our knockdown experiments was relatively small and no rescue assays were conducted, we were able to show simultaneous inhibition of *dlx1a/dlx2a* or *dlx5a/dlx6a* function in our transgenic lines resulted in a significant loss of transgene expression in the diencephalon and the midbrain tectum as well as a lesser extent of reduction in the telencephalon, suggesting that functional redundancy also exists between zebrafish *dlx* genes. The decreased transgene activity in the morphants is most likely due to the loss of dlx protein binding to I56i and/or I56ii enhancers, suggesting that zebrafish *dlx* genes from distinct bigene clusters are also under auto- and cross-regulation via their corresponding CREs, consistent with previous observations made for their mouse orthologs (Zerucha et al., 2000; Zhou et al., 2004; Poitras et al., 2007; Potter et al., 2009). For example, previous studies have shown that the Dlx1 and Dlx2 proteins may bind to the I56i enhancer and regulate *Dlx5* and *Dlx6* expression in the developing mouse forebrain (Anderson et al. 1997b; Zerucha et al., 2000; Stuhmer et al., 2002b). Recently, both *in vitro* and *in vivo* evidence has also been provided for the auto-regulation of the *Dlx* genes as both URE2 and I12b CREs have been found to be direct targets of Dlx1 and Dlx2 proteins (Poitras et al., 2007; Potter et al., 2009). Moreover, *Dlx1*/*Dlx2* expression plays essential roles in regulating the activities of these CREs (Poitras et al., 2007; Potter et al., 2009). Interestingly, telencephalic expression of the *1.4kbdlx5a/dlx6a:GFP* and *1.1kbI56i:GFP* transgenes was less affected by the absence of Dlx proteins when compared to the diencephalic expression, which contrasts sharply with the situations seen in mice (Anderson et al., 1997a; Anderson et al., 1997b; Wang et al., 2010). These findings suggest the existence of parallel and possibly redundant genetic pathways

responsible for *dlx* expression in different contexts in the developing zebrafish forebrain via the interactions with the I56i and/or I56ii enhancers.

Although the overall activities of orthologous *Dlx* CREs appear well conserved in distinct vertebrate species, they may also serve slightly different functions. The mouse I56i sequence targets *lacZ* reporter gene expression to the pharyngeal arches of transgenic mice (Zerucha et al., 2000; Park et al., 2004), whereas its zebrafish counterpart is not able to do the same as shown in the current study. Likewise, orthologous URE2 sequences from zebrafish, mouse and elephant shark were shown to have differential roles in regulating the mandibular arch development (MacDonald et al., 2010b). Functional conservation and divergence of the *cis*-regulatory networks of the orthologous *Hoxa2* gene and the *HoxD* cluster have also been observed in many distantly related vertebrates (Tumpel et al., 2002; Spitz et al., 2003). It has been proposed that the regulatory elements in the same gene locus in different species may have gradually acquired certain differential mechanisms over evolution to modify their protein-binding activity (Tautz, 2000; Ludwig, 2002; Gibert and Simpson, 2003). The modifications that are specific to certain vertebrate lineages, such as the mammal-specific loss or teleost-specific gain of some upstream signaling, may also contribute to differential activities of orthologous regulatory elements, at least in certain tissue types (Wittkopp, 2005). These modifications perhaps have emerged following the teleost-specific genome duplication event after they undergo divergence from mammals, which may produce many paralogous key regulatory genes that could subsequently act as novel modulators interacting with enhancer elements (Wittkopp, 2005).

3.4.4. Functional implication of *dlx* genes in zebrafish GABAergic neuron differentiation

In the developing mouse forebrain, *Dlx* genes amongst other key regulatory genes (e.g. *Mash1, Necdin, Arx* and *Smad*) play essential roles in regulating the production and differentiation of GABAergic neurons (Cobos et al., 2005b; Kuwajima et al., 2006; Maira et al., 2010; Long et al., 2009a). Various *Dlx* CREs have been shown to mark GABAergic neuron progenitors in the ganglionic eminences in the subpallial telecephalon as well as diverse subtypes of GABAergic interneurons in the adult cortex (Ghanem et al., 2007). While the distribution of GABAergic neurons has been well described in the developing and adult zebrafish brain (Doldan et al., 1999; Kim et al., 2004; Mueller et al., 2006), very little is known about their differentiation process as well as the underlying mechanisms. Due to the lack of antibodies specifically recognizing zebrafish dlx proteins, we examined GFP expression driven by individual *dlx* CREs in immunohistochemical analyses of developing GABAergic neurons. Based on GABAergic interneuron markers that were mainly used in mice, we found that the *dlx5a/dlx6a* regulatory elements are inactive in a large population of differentiated GABAergic neurons in the early developing forebrain. However, colocalization of GFP and interneuron marker expression was observed in some subpopulations of cells in the ventral telencephalon and diencephalon as well as in the optic tectum. These findings are in contrast with previous observations in mice and lead us to propose that the differentiation of zebrafish GABAergic neurons might be also regulated by other *dlx* forebrain CREs (e.g. URE2 and I12b) or the genetic pathways that are not identical with

those in mice, likely due to different microenvironment between the developing zebrafish and mouse forebrain. Indeed, *fgf19* and *her6* genes were reported to participate in regulating differentiation and survival of zebrafish GABAergic neurons in the developing telencephalon and diencephalons (Miyake et al., 2005; Scholpp et al., 2009). The hairy-related gene *her6* has been found to serve as an important regulatory gene responsible for maintaining the neurogenic gradient in the caudal thalamus and it also mediates the cell fate switch between glutamatergic and GABAergic neurons (Scholpp et al., 2009). In addition, sequence divergence of orthologous I56i/I56ii elements in mice and zebrafish (Ghanem et al., 2003) may also possibly contribute to their ability to target distinct populations of GABAergic neurons in these two species. It is also worth noting a challenge for studying GABAergic neuron differentiation in zebrafish, because of the differential cross-reactivity of commercially available antibodies used to detect the different GABAergic neuron subtypes. In our case, even though the efficiency of the antibodies we used as interneuron markers have been tested in other species including mouse and human, they may be less effective or ineffective in labeling all isoforms of the studied protein when tested in zebrafish, especially considering the fact that the zebrafish genome contains a large number of duplicated genes. Liu et al. (2010) showed that there are four and six SOM genes functionally expressed in the zebrafish telecephalon and hypothalamus, respectively. Using an analysis of existing genomic information, Friedberg (2005) identified a total of nine isoforms that encode PV in zebrafish. By carrying out similar studies in transgenic lines made with other *dlx* CREs (MacDonald et al., 2010b; MacDonald et al., 2010c), by using additional GABAergic interneuron markers (e.g. SOM and NPY), and by extending our observations to adult fish, we should

better understand the roles of *dlx* genes and their CREs in zebrafish GABAergic interneuron differentiation.

In summary, our data demonstrate that zebrafish *dlx5a/dlx6a* regulatory elements exhibit partially conserved activity when compared to their mouse orthologs and these CREs may be involved in the genetic pathways responsible for the production of some populations of GABAergic neurons. I56i and I56ii CREs are likely to exert differential function in modulating this process in distinct populations of cells. Similar to the mouse *Dlx* genes, auto- or cross-regulatory mechanisms may also exist between different zebrafish *dlx* genes, even though *dlx* genes may be regulated in a different way between the developing telencephalon and diencephalon. Besides the *dlx5a/dlx6a* regulatory elements, other *dlx* CREs or regulatory factors may also contribute to the differentiation of GABAergic interneuron subtypes in the developing zebrafish forebrain. Further examination, throughout development and in adult animals, of the activities of various individual *dlx* CREs will provide us with additional clues with respect to the evolution of regulatory mechanisms responsible for *dlx* forebrain expression and further improve our understanding about the implications of *dlx* genes during vertebrate forebrain development.

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4. Targeted deletion of a *Dlx* intergenic enhancer I12b reduces *Dlx1/Dlx2* expression and inhibits cell proliferation in the developing mouse forebrain

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Abstract

Dlx homeobox genes encode a family of transcription factors that are necessary for the proper differentiation, migration and survival of GABAergic interneurons. So far, at least four well conserved *cis*-regulatory elements (CREs) have been identified to be important for regulating *Dlx* transcriptional control in the developing telencephalon and diencephalon: I12b and URE2 in the *Dlx1*/*Dlx2* bigene cluster, and, I56i and I56ii in the *Dlx5*/*Dlx6* bigene cluster. However, how *Dlx* regulation is linked to these various individual CREs and how these CREs maintain correct *Dlx* forebrain expression are still not fully characterized. Towards exploring the specific contribution of I12b CRE to overall *Dlx* function as well as directly addressing its functional role *in vivo*, we have used homologous recombination in mouse embryonic stem (ES) cells to generate mutant mice in which I12b is deleted from its endogenous genomic locus. Here, we report that mice completely lacking I12b CRE are viable and fertile and that I12b enhancer loss fails to result in notable abnormalities when assayed for a variety of phenotypes including brain size and forebrain morphology. Upon visual observation, tangential migration of GABAergic neurons from the subpallial telencephalon to the neocortex in $I12b^{-/-}$ mutant embryos generally appear to be comparable to that in the wild-type littermates; instead, our initial observations suggested a possibly reduced cell proliferation in the basal telencephalon, particularly in the ventricular and subventricular zones (VZ and SVZ) of the lateral/medial ganglionic eminences (LGE/MGE). Although expression patterns of *Dlx1*, *Dlx2* and *Dlx5* revealed by *in situ* hybridization assay at E13.5 displayed no significant difference between I12b homozygous mutants and their wild-type littermates, our real-time RT-PCR quantification assays at this time point identified a moderate

reduction (~15-25%) of both *Dlx1* and *Dlx2* transcripts (*Dlx5/Dlx6* mRNA levels remain intact) in the forebrain of I12b$^{-/-}$ mutants. Interestingly, this alteration coincides with a ~10% increase in the mRNA levels of *Mash1*, a direct upstream regulator of *Dlx1* and *Dlx2*. Taken together, our findings, while not inclusive of all possible phenotypic impacts of I12b deletion, indicate that I12b enhancer may play an important role in controlling *Dlx* expression as well as in GABAergic neurogenesis in the developing forebrain. These data will lay the basis for future *in vivo* studies on the genetic networks involving Dlx genes and their various CREs during vertebrate forebrain development.

4.1. Introduction

The forebrain is composed of a large diversity of neuronal cells with distinct functions and morphologies and is thought to be one of the most complicated anatomical structures in mammals. GABAergic interneurons play key roles in modulating and maintaining the balanced activity of many forebrain areas, such as the cortex and hippocampus, by directly exerting their inhibitory impacts upon excitatory glutamate-releasing projection neurons (Owens and Kriegstein, 2002; Whittington and Traub, 2003). Improper balance between inhibitory and excitatory signaling has been proposed to be relevant to many common human neurological disorders, such as autism and epilepsy (Di Cristo, 2007). GABAergic interneurons are originally born in various progenitor domains in the developing subpallium, specifically in the ganglionic eminences, anterior entopeduncular area (AEP) and preoptic area (POA), and tangentially migrate and then populate different layers in the developing cortex (Marin et al., 2000; Xu et al., 2004; Wonders and Anderson, 2006). A number of transcription factors (e.g.

Mash1, *Nkx2.1* and *Gsh1/Gsh2*) and signaling molecules, including retinoid acid (RA), Sonic hedgehog (Shh) and fibroblast growth factors (FGFs), are responsible for establishing regionalization of the developing subpallium (Medina et al., 2009; Moreno et al., 2009; Nobrega-Pereira and Marin, 2009). Some of these molecules are also involved in the genetic pathways important for GABAergic neuron generation. For instance, *Nkx2.1*$^{-/-}$ mutants have a significantly decreased number (~50%) of cortical interneurons when compared to wild-type mice (Sussel et al., 1999). The sequences of *Dlx* homeobox genes are related to that of the *distal-less* gene in *Drosophila* (Cohen et al., 1989). Four *Dlx* genes (*Dlx1*, *Dlx2*, *Dlx5* and then *Dlx6*) are sequentially expressed in the developing mouse forebrain, where they mainly promote GABAergic interneuron progenitors to proliferate and differentiate into different subtypes (Panganiban and Rubenstein, 2002). While single *Dlx* gene knockouts exhibit relatively milder abnormal phenotypes when compared to that of double *Dlx* null mutants (e.g. *Dlx1/2*$^{-/-}$ and *Dlx5/6*$^{-/-}$) (Qiu et al., 1997; Acampora et al., 1999; Cobos et al., 2005a), inactivation of both *Dlx1* and *Dlx2* genes causes severe deficiencies in the generation, differentiation and migration of GABAergic neurons starting at early embryonic stages (Anderson et al., 1997a; Anderson et al., 1997b; Marin et al., 2000; Pleasure et al., 2000; Long et al., 2007). Simultaneous inactivation of *Dlx5* and *Dlx6* function leads to exencephaly of the anterior portion of the embryonic forebrain and the mutants have a preferentially decreased number of PV-expressing cortical interneurons with increased dendrite branching (Wang et al., 2010).

Vertebrate *Dlx* genes are generally organized as convergently transcribed bigene clusters on different chromosomes and the two *Dlx* genes consisting of a bigene pair are separated by a short intergenic sequence (~3.5 to 16 kb) (Zerucha and Ekker, 2000;

Sumiyama et al., 2003). The two *Dlx* genes from the same bigene cluster are expressed with highly overlapping patterns in many tissue types possibly because they are under the same regulatory mechanisms (Liu et al., 1997; Eisenstat et al., 1999). Through phylogenetic footprinting in five distantly related vertebrate species and through transgenic animal analyses, our laboratory has previously identified and characterized four highly conserved *cis*-regulatory elements (CREs) that act as enhancers responsible for regulating *Dlx* forebrain expression: I56i and I56ii within the *Dlx5/Dlx6* loci (Zerucha et al., 2000), and, URE2 and I12b within the *Dlx1/Dlx2* loci (Ghanem et al., 2003; Ghanem et al., 2007). Although their sequences vary greatly, these CREs all target reporter transgene expression to the ventral telencephalon and diencephalon in a pattern that faithfully mimics that of endogenous *Dlx* forebrain expression (Zerucha et al., 2000; Ghanem et al., 2003; Ghanem et al., 2007). Further analyses of these CREs have provided evidence that URE2, I12b and I56i are active in GABA-expressing progenitor cells in the ganglionic eminences in the subpallial telecephalon as well as in diverse subtypes of GABAergic interneurons in the adult cortex (Ghanem et al., 2007). I56ii is specifically active in a subpopulation of GABAergic striatal projection neurons between E11.5 and E13.5 during early embryogenesis (Ghanem et al., 2008). Moreover, more recent studies demonstrated that in addition to being as a direct target of Dlx proteins, the ~400 bp I12b enhancer is also directly targeted by Mash1, a proneural bHLH transcription factor (Poitras et al., 2007; Potter et al., 2009). Mash1 may regulate *Dlx1/Dlx2* expression by acting through I12b enhancer (Poitras et al., 2007; Potter et al., 2009). Together, these data indicate that the genetic pathway involving *Mash1* and *Dlx*

genes may control GABAergic neuron development through their dynamic interactions with various *Dlx* CREs.

To date, our understanding of how *Dlx* forebrain expression is precisely regulated and maintained through their various individual CREs is still very limited. In order to investigate the relative contribution of I12b CRE to overall *Dlx* expression and to evaluate its full functional potential *in vivo*, we applied an embryonic stem (ES) cell-based approach to remove this enhancer from its native chromosomal context and generated a mutant mouse model carrying a targeted I12b deletion. Here, we first determine whether I12b enhancer deletion affects the survival of the animals as well as the size and morphology of the forebrain, with a particular focus on the regions of the olfactory bulb, neocortex and hippocampus, where I12b is highly active. Subsequently, to define the potential implication of I12b CRE in GABAergic neurogenesis, we examined cell proliferation and tangential migration streams of GABAergic neurons in $I12b^{-/-}$ homozygous mutants using a set of immunohistochemical analyses. In addition, the effects of I12b enhancer deletion on the transcript levels of *Dlx* genes and other regulatory genes (e.g. *Mash1, Nkx2.1* and *Gsh2*) essential for GABAergic neuron production and differentiation were also evaluated by quantitative real-time RT-PCR assays and/or *in situ* hybridization at various developmental stages.

4.2. Materials and Methods

4.2.1. Animal maintenance

Mouse colonies maintained on a C57BL/6 background were housed with free access to standard chow and water in a pathogen-free environment under a 12h light/dark

cycle, according to the guidelines of the Canadian Council on Animal Care (CCAC). For staging of embryos, the day on which a vaginal plug was detected was calculated as embryonic day 0.5 (E0.5). All experimental procedures were approved by the University of Ottawa Animal Care Ethics Committee. Wild-type littermates were used as controls for all comparative studies.

4.2.2. Construction of I12b CRE targeting vector

In brief, a Bacterial Artificial Chromosome (BAC) clone (#510G1) containing a ~200 kb *Dlx1/Dlx2* locus was obtained by screening a BAC library (BACPAC Resources Center) made from the liver tissue of strain 129/Sv mice. To generate the I12b targeting vector (ΔI12b-510G1), a modified LoxP-flanked neomycin (neo) selection cassette driven by a murine PGK promoter was inserted into the place of the wild-type I12b enhancer present on the isolated BAC clone, which was accomplished by homologous recombination in *E. coli* EL250 at 32°C as described previously (Lee et al., 2001). The recombined BAC clones were then verified for sequence accuracy at the site of recombination prior to electroporation into ES cells. The organization of the BAC targeting plasmid is shown in Figure 4.1A and details of the vector construction are available upon request.

4.2.3. Production of I12b null mice

The circular targeting construct was electroporated into mouse R1 ES cells (129/Sv strain background, Transgenic Mouse Core Facility, McGill Cancer Center), which were then allowed to grow on mitomycin-treated neo-resistant STO fibroblast feeder cells.

Two days following electroporation, ES cells were selected for neo-resistance in 300 µg/ml of G418 (Gibco) for one week. Recombinant ES clones were selected, expanded, trypsinized and divided into two separate 96-well plates for further culture: one was used for genomic DNA extraction and genotyping, while the other one was frozen for later use. Genotyping of correctly targeted clones that have undergone homologous recombination was completed using a quantitative real-time PCR assay as previously described by Valenzuela et al. (2003). The following two primer-probe pairs were used: I12b CRE forward: 5'-GGC CCA TCA AAC ACA ACA-3', I12b CRE reverse: 5'-GGC TTT CAG TGC GTT ATC TCT T-3', probe: FAM-GTT TCC CCA TCT CTC T-TAMRA; I56i CRE (internal control) forward: 5'-TAC AGC GTT TTT TAC CGT CAA AG-3', I56i CRE reverse: 5'-AGG TCC TAC GTC TCT GCA AT-3', probe: Yakima Yellow-GAA CAG GAA AGG CGA AA-TAMRA. The real-time PCR conditions are an initial "Hot Start" activation step at 95°C for 15 min, followed by 30 cycles of 15 s at 95°C, 30 s at 56°C and 30 s at 72°C. Two independent positive ES clones (#43 and #86) containing a neo-resistant mutant allele in place of I12b CRE (Figure 4.1A) were microinjected into blastocysts isolated from C57BL/6 mice, which were subsequently reimplanted into the uteri of pseudo-pregnant CD1 mice. The eight resultant chimeras were then bred with C57BL/6 mice and successful germline transmission of the I12b disrupted allele was assessed by the presence of the agouti coat color and PCR verification. Agouti F1 offspring heterozygous for I12b CRE deletion were intercrossed to generate $I12b^{-/-}$ homozygous mutant progenies. Genotyping of I12b mutant mice were performed using a standard PCR assay described in details below.

4.2.4. Genotyping of embryos and mice

I12b mutant embryos and newborn progeny were screened by regular PCR assays using genomic DNA isolated from tail biopsies. Briefly, tissue samples were lysed in appropriate volumes of digestion solution containing 100 mM NaCl pH 7.4, 10 mM Tris-HCl, 5 mM EDTA, 0.5% SDS and 0.2 mg/ml proteinase K (Invitrogen) O/N at 55°C, followed by phenol/chloroform extraction and an ethanol precipitation at -20°C according to standard protocols. The final DNA pellets were dissolved in sterile Milli-Q water, quantified on a Nanodrop spectrophotometer and kept at 4°C. Genotyping analyses were performed by PCR using two specific primer pairs: I12b-forward: 5'-CCA AGA GGG TCA GCA TCA TT-3', I12b-reverse: 5'-CAG AGA GAT GGG GAA ACT GC-3'; neo-forward: 5'-AGA CAA TCG GCT GCT CTG AT-3', neo-reverse: 5'-CTC GTC CTG CAG TTC ATT CA-3'. The PCR reaction yielded a 122 bp band for the wild-type allele and a 110 bp product when the floxed PGK-neo cassette was present in place of the I12b CRE. The PCR reaction was carried out in a total volume of 50 µl mixture consisting of 100 ng DNA template, 200 µM of each dNTP, 20 pmol of each primer, 5 µl of 10×PCR buffer and 1 U Taq polymerase using the following cycling conditions: an initial denaturation step at 95°C for 10 min, followed by 30 cycles of 30 s at 95°C, 30 s at 58°C and 1 min at 72°C, and a final extension step at 72°C for 10 min. Amplified PCR fragments were checked by 0.8% agarose gel electrophoresis and a negative control was included in each experiment to ensure the specificity of PCR amplification.

4.2.5. Tissue preparation and sectioning

E13.5 and E15.5 embryos were anesthetized by cooling, dissected, and fixed O/N in cold 4% paraformaldehyde (PFA)/1×PBS at 4°C. Neonatal pups were anesthetized with an exposure to CO_2 in a chamber and sacrificed by decapitation. P0 brains were dissected and immersed in cold 4% PFA/1×PBS O/N at 4°C. Fixed brain tissues were then cryoprotected by sequentially immersing in 12%, 16% and 22% sucrose in 1×PBS (E13.5 and E15.5) or by overnight immersion in 30% sucrose/1×PBS (P0) at 4°C, followed by embedding in Tissue Freezing Medium (Triangle Biomedical Sciences) and snap-frozen at −80°C. Frozen blocks were transversely sectioned at the thickness of 20 µm (E13.5 and E15.5) or 40 µm (P0) onto Superfrost Plus slides (Fisher) using a Leica CM1850 standard cryostat (Leica Microsystems) at -20°C and stored at -80°C until further use.

4.2.6. Nissl staining

To compare the general morphology between P0 I12b$^{-/-}$ mutant and wild-type forebrain, cresyl violet staining (Nissl staining) was performed as per a standard protocol. Briefly, sections were air-dried at RT for 30 min, dehydrated in serial ethanol dilutions with descending concentration (70–0%) for 1 min each, and immersed in a staining solution (5g/l cresyl violet acetate in 0.3% acetic acid, Sigma) for 3 min. After rinsing with water for 2 min, stained sections were subsequently dehydrated in ethanol dilutions with increasing concentration (30–100%) for 1 min each, cleared in xylene and mounted. Images were taken on a Nikon SMZ 1500 dissecting microscope by using a Nikon DXM 1200C digital camera.

4.2.7. Immunohistochemistry

Immunohistochemistry on cryostat sections was carried out based on standard methods described in Ghanem et al. (2007). In brief, frozen sections were dried for 1h at RT and washed 3×10 min in 1×PBS to eliminate residues from tissue cryoprotectant. Sections were then incubated with a blocking solution containing 1% bovine serum albumin (BSA), 10% newborn calf serum and 0.1% Tween 20 for 2h at RT. The solution was subsequently replaced with a fresh aliquot containing the primary antibody and incubation was processed in a humid chamber O/N at 4°C. Following 3×15 min washes in 1×PBS, sections were reacted with the appropriate secondary fluorescence-coupled antibody for 2h at RT in the dark. For fluorescent imaging, histological sections were cover-slipped with a Vectashield mounting medium (Vector Labs), analyzed with a Zeiss Axiophot fluorescence microscope using the appropriate filter sets and photographed with a Zeiss Axiocam digital camera. The following primary antibodies were used in this study: rabbit polyclonal anti-GABA (1:1000, Sigma), rabbit polyclonal anti-calbindin (anti-CB, 1:1000, Swant), rabbit polyclonal anti-phospho-histone H3 (anti-PH3, 1:100, Upstate), rabbit polyclonal anti-active caspase 3 (1:500, BD Pharmingen). Alexa Fluor 488® goat anti-rabbit IgG (H+L) (1:300, Invitrogen) was applied as the second antibody in all the experiments.

4.2.8. *In situ* RNA hybridization on sections

Antisense RNA probes for *Dlx1*, *Dlx2* and *Dlx5* were prepared as previously described (Cobos et al., 2005a; Long et al., 2007). Briefly, the probes were synthesized by *in vitro* transcription with T7 or Sp6 RNA polymerase (Promega) and labeled with

digoxigenin (DIG)-UTP using DIG RNA Labeling Mix (Roche). *In situ* hybridization experiments on 20 µm frozen sections were performed according to the following procedure (Smith et al., 2008). After defrosting at RT for 1h, the slides were transferred to a sealed humid chamber and incubated for 1h at 65°C with 500 µl of a pre-hybridization solution consisting of 50% formamide, 10% dextran sulfate, 1 mg/ml yeast tRNA (Invitrogen), 1×Denhardts solution (Sigma), and 1×salt solution (0.2 M NaCl, 10 mM Tris-HCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1 mM Tris-base, 5 mM EDTA pH 7.5). DIG-labeled RNA probes were denatured at 70°C for 10 min, cooled in ice, and added into the prewarmed (65°C) prehybridization buffer at a final concentration of 200–400 ng/ml. The prehybridization solution was then replaced with ~300 µl of the probe mix, covered with a coverslip and incubated O/N at 65°C. In the following day, after removal of the coverslips, slides were washed 3×30 min in solution A (1×SSC, 50% formamide, and 0.1% Tween 20) at 65°C, followed by 2×30 min washes in 1×TBST at RT. Slides were then blocked in 10% heat-inactivated calf serum in 1×TBST (blocking solution) for 2h at RT, followed by incubation with a fresh aliquot of block solution containing the anti-DIG-AP Fab fragment antibody (1:5000 dilution) O/N at 4°C. After washing 5×20 min in 1×TBST at RT, slides were incubated for 2×10 min in NTMT staining buffer (100 mM NaCl, 100 mM Tris HCL pH 9.5, 50 mM $MgCl_2$, and 0.1% Tween 20). For staining, slides were transferred into large Petri dishes protected from light. The anti-DIG-AP on each slide was visualized by incubating with 400 µl of NTMT containing 1.4 µl of BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and 2.7 µl of NBT (nitrobluetetrazolium chloride). Slides were checked periodically for signal detection and the reaction was stopped by 2×10 min washes in distilled water, followed by fixation in 4% PFA O/N at

4°C. Slides were finally washed by distilled water and allowed to dry before cover-slipping with Aqua-Mount^{TM} aqueous mounting medium (Fisher). Staining was examined under a Nikon SMZ 1500 dissecting microscope and images were taken using a Nikon DXM 1200C digital camera.

4.2.9. Quantitative real-time RT-PCR assay

To detect changes in gene expression levels, we dissected the telencephalon from E13.5 I12b homozygous (n = 10) and heterozygous mutants (n = 9) as well as eight wild-type littermates. Total RNA was then extracted and purified using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration was quantified at A260/280 nm on a Nanodrop spectrophotometer (Thermo Scientific) and 3 µg of total RNA were reverse transcribed for the synthesis of single-strand cDNA by using Oligo$(dT)_{15}$ primer and SuperScript II Reverse Transcriptase (Invitrogen). All solutions were prepared with diethylpyrocarbonate (DEPC)-treated water. RNase-free instruments and RNaseZap (Ambion) were applied during all the procedures. Quantitative real-time RT-PCR was conducted on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) in a 50 µl reaction mixture containing 4 µl of cDNA template, 25 µl QuantiTect SYBR Green PCR Master Mix (Qiagen) and 20 pmol of each primer. The following primer sets were used: *Dlx1*-forward: 5′-CAG TTG CAG GCT TTG AAC C-3′, *Dlx1*-reverse: 5′-ACT TGG AGC GTT TGT TCT GG-3′; *Dlx2*-forward: 5'-GCC TCA CCC AAA CTC AGG-3', *Dlx2*-reverse: 5'-GCC GCT TTT CCA CAT CTT C-3'; *Dlx5*-forward: 5'-CGA CTT CCA AGC TCC GTT C-3', *Dlx5*-reverse: 5'-TTC TTT CTC TGG CTG GCT G-3'; *Dlx6*-forward: 5'-CGG ACC ATT TAT TCC AGC C-3', *Dlx6*-

reverse: 5’-CGC TTA TTC TGA AAC CAT ATC-3’; *Gsh2*-forward: 5’-CTT CCA ATA TGT ACC TGT CCC-3’, *Gsh2*-reverse: 5’-GCT TGT GTG ATT GTT CCT CG-3’; *Mash1*-forward: 5’-TCT CCT GGG AAT GGA CTT TG-3’, *Mash1*-reverse: 5’-AGG TTG GCT GTC TGG TTT G-3’; *Nkx2.1*-forward: 5’-AGC ACA CGA CTC CGT TCT CA-3’; *Nkx2.1*-reverse: 5’-CCC TCC ATG CCC ACT TTC TT-3’. To quantify the transcript levels of *Dlx* genes and other regulators, we detected and used the mRNA levels of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as an internal control. The primers for *GAPDH* amplification were: forward, 5′-TGC ACC ACC AAC TGC TTA GC-3′; reverse, 5′-GGC ATG GAC TGT GGT CAT GAG-3′. The transcripts of the target genes and *GAPDH* were amplified in the same tubes using the following protocol: initial “Hot Start” activation step for 10 min at 95°C and 40 cycles of 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C. For each PCR reaction, standard curves for the target genes and *GAPDH* were established to determine quantitative mRNA levels by using five serial dilutions of cDNA as templates. The transcript levels of the target genes were normalized against that of *GAPDH* to give the relative values of their expression levels. Real-time RT-PCR assays were repeated at least twice in triplicate for each sample and a non-template control was included in each experiment. All data quantification and statistical analysis was performed with Microsoft Excel 2010. Data were calculated in relative units and presented as the mean ± SEM. The differences in gene expression levels between I12b homozygous mutant, heterozygous mutants and wild-type littermates were evaluated by using the Student *t*-test. *P* values < 0.05 were considered as statistically significant.

4.3. Results

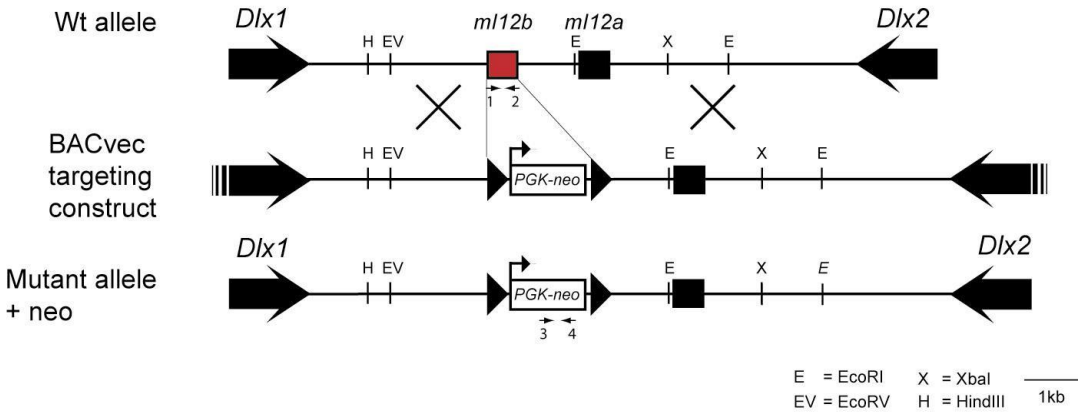
4.3.1. Production of mutant mice carrying a targeted deletion of I12b CRE

In an effort to precisely define the role of I12b CRE *in vivo*, an ES cell-based gene targeting technique was used to establish a strain of mice in which the individual I12b enhancer was deleted from its genomic locus. Specifically, we first built a mutant allele where a ~400 bp region of DNA containing the entire I12b sequence was replaced with a LoxP-flanked PGK-neo-resistance cassette conferring resistance to the selection drug G418. The general strategy for removal of the I12b enhancer from the *Dlx1/Dlx2* locus by homologous recombination in ES cells is depicted in Figure 4.1A. The neo-resistant ES cells harboring an appropriate recombination event were identified among a total of 96 clones by a quantitative real-time PCR screening. Two independent positive ES clones were injected into host blastocysts and the resulting eight highly chimeric mice were subsequently bred to generate heterozygous progenies ($I12b^{+/-}$) that carry the proper I12b deletion in the germline. Intercrosses between heterozygotes gave birth to homozygous mutants ($I12b^{-/-}$) at the predicted Mendelian segregation ratios (more than 200 embryos genotyped), demonstrating that disruption of I12b CRE does not cause embryonic lethality either in utero or shortly after birth. Regular PCR assessment of different genotypic categories is shown in Figure 4.1B. When housed under standard conditions, I12b null mice are viable and proved to be fertile, in contrast to *Dlx1* and *Dlx2* single mutants and *Dlx1/Dlx2* double knockouts, which all die perinatally from a spectrum of severe central nervous system and/or craniofacial defects (Anderson et al., 1997a; Anderson et al., 1997b; Qiu et al., 1995; Qiu et al., 1997; Acampora et al., 1999; Cobos et

Figure 4.1. Experimental strategy for targeted deletion of I12b CRE in ES cells and genotyping analysis of I12b mutant offsprings

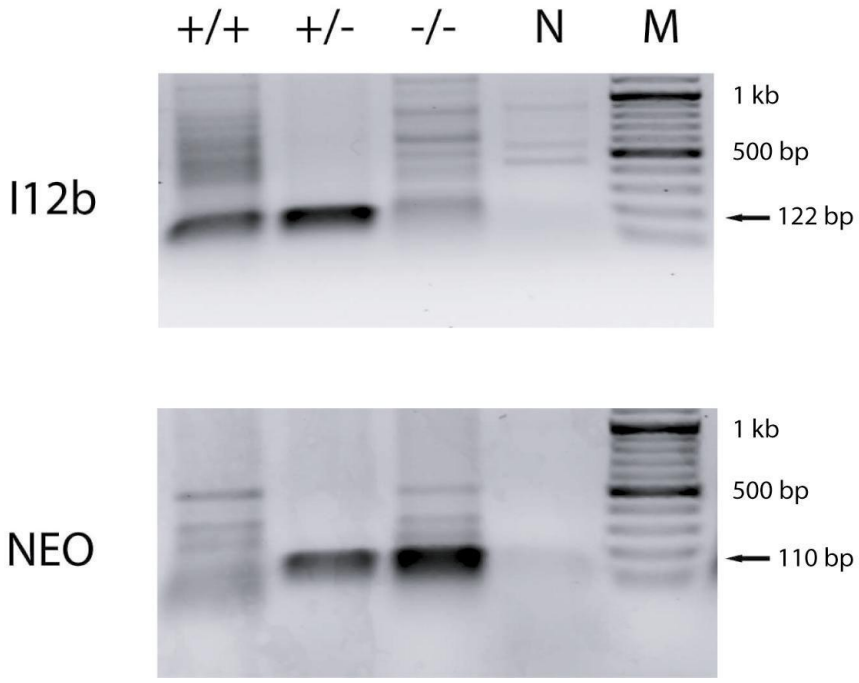
(**A**) A schematic diagram of targeting strategy for I12b CRE deletion within the *Dlx1/Dlx2* locus. The I12b BAC targeting vector containing a neomycin selection cassette (PGK-neo) flanked by loxP sites (black triangles) undergoes homologous recombination with one allele of the endogenous I12b CRE (red box) and results in the replacement of the ~400 bp I12b sequence with the neo-resistant cassette and the formation of an expected I12b deleted mutant allele. Cross lines represent the homologous recombination regions. The arrows indicate the location and transcriptional orientation of *Dlx1* and *Dlx2* genes. Restriction endonuclease recognition sites are abbreviated as follows: E, EcoRI; EV, EcoRV; H, HindIII; X, XbaI. TK: thymidine kinase promoter; Black box: I12a enhancer. (**B**) A representative PCR assay with mouse tail genomic DNA depicting different genotypes of three progenies from I12b heterozygous intercrosses. Two primer pairs (indicated as 1,2 and 3,4 in **A**) specific for the detection of the wild-type and neo+ mutant allele were used for PCR screening, which can distinguish between all three genotypes ($I12b^{+/+}$, $I12b^{+/-}$, and $I12b^{-/-}$) based on the presence and size of the PCR products resolved on a 1% agarose gel. The primer set 1 and 2 amplifies a 122 bp fragment for the wild-type allele, while amplification with primers 3 and 4 yields a band of 110 bp corresponding to the neo gene in the mutated I12b enhancer locus. The genotype is listed on the top of each lane. N: negative control without template DNA; M: molecular weight standards, along with their sizes at right.

(A)



Strategy for the deletion of I12b enhancer in ES cells

(B)



al., 2005a). In addition, mice homozygous for I12b loss develop normally and have body weight and size (n > 20) that are comparable to that of heterozygotes and wild-type mice and their gross appearance are phenotypically indistinguishable from wild-type and heterozygous littermates during growth and maturation (Figure 4.2B,E and data not shown). These initial findings suggest that I12b deletion does not markedly affect overall growth and survival of the animals.

4.3.2. Morphological analyses of brain structures in I12b mutants

We next attempted to phenotype I12b homozygous mutants for potential morphological abnormalities in the brain. Upon visual inspection, we failed to detect any notable morphological deficits in the I12b null brain at the various time points examined (E13.5, E15.5 and P0, Figure 4.2A,C,F,G). In addition, visual observation showed that the size of $I12b^{-/-}$ mutant brains are comparable to that of their wild-type littermates and appear grossly normal (Figure 4.2A,C,F,G). We also performed a histological analysis of Nissl-stained sections from the forebrain of animals sacrificed on the day of birth (P0). As shown in Figure 4.3, morphological structures of the olfactory bulb, neocortex and hippocampus, where I12b CRE is highy active (Ghanem et al., 2007; Ghanem and Ekker, unpublished data), are generally comparable between $I12b^{-/-}$ mutants and wild-type controls, despite that the thickness of the neocortex in a few I12b null mice is slightly smaller as compared to that of wild-type littermates (Figure 4.3C, C', arrow and data not shown).

4.3.3. I12b CRE deletion impacts normal cell proliferation in the basal ganglia

Figure 4.2. Histological analysis of I12b$^{-/-}$ mutants at various developmental stages (E13.5, E15.5 and P0)

(**A**,**C**,**F**,**G**) At various time points examined, the dissected I12b null brains are of almost the same size as that of their wild-type littermate controls and appear grossly normal. The images are shown in dorsal views, anterior upward. (**B**,**D**,**E**) Sagittal views of E13.5 and E15.5 I12b homozygous mutants also reveal normal morphology as compared to the corresponding wild-type embryos. Scales (1mm) are show on the left in all the panels.

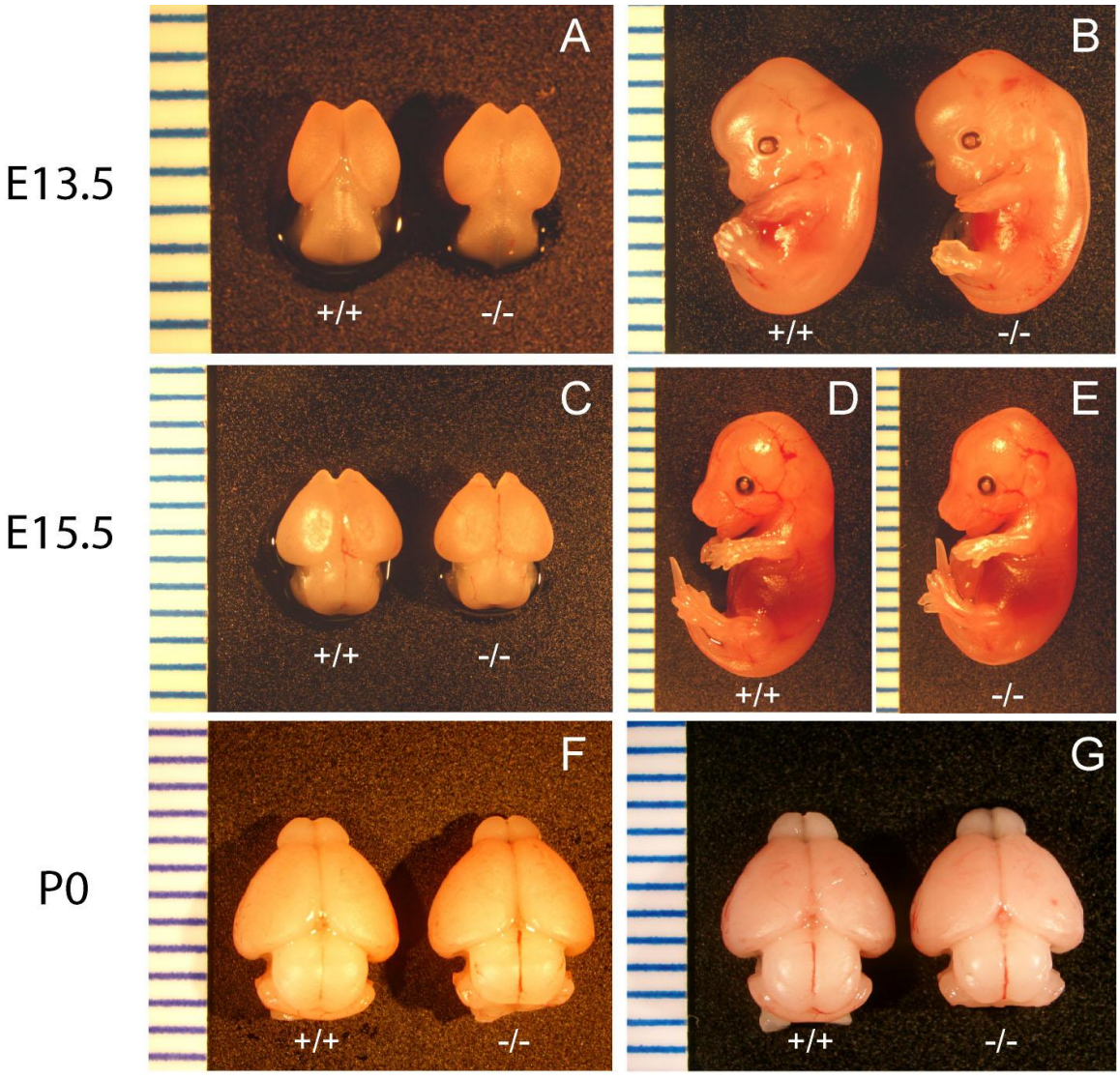
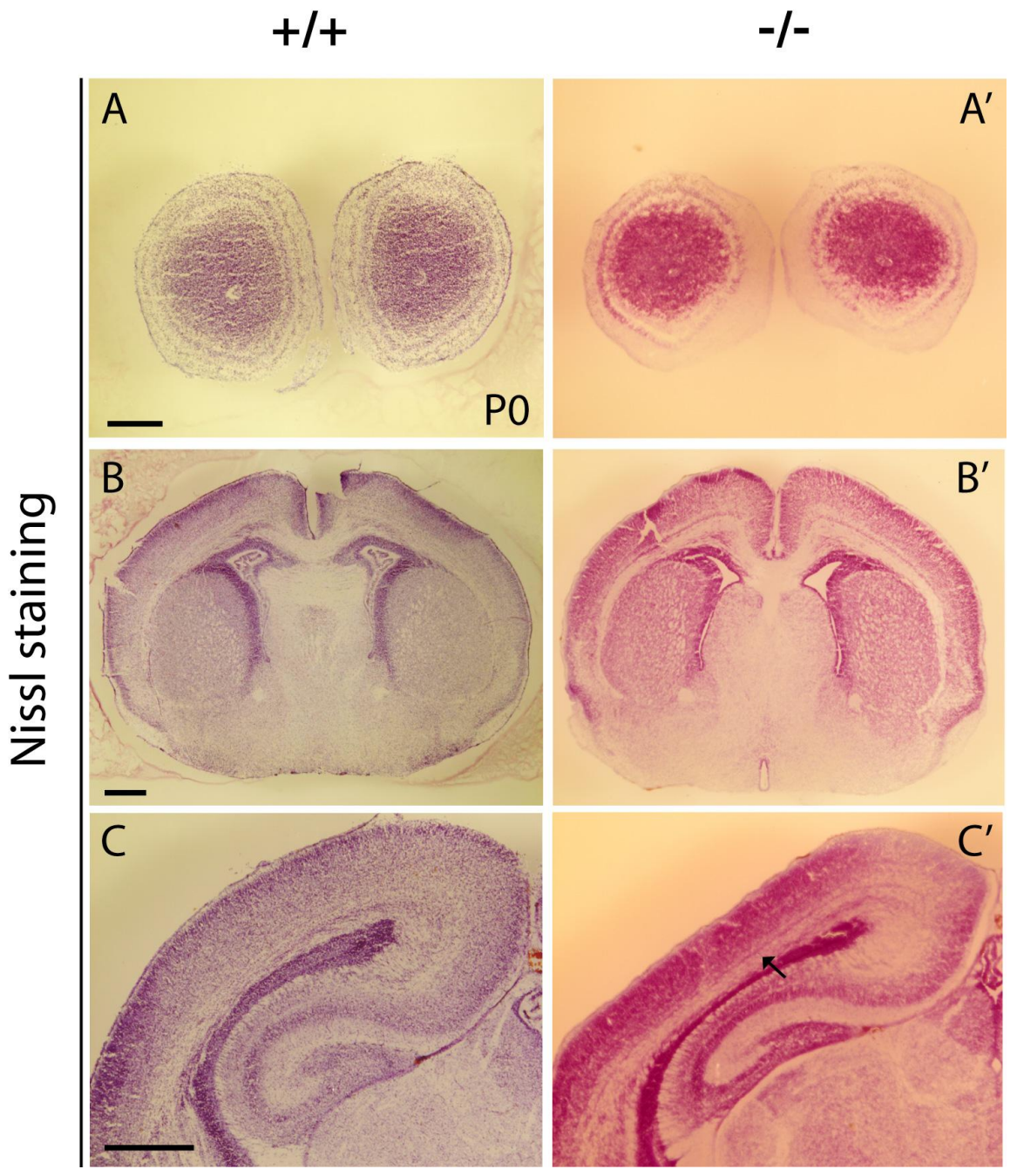

E13.5
E15.5
P0
A
B
C
D
E
F
G
+/+
-/-
+/+
-/-
+/+
+/+
-/-
+/+
-/-

Figure 4.3. Coronal sections through the brains of newborn I12b null and wild-type mice stained with cresyl violet

Nissl staining shows that morphological structures and organization of the olfactory bulb (**A**,**A'**), neocortex (**B**,**B'**) and hippocampus (**C**,**C'**) are generally comparable between P0 I12b$^{-/-}$ mutants and wild-type littermate controls. Interestingly, the thickness of the neocortex in a few I12b$^{-/-}$ mice is slightly smaller (**C'**, arrow) in comparison with that of the wild-type mice. Representative pictures from five independent experiments are shown here. Scale bars: 200μm (**A**,**A'**,**B**,**B'**) and 500μm (**C**,**C'**).


+/+
-/-
Nissl staining
A
A′
P0
B
B′
C
C′

To investigate whether I12b elimination affects tangential migration of early-born GABAegic neurons in the developing forebrain similar to the phenotypes seen in compound *Dlx* mutants (Anderson et al., 1997a; Wang et al., 2010), we observed the migration streams of GABA- and calbindin (CB)-expressing cells from the subpallial telencephalon to the neocortex at E13.5 and E15.5, respectively. Although a detailed quantitative analysis was not conducted, immunohistochemical staining for GABA showed that the migration streams of GABAergic neurons in E13.5 I12b null mice are generally comparable to that of the wild-type littermates (Figure 4.4). In parallel, we used CB as a molecular marker to trace the tangential migration of a subpopulation of GABAergic interneurons primarily moving from the subpallium to the developing neocortex via the intermediate zone (Jimenez et al., 2002). Upon visual observation, we found that the streams of CB-expressing tangential migratory cells in E15.5 I12b mutants generally appear to be comparable to that in the wild-type littermates (Figure 4.5). To further check whether I12b deletion influences normal proliferation of neural progenitor cells in the developing telencephalon, we immunostained proliferating cells that have entered the G2/M phase using a mitotic marker, anti-phospho-histone H3 (PH3) antibody. Upon visual inspection, we noticed that the number of cycling cells in the subpallium of E13.5 I12b$^{-/-}$ telencephalon seems to be decreased compared to that in the wild-type controls, especially along the regions of ventricular and subventricular zones (VZ and SVZ) in the lateral/medial ganglionic eminences (LGE/MGE) (Figure 4.6A-H), a mutant phenotype resembling that reported in *Dlx1/Dlx2* double knockouts (Anderson et al., 1997b). However, the retarded cell growth was not accompanied by an increased expression of active caspase 3 in LGE, MGE and CGE cells (Figure 4.6I-P), suggesting

Figure 4.4. Comparison of tangential migration streams of GABA-expressing cells between E13.5 I12b$^{-/-}$ and wild-type telencephalon

Transverse telencephalic sections from E13.5 I12b$^{-/-}$ mutant embryos (**E-H**) and their corresponding wild-type littermates (**A-D**) were immunoreacted with an antibody against GABA in order to evaluate the tangential migration streams of GABAergic progenitors. The migration streams of GABAergic neurons at the rostral/medial (F, arrow) and caudal levels (**H**, arrows) in E13.5 I12b null mice are generally comparable to that of the wild-type littermates (**B**,**D**). Dorsal is upward in all the panels. The boxed regions in **A**,**C**,**E**,**G** are shown at higher magnification in **B**,**D**,**F**,**H**. Scale bars: 300 μm (**A**,**C**,**E**,**G**) and 200 μm (**B**,**D**,**F**,**H**).

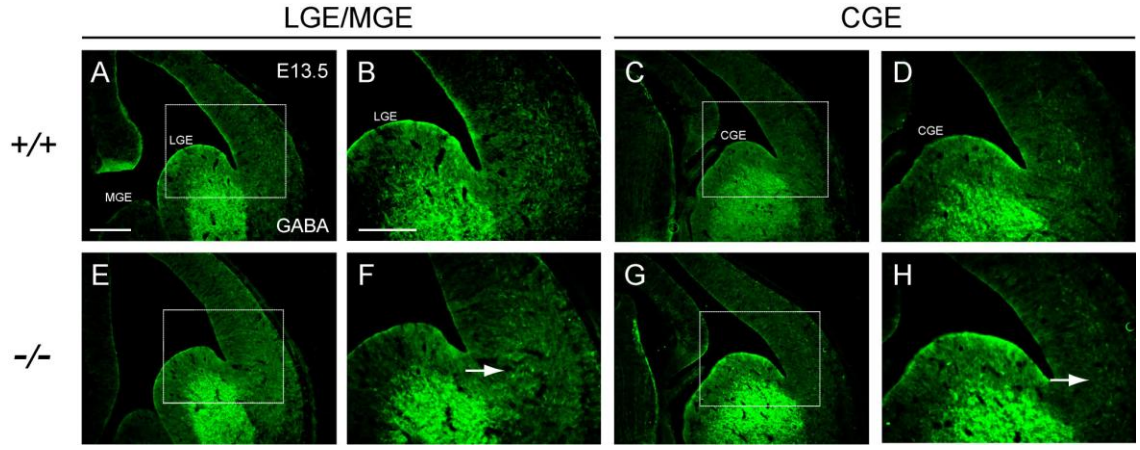

LGE/MGE
CGE
+/+
-/-
A
E13.5
LGE
MGE
GABA
B
LGE
C
CGE
D
CGE
E
F
G
H

Figure 4.5. Comparison of tangential migration streams of CB-expressing cells between E15.5 I12b$^{-/-}$ and wild-type telencephalon

Transverse telencephalic sections from E15.5 I12b-deficient embryos (**C**,**D**) and their corresponding wild-type littermates (**A**,**B**) were immunostained with an antibody against CB to label tangentially migrating cells. Streams of CB-positive neurons were observed leaving from the ganglionic eminence (GE) towards the developing cortex in I12b$^{-/-}$ mutants (**D**, arrow), which in general appears to be comparable to that detected in the wild-type controls (**B**). Dorsal is upward in all the panels. The boxed regions in **A**,**C** are shown at higher magnification in **B**,**D**. Scale bars: 300 μm (**A**,**C**) and 200 μm (**B**,**D**).

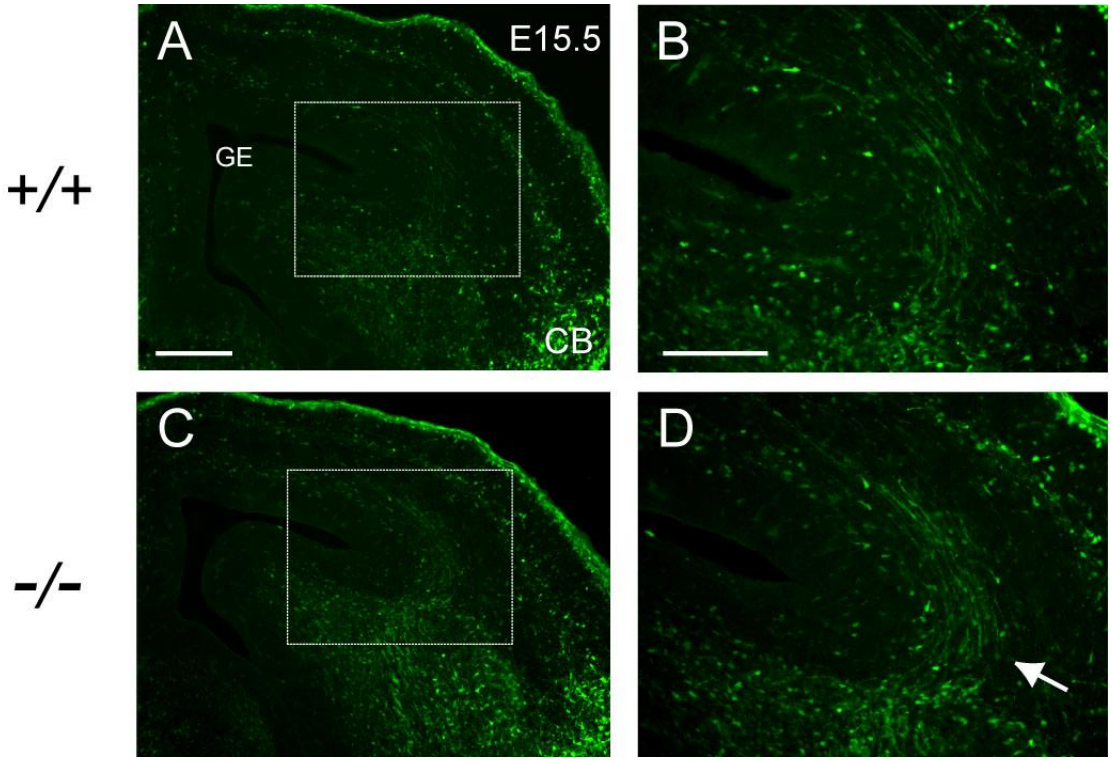
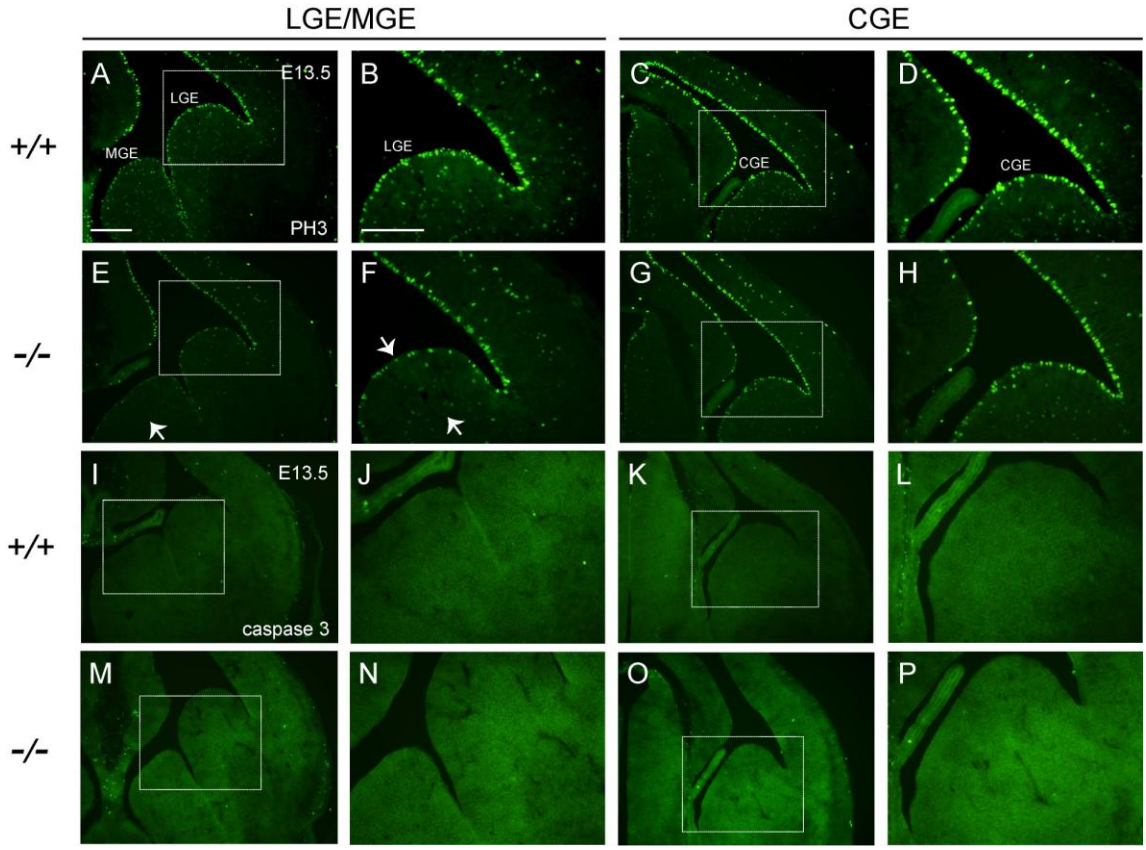

A
E15.5
GE
CB
B
+/+
C
D
-/-

Figure 4.6. I12b CRE deletion may potentially affect cell proliferation in the subpallium

Immunolocalization of PH3 reveals that the number of cycling cells may be decreased in the subpallium of E13.5 I12b$^{-/-}$ telencephalon (**E**,**F**,**G**,**H**) when compared to the wild-type littermate controls (**A**,**B**,**C**,**D**), particularly along the areas of ventricular and subventricular zones in the LGE/MGE (**E**,**F**, arrows). However, the decreased cell proliferation in I12b$^{-/-}$ mutants is not accompanied with an upregulation of caspase 3 expression at the levels of LGE/MGE and CGE (**I**-**P**). Dorsal is upward in all the panels. The boxed regions in **A**,**C**,**E**,**G**,**I**,**K**,**M**,**O** are shown at higher magnification in **B**,**D**,**F**,**H**,**J**,**L**,**N**,**P**. Scale bars: 300 μm (**A**,**C**,**E**,**G**,**I**,**K**,**M**,**O**) and 200 μm (**B**,**D**,**F**,**H**,**J**,**L**,**N**,**P**). Abbreviations: CGE, caudal ganglionic eminence; LGE, lateral ganglionic eminence; MGE: medial ganglionic eminence.


LGE/MGE
CGE
+/+
-/-
+/+
-/-
A
E13.5
LGE
MGE
PH3
B
LGE
C
CGE
D
CGE
E
F
G
H
I
E13.5
caspase 3
J
K
L
M
N
O
P

that this phenotype is not resultant from apoptotic death of subsets of subpallial progenitor cells.

4.3.4. Removal of I12b leads to a reduction of *Dlx1/Dlx2* mRNA levels in the forebrain

In an effort to ascertain the necessity of I12b CRE for overall *Dlx* regulation *in vivo*, we sought to examine the expression pattern and transcript levels of four *Dlx* genes (*Dlx1*, *Dlx2*, *Dlx5* and/or *Dlx6*) in E13.5 I12b null telencephalon. Our *in situ* hybridization assays demonstrated that knockout of I12b element did not make significant impact upon the expression pattern of *Dlx1* and *Dlx2*, two genes in close proximity to I12b sequence, in the developing forebrain (Figure 4.7A-F'). As seen with *Dlx1/Dlx2*, I12b deletion did not delay the onset of *Dlx5* expression and the spatial distribution of transcripts appeared to be highly similar when homozygous I12b mutants were compared with wild-type littermates (Figure 4.7G-I'). Normal *Dlx* expression pattern identified in the $I12b^{-/-}$ forebrain prompted us to carry out a real-time RT-PCR analysis in order to directly address the potential changes in expression levels. Interestingly, we found a ~25% reduction in relative levels of *Dlx1* mRNA (0.74 ± 0.02, $P < 0.01$) and a ~15% decrease in relative levels of *Dlx2* mRNA (0.86 ± 0.03, $P < 0.01$) in the telencephalon of $I12b^{-/-}$ mutants after normalization with that in wild-type controls (set as 1, Figure 4.8). We then determined what happens to the expression level of *Dlx5* and *Dlx6* mRNA since these two genes were previously shown to be down-regulated in the ventral telencephalon of mice lacking *Dlx1* and *Dlx2* function (Anderson et al., 1997b; Zerucha et al., 2000). *Dlx5* and *Dlx6* transcript levels appeared to be slightly

elevated to different degrees in E13.5 I12b null telencephalon as compared to that in the corresponding wild-type animals (Figure 4.8). In addition to Dlx putative binding sites, the I12b enhancer also contains a number of consensus-binding motifs for several other transcription factors required for telencephalic development, such as Meis/Msx/Nkx homeodomain proteins and Mash1 (Poitras et al., 2007). Hence, we looked at whether there is any change in the quantitative levels of some of these key regulators as a consequence of I12b deletion. Although the levels of *Nkx2.1* and *Gsh2* mRNA remained intact, the normalized amount of *Mash1* transcript, a direct upstream regulator of *Dlx1/Dlx2*, increased by ~10% (1.09 ± 0.02, $P < 0.01$) in E13.5 I12b$^{-/-}$ mutants relative to wild-type controls (set as 1, Figure 4.8). These findings provide the first evidence suggesting that I12b CRE deletion possibly does not lead to changes of *Dlx* expression pattern; however, it is sufficient to alter the basal levels of some genes (such as *Dlx1/Dlx2* and *Mash1*) involved in the *Dlx*-mediated regulatory pathway.

4.4. Discussion

4.4.1. Functional redundancy between *Dlx* forebrain CREs

The sequences of I12b enhancer and of their orthologs in distantly related vertebrate species are highly conserved with a sequence similarity above ~80% (Ghanem et al., 2003). In transgenic mice, I12b specifically targets *lacZ* reporter gene expression to the ventral telencephalon and diencephalon in a pattern that faithfully resembles endogenous *Dlx* forebrain expression (Ghanem et al., 2003; Ghanem et al., 2007), indicating that it may be a key regulatory element responsible for *Dlx* regulation. It has been proposed that highly conserved or ultraconserved regulatory elements, which show

Figure 4.7. *In situ* hybridization analysis of *Dlx* expression in the telencephalon of I12b-deficient mouse embryos at E13.5

Coronal sections from E13.5 wild-type (**A-I**) and I12b-deficient mutant telencephalon (**A'-I'**) show no significant difference of *Dlx1* (**A-C'**), *Dlx2* (**D-F'**) and *Dlx5* (**G-I'**) mRNA expression patterns at the rostral, medial and caudal levels. Dorsal is upward in all the panels. Control: left hemisection; $I12b^{-/-}$: right hemisection. Representative images from three independent experiments are provided here. Scale bar: 100 μm.

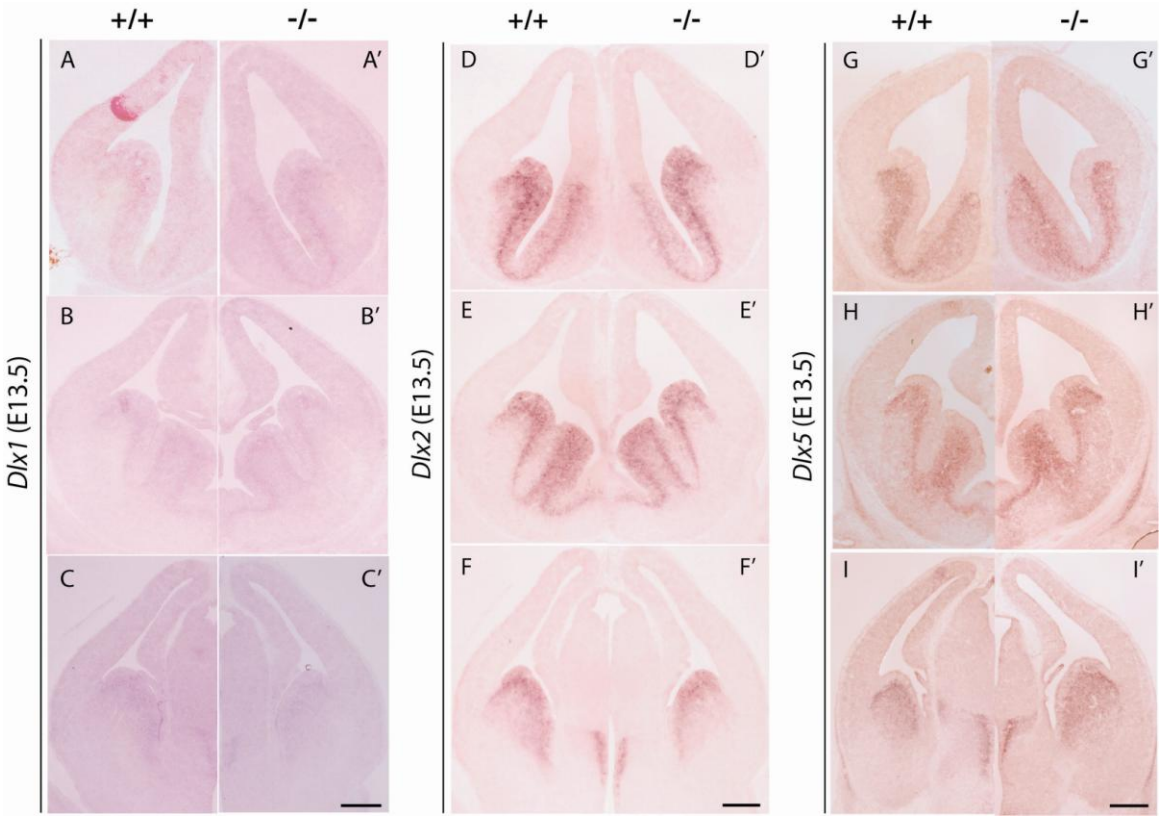
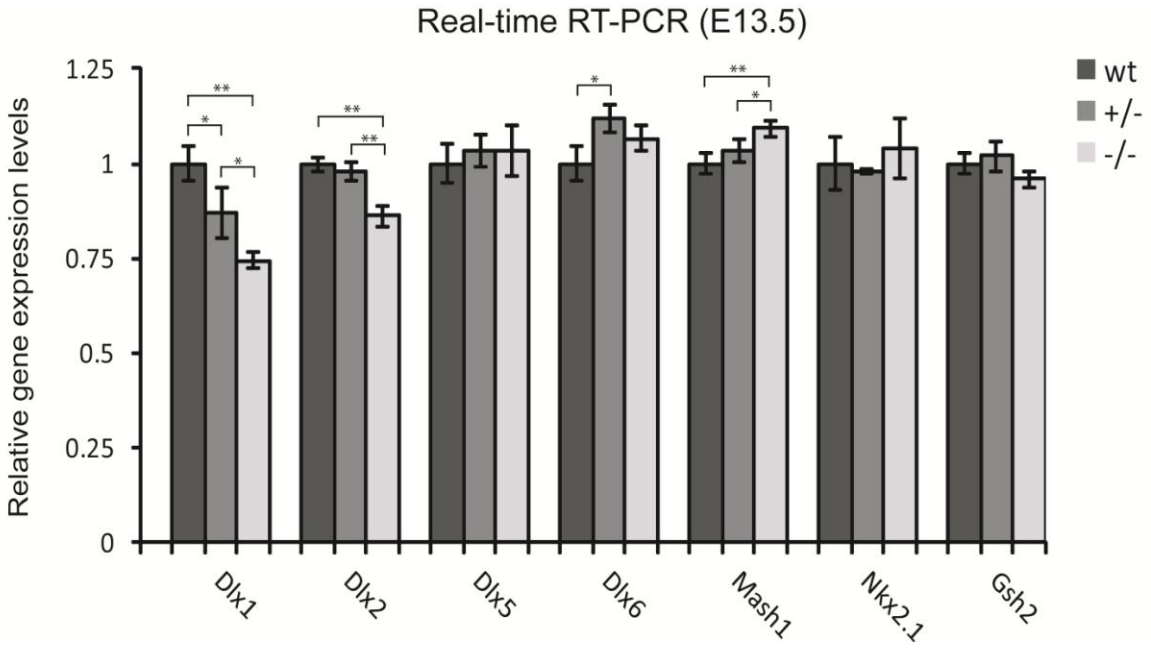

+/+
-/-
+/+
-/-
+/+
-/-
Dlx1 (E13.5)
Dlx2 (E13.5)
Dlx5 (E13.5)
A
A′
B
B′
C
C′
D
D′
E
E′
F
F′
G
G′
H
H′
I
I′

Figure 4.8. Real-time RT-PCR assay displaying the relative expression levels of *Dlx1/Dlx2, Dlx5/Dlx6, Mash1, Nkx2.1* and *Gsh2* transcripts in the telencephalon of E13.5 I12b heterozygous and homozygous mutants

Although the relative quantitative amounts of *Dlx5/Dlx6*, *Nkx2.1* and *Gsh2* mRNA are not significantly altered, deletion of I12b enhancer results in a ~15-25% reduction of *Dlx1* (0.74 ± 0.02) and *Dlx2* (0.86 ± 0.03) as well as an ~10% increase of *Mash1* (1.09 ± 0.02) at the transcriptional levels in the telencephalon of $I12b^{-/-}$ mutants relative to that in the wild-type littermate controls (defined as 1). Data are presented as the average relative unit ± SEM and the transcript level of *GAPDH* in the same sample was used as a normalization control. The differences in gene expression levels between three groups were examined by using the Student-*t* test. All the experiments were repeated three times in triplicate. *: $P < 0.05$; **: $P < 0.01$.


Real-time RT-PCR (E13.5)
Relative gene expression levels
1.25
1
0.75
0.5
0.25
0
wt
+/-
-/-
Dlx1
Dlx2
Dlx5
Dlx6
Mash1
Nkx2.1
Gsh2

little or even no sequence variations across different species, are preserved during the evolutionary process due to their essential biological activities (Meireles-Filho and Stark, 2009; Paixao and Azevedo, 2010). If the function of these regulatory elements is truly important as hypothesized, deleting these elements from their endogenous genomic loci would cause significant phenotypic effects on the host organisms, such as severe morphological defects leading to embryonic lethality. For example, deleting an evolutionarily conserved enhancer element which regulates *Pax3* expression in the neural crest expression leads to exencephaly and spina bifida during early embryogenesis (Degenhardt et al., 2010). Similarly, targeted deletion of an enhancer responsible for *dHAND* branchial arch expression results in many severe defects in the craniofacial structures and mutant mice die soon after birth (Yanagisawa et al., 2003). In contrast, in our case, I12b$^{+/-}$ and I12b$^{-/-}$ mutant mice all survive and reproduce as anticipated. The viability and litter size of their offspring exhibit no significant differences when compared to that of their wild-type littermates. Despite that the thickness of the neocortex in I12b null mice seems to be a bit reduced compared to wild-type controls, no apparent morphological and histological defects were found in the I12b$^{-/-}$ forebrain. These observations indicate that while the I12b sequence is well conserved during evolution and its enhancer activity is detectable in our transgenic assays, targeted removal of this enhancer *in vivo* does not necessarily result in pronounced effects on survival, fertility and brain morphology, which is consistent with other enhancer deletion studies (Chen et al., 2002; Chen and Goldhamer, 2004; Ahituv et al., 2007). One possible reason for this phenomenon could be a functional redundancy among various *Dlx* CREs. Redundant activities among different regulatory elements have been suggested as one of the

protective mechanisms in host organisms when dealing with random mutations occurring within the critical sequences that otherwise might trigger detrimental phenotypic consequences (Piano et al., 1999; Frankel et al., 2010; Paixao and Azevedo, 2010). As such, when I12b is absent, the remaining *Dlx* forebrain enhancers, such as URE2 upstream of *Dlx1*, may step in and exert their activities to maintain proper *Dlx* gene regulation and avoid the pathologies in I12b mutants. This point may be particularly true for I56i CRE within the *Dlx5/Dlx6* intergenic sequence. Our previous transgenic assays have demonstrated that I56i and I12b are active in almost identical populations of GABAergic neurons *in vivo* (Ghanem et al., 2007). In addition, I56i was found to play a role in the cross-regulation between *Dlx1/Dlx2* and *Dlx5/Dlx6* genes (Zerucha et al., 2000). It should be emphasized here that the various individual *Dlx* CREs contain different putative binding motifs for distinct *trans*-acting factors, which may contribute to at least some of their differential functional activities.

4.4.2. Enhancer sharing between the *Dlx* genes

One typical feature of the *Dlx* gene family is the highly overlapping expression patterns between different members and this property of *Dlx* genes appears well conserved across various vertebrate species (Panganiban and Rubenstein, 2002; Sumiyama et al., 2003). Thus, the same cells may show expression of several *Dlx* genes at the same time. To date, it remains to be determined whether the two *Dlx* genes on the same cluster are simultaneously under the control of each individual enhancer (enhancer sharing) or if one of them is specifically linked to one *Dlx* gene, possibly through an insulator mechanism which blocks its interaction with the other *Dlx* gene. The question

could become more important when taking into consideration that enhancer sharing between the two *Dlx* genes from the same cluster may be at least partially responsible for their overlapping expression pattern (Krumlauf, 1994; Gould et al., 1997). It could be also one contributing factor maintaining the conserved organization of *Dlx* bigene clusters during the evolutionary process (Krumlauf, 1994; Gould et al., 1997). The relative levels of *Dlx1* and *Dlx2* transcripts are significantly reduced by ~3-13% and ~15-25% in I12b heterozygous and homozygous mutants respectively, providing some evidence that these two neighboring genes may be regulated in the same I12b-dependent manner *in vivo*. The sharing of regulatory elements between clustered genes has been previously documented in chicken *β–globin* genes and in the *Hox* gene complex (Choi and Engel, 1988; Gerard et al., 1996; Zakany et al., 1997). Specifically, Zakany et al. (1997) identified an evolutionarily conserved enhancer named RVIII within the mouse *Hoxd10*/*Hoxd11* loci and this element can be shared by the two genes for regulating and maintaining their proper expression in the trunk. In the future, it will be interesting to further explore whether other *Dlx* CREs also interact with both *Dlx* genes from the same cluster in their surrounding genome by employing our other enhancer deletion mice (e.g. I56i$^{-/-}$ and I56ii$^{-/-}$ mutants).

4.4.3. Contribution of I12b to the regulation of the genetic pathway involving *Mash1* and *Dlx1/Dlx2*

To date, how different lineages of GABAergic neurons are generated and differentiate in the developing subpallium still remains largely obscure. During early embryogenesis, *Dlx1* and *Dlx2* gene expression begins in the VZ and SVZ of the

ganglionic eminences at ~E9.5 and these two genes are thought to play a role in regulating the proliferation and differentiation of early progenitor cells in these regions (Bulfone et al., 1993a; Anderson et al., 1997b). Indeed, *Dlx1/Dlx2* double knockouts exhibit significantly decreased cell proliferation and deficient neurogenesis in the progenitor domains of the LGE and MGE (Anderson et al., 1997b). Consistent with these findings, we noticed that cell growth in the E13.5 I12b$^{-/-}$ telencephalon may be affected in the VZ/SVZ regions of the LGE and MGE, likely due to reduced *Dlx1* and *Dlx2* transcript levels in these areas or to yet to be determined alterations in the expression of some key regulatory genes involved in the *Dlx1/Dlx2*-mediated genetic pathway. In the developing mouse forebrain and, very likely, in other tissues, expression of *Dlx* genes is thought to be modulated by both auto- and cross-regulation through their dynamic interactions with the CREs at their own genomic loci or those from other *Dlx* bigene clusters (Zerucha et al., 2000; Zhou et al., 2004; Bond et al., 2009; Potter et al., 2009). Mutations of the putative Dlx binding sites in the *Dlx* CREs (e.g. I12b and I56i) result in markedly reduced reporter gene expression in the developing forebrain of transgenic animals and also impact on the binding of Dlx proteins to these enhancers (Zerucha et al., 2000; Poitras et al., 2007). Of interest, expression patterns *Dlx5* and *Dlx6* remain largely unchanged and their mRNA levels are only slightly affected in the embryonic forebrain of I12b$^{-/-}$ mutants, suggesting that *Dlx5* and *Dlx6* expression may be independent on the altered levels of *Dlx1* and *Dlx2* due to I12b deletion. Moreover, the transcript levels of *Mash1*, a direct upstream modulator of *Dlx1/Dlx2*, are slightly elevated following I12b enhancer deletion probably as a result of a compensation effect, although the molecular mechanisms underlying this event needs to be further investigated. These data support a

notion that I12b enhancer might be functionally involved in regulating and maintaining the genetic network in which *Mash1* and *Dlx1/Dlx2* genes are involved in the developing mouse forebrain.

4.4.4. Possible implication of I12b in cell-fate determination in the developing forebrain

Molecular mechanisms of cell-fate determination have been extensively studied in the developing mammalian forebrain, where *Dlx* genes as well as a number of bHLH transcription factors including *Mash1*, *Ngn2*, and *Olig1/Olig2* have been shown to exert important regulatory functions (Kondo and Raff, 2000; Bertrand et al, 2002). In the progenitor domains of the developing subpallium and in the SVZ of the postnatal mouse brain, *Mash1* plays key roles in promoting the commitment of multipotent neural progenitor cells to a neuronal fate through inhibition of astrocyte proliferation and differentiation (Parras et al., 2004; Parras et al., 2007). Previous studies also showed that *Mash1* is responsible for regulating the production of oligodendrocyte precursor cells (OPCs) (Parras et al., 2004; Parras et al., 2007). Both *Mash1* single and *Mash1;Ngn2* double knockouts have been found to have significantly decreased number of precursors that are committed to an astrocytic fate in the developing subpallium when compared to that of the wild-type littermates (Casarosa et al, 1999; Horton et al, 1999; Nieto et al, 2001; Yun et al., 2002). Reduced numbers of OPCs were also observed in *Mash1* mutant embryos as well as in *Mash1* mutant cells cocultured with wild-type cells, further supporting a role of *Mash1* in oligodendrogliogenesis (Bertrand and Guillemot, unpublished data). Interestingly, *Dlx1/Dlx2*, the downstream targets of *Mash1*, were

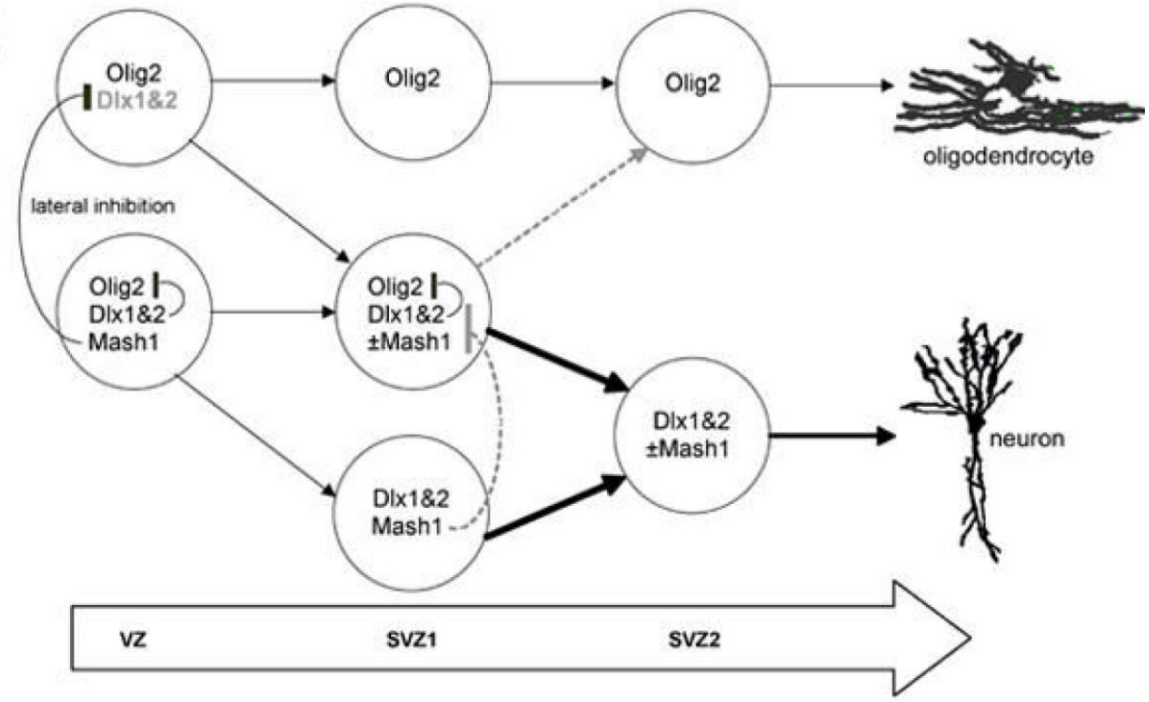
found to be expressed in the subpallial-originated OPCs, indicating that the genetic pathway involving *Mash1* and *Dlx1/Dlx2* may be also implicated in regulating the production and/or differentiation of oligodendrocytes, in addition to GABAergic neurons (He et al., 2001; Marshall et al., 2003). Petryniak et al. (2007) recently reported that *Dlx1/Dlx2* can negatively regulate the expression of *Olig2*, a key factor regulating OPC specification and maturation (Lu et al., 2002), in progenitor cells in order to promote the generation of GABAergic neurons, while *Mash1* can inhibit the process of neurogenesis and induce the production of oligodendrocytes by downregulating *Dlx1/Dlx2* expression (Supplementary Figure 4.1). Because the expression levels of *Dlx1/Dlx2* and *Mash1* are significantly altered in the I12b$^{-/-}$ forebrain, we are presently studying the expression of *Olig1* and *Olig2* in I12b mutants in order to test if this enhancer is involved in the regulatory network modulating neuronal and oligodendrocytic cell fate specification.

4.4.5. Potential importance of I12b to GABAergic interneuron differentiation

Dlx genes are essential players in the molecular and genetic pathways responsible for the production of neuronal diversity, particularly for GABAergic interneurons, during mouse forebrain development (Panganiban and Rubenstein, 2002). As noted, disruption of both *Dlx1* and *Dlx2* function leads to major defects in the generation and differentiation of GABAergic interneuron progenitors born in the embryonic ganglionic eminences (Anderson et al., 1997a; Bulfone et al., 1998; Pleasure et al., 2000). These cells accumulate in the basal ganglia and fail to migrate to the neocortex, olfactory bulb and hippocampus through different migratory routes (Anderson et al., 1997a; Bulfone et al., 1998; Pleasure et al., 2000). However, our initial phenotype analysis showed the

Supplementary Figure 4.1. A proposed model showing the *Dlx1&2/Mash1/Olig2*-mediated pathway that control the switch between neurogenesis and oligodendrogliogenesis in the developing telencephalon

Dlx1 and *Dlx2* repress the expression of *Olig2* in progenitor cells in order to promote the generation of GABAergic neurons. *Mash1* can inhibit the process of neurogenesis and induce the production of oligodendrocytes via downregulating *Dlx1/Dlx2* expression. Adapted from Petryniak et al., 2007.


Olig2
Dlx1&2
Olig2
Olig2
oligodendrocyte
lateral inhibition
Olig2
Dlx1&2
Mash1
Olig2
Dlx1&2
±Mash1
Dlx1&2
±Mash1
neuron
Dlx1&2
Mash1
VZ
SVZ1
SVZ2

tangential migration streams of GABAergic neurons in the forebrain of I12b$^{-/-}$ mutant embryos are generally comparable to those seen in wild-type mice. The underlying reason(s) for this observation is still unknown at this moment but might be linked to functional redundancy among Dlx proteins or different *Dlx* CREs as discussed earlier. Alternatively, there may be other undiscovered mechanisms, such as those underlying cell movement or extracellular environment changes, which can compensate for the potential effects induced by I12b enhancer deletion (Rakic and Komuro, 1995). A detailed quantitative analysis is in progress to confirm this observation. Although we have not observed obvious impacts of I12b deletion on GABAergic interneuron development at early embryonic stages, its potential effects may become more evident at later time points throughout development or in adult animals. Adult *Dlx1*$^{-/-}$ mutants maintained in a C57BL/6J×CD1 background display a specific loss of several GABAergic interneurons subtypes (CR-, SOM- and NPY-expressing cells) in the cortex and hippocampus in a time-dependent manner (Cobos et al., 2005a). These mutant mice also exhibit an epileptic phenotype and have a reduced response to fear conditioning (Cobos et al., 2005a; Mao et al., 2009). In addition, *Dlx5/Dlx6*$^{-/-}$ mutants have a selectively reduced number of PV-expressing cortical interneurons and adult *Dlx5/Dlx6*$^{+/-}$ mice exhibit spontaneous electrographic seizures probably due to improper function of PV-positive neurons (Wang et al., 2010). These findings suggest that various individual *Dlx* genes may play specific roles in regulating the development and/or maturation of distinct subtypes of GABAergic interneurons starting from the early embryonic stages until adulthood. Hence, it is reasonable to infer that while certain alterations in *Dlx* regulation/function as a result of aberrant regulatory activities of their CREs may only

have subtle effects at early embryonic stages, they may induce a severe later-onset phenotype in adult animals. To test this possibility, we are presently exploring whether the somatosensory cortex and hippocampus of 1-month-old I12b$^{-/-}$ mice have loss of some molecular markers for GABAergic neurons (Gad1/Gad2, CB, CR, PV, SOM, NPY, reelin). Given that I12b deletion leads to ~20% decrease in *Dlx1/Dlx2* expression and adult *Dlx1*$^{-/-}$ mutants have marked loss of CR-, SOM- and NPY-expressing interneurons (Cobos et al., 2005a), we expect that I12b enhancer deletion will make an impact at least on the development of these three interneuron subtypes. In the long term, it will be of important significance to study whether I12b$^{-/-}$ mutants harbor some behavioral abnormalities associated with human neurological disorders by employing the tests previously described in (Cobos et al., 2005a; Mao et al., 2009).

In summary, our results demonstrate that mice homozygous for I12b enhancer deletion are viable, develop normally, and display no apparent morphological abnormalities in the forebrain. Targeted deletion of I12b leads to a significant reduction of *Dlx1* and *Dlx2* expression levels and may potentially interfere with normal cell proliferation in the developing subpallium. These data provide the first evidence regarding the specific contribution of an individual CRE to overall *Dlx* expression *in vivo* and indicates a complex transcriptional regulation of *Dlx* genes during forebrain development. Further studies on the underlying molecular mechanisms regulating *Dlx* expression by probing the function of their various CREs will ultimately contribute to our understanding of the genetic pathways involved in controlling GABAergic neuron development. This novel mouse model may also set a stage for future investigations of

potential functional redundancy or their dynamic interactions among different individual *Dlx* CREs when mediating *Dlx* forebrain expression.

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5. Conclusions

The *Dlx* genes encode a group of homeobox-containing transcription factors, which are responsible for proper development of various embryonic structures in many tissues/organs during the period of mid to late gestation. In the developing mouse forebrain, *Dlx* genes are expressed in a highly overlapping but distinct pattern and are critically involved amongst other master regulatory genes in a genetic cascade necessary for the normal differentiation, migration and survival of GABAergic neurons. These neurons produce inhibitory signals in order to maintain the balance of neuronal activities in many important regions of the forebrain, such as the cerebral cortex and hippocampus. Spatio-temporal expression of *Dlx* genes, at least in the developing forebrain, is modulated by a number of highly conserved regulatory elements that have been identified within the *Dlx* loci. In my PhD thesis, I have investigated the potential implications of some of these regulatory elements in the developing mouse and zebrafish forebrain. Specifically, I have characterized in detail the activity of the I56ii enhancer in the developing telencephalon through a comparative analysis of the activities of the other three forebrain-specific *Dlx* CREs (I12b, URE2 and I56i) using transgenic mice and have thus contributed to the understanding of the roles played by I56ii in the overall *Dlx* regulation during mouse telencephalic development. I have also provided evidence that zebrafish I56i and I56ii CREs may have conserved activity in populations of GABAergic neurons within the zebrafish telencephalon and diencephalon which mimics the situation we have seen in mice and knockdown of *dlx* genes significantly interferes with enhancer activity, strongly indicating a possible involvement of *dlx* genes in controlling zebrafish GABAergic neuron production potentially through an evolutionarily conserved

regulatory mechanism, at least in certain domains of the developing forebrain. Finally, I have successfully established a mouse model harboring a targeted deletion of I12b CRE and have initiated the preliminary phenotypic analyses. This study will provide more insight into the specific contribution of individual *Dlx* CREs to overall *Dlx* regulation during mouse forebrain development.

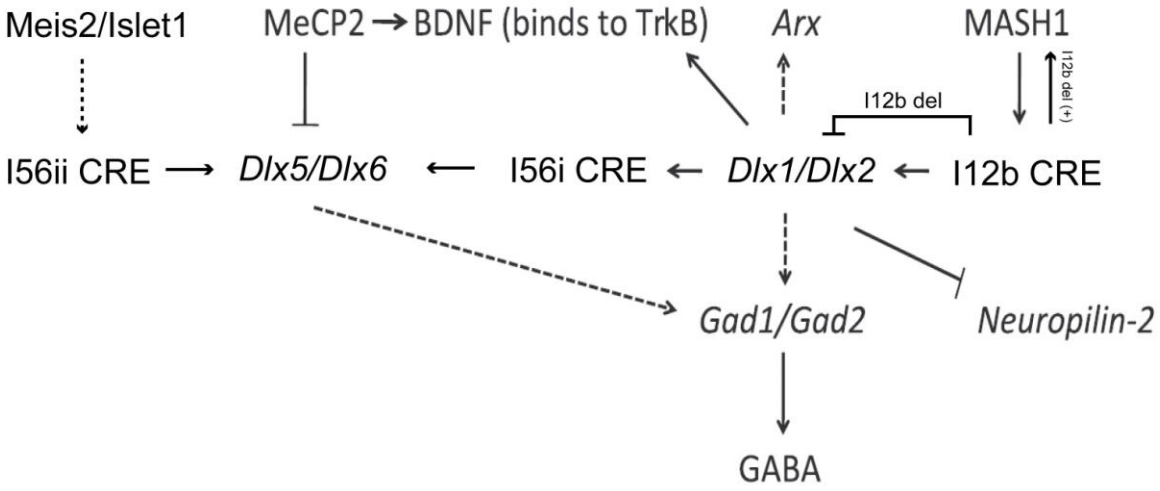
Although some aspects of *Dlx* regulation and function in the developing forebrain have been addressed in my PhD projects (see Figure 5.1 for a model of *Dlx*-mediated genetic network), there are many interesting issues that deserve further investigation. In the following text, I will discuss some of the pertinent questions that still remain unanswered and may be the goals of future studies.

5.1. Investigation of specific contribution of I56ii CRE to overall *Dlx* regulation

Although the expression patterns of reporter transgenes under the control of various CREs in the developing mouse forebrain faithfully recapitulate that of endogenous *Dlx* genes, our previous findings indicate that these distinct CREs are most likely involved in regulating *Dlx* expression in overlapping but different subpopulations of subpallium-derived GABAergic interneuron progenitor cells as well as in different subsets of cortical GABAergic interneurons in adult animals (Ghanem et al., 2007). This possibility is greatly strengthened by the fact that the activity of I56ii CRE is restricted to a subpopulation of *Dlx*-expressing GABAergic projection neurons lining the superficial mantle of the ganglionic eminences between E10.5 and E13.5 and does not overlap with the other forebrain enhancers (Ghanem et al., 2008). Despite these interesting findings, the specific effects of each individual CRE on overall regulation of *Dlx* genes during

Figure 5.1. A model of the genetic network mediated by *Dlx* genes and various forebrain-specific *Dlx* CREs.

Solid arrows represent upstream and downstream targets of the *Dlx* genes which have been validated by chromatin immunoprecipitation or other protein-DNA interaction experiments, where broken arrows indicate potential candidate genes upstream and downstream of the *Dlx* genes. BDNF, brain-derived neurotrophic factor. Modified from Wigle and Eisenstat, 2008.


Meis2/Islet1
MeCP2 → BDNF (binds to TrkB)
Arx
MASH1
I12b del
I12b del (+)
I56ii CRE
Dlx5/Dlx6
I56i CRE
Dlx1/Dlx2
I12b CRE
Gad1/Gad2
Neuropilin-2
GABA

forebrain development as well as their specific involvement in generating GABAergic interneuron diversity remains largely unknown. This issue can be directly demonstrated by targeted deletion of one enhancer or simultaneous knockout of many enhancers in mice. Using the same technique as I describe in Chapter IV for generating I12b$^{-/-}$ mutant mice, our laboratory has recently generated a mutant mouse model carrying a targeted deletion of I56ii CRE. This mutant mouse model differs from *Dlx5*/*Dlx6* single or double knockout mice (Beverdam et al., 2002; Depew et al., 2002; Wang et al., 2010), since in this case, *Dlx5* and/or *Dlx6* function is only inactivated in a subset of cells in the ventral telencephalon and thus it will result in a phenotype specifically linked to improper function of these cells. The expression patterns and quantitative levels of *Dlx1/Dlx2* and *Dlx5/Dlx6* in the I56ii$^{-/-}$ mutants are currently being examined by using both *in situ* hybridization and real-time RT-PCR assays since it has been shown that *Dlx* family members can cross-regulate each other (Zerucha et al., 2000). Hence, altered expression patterns and/or levels of specific *Dlx* genes as a result of enhancer deletion may influence the expression of other family members. Given that I56ii CRE is specifically active in a group of GABAergic projection neurons, the expression of several striatal projection neuron markers (*Islet1, Meis2, Ebf1* and *DARPP-32*) will also be examined in I56ii heterozygous and homozygous mutants.

As discussed in Chapter II, the position of I56ii-positive cells in the ventral telencephalon corresponds to that of a group of cells, termed “corridor cells”, which contribute to the establishement of a permissive route necessary for the guidance and pathfinding of thalamocortical axons (Lopez-Bendito et al., 2006). Perhaps, I56ii is involved in a genetic cascade important for maintaining proper *Dlx5/Dlx6* expression that

play key roles in establishing the "corridor" cells during forebrain formation. To answer this question, I56ii$^{-/-}$ embryos will be examined for formation of thalamocortical projections by using a series of molecular markers, such as CR. Moreover, because thalamocortical projection system is one of the most essential processing connections in the mammalian brain that conveys multiple important sensory and motor signals to the cortex (Dickson 2002; Dufour et al., 2003), we also expect to observe some behavioral changes in I56ii$^{-/-}$ adult mice.

5.2. Identification of upstream activators regulating *Dlx* CRE activities

Interestingly, the sequences of various *Dlx* forebrain-specific CREs only share limited similarity, despite the fact that three out of the four CREs (I12b, I56i and URE2) display high overlapping activities when targeting reporter transgene to populations of progenitors in the basal ganglia as well as when marking adult cortical GABAergic interneurons (Ghanem et al., 2003; Ghanem et al., 2007). The length of these three *Dlx* CREs, the degree of conservation among different vertebrate species for each individual CRE, and their sequence divergence suggest that *Dlx* expression in intermingled populations of GABAergic neurons or in their progenitors may be obtained and maintained through distinct regulatory mechanisms, such as the binding of distinct combinations of *trans*-acting factors to the various CREs. However, our current knowledge regarding direct upstream modulators or signaling molecules influencing *Dlx* expression is very poor. Using a combination of DNase I footprinting, transfection assays, electrophoretic mobility shift assay (EMSA) and ChIP, Poitras et al. (2007) provided evidence that the proneural transcription factor Mash1 directly interacts with

I12b and regulates *Dlx* expression, further supporting a notion that there exists a *Mash1/Dlx/Gad* genetic cascade regulating GABAergic neuron development. In addition to the putative Dlx binding sites (A/C/G)TAATT(G/A)(C/G), comparisons between sequences of *Dlx* CREs and transcription factor binding site databases have revealed a list of other potential binding motifs within I56i (6-8 sites identified thus far) and URE2 (10 sites) CREs. Ongoing work in the laboratory are aimed at further determining whether some of the candidate transcription factors can physically bind to these CREs and mediate *Dlx* expression, using the molecular approaches previously adopted in (Poitras et al., 2007). As noted in Chapter II, Meis2 and Islet1 may be potential upstream regulators of *Dlx* genes in corridor cells by acting upon I56ii enhancer during early embryogenesis. One weakness of this study is that physical interactions between I56ii and these transcription factors have not yet been directly proved. It would be important in further experiments to study the affinity of Meis2 and Islet1 putative binding sites in I56ii using competition EMSA and whether Meis2 and Islet1 can directly bind to I56ii *in vivo* by employing ChIP. It would also be interesting to investigate possible impact of loss or disruption of Meis2 and Islet1 binding sites on the I56ii enhancer activity by establishing transgenic mice with reporter constructs containing mutations in the putative Meis2 and Islet1 binding sites. Such experiments will definitely help us better understand the key upstream factors in the regulatory pathways involving *Dlx* genes and shed more light on their specific interaction with various individual *Dlx* CREs.

5.3. Conserved activity of *Dlx* CREs in the forebrain of other vertebrate species

Dlx forebrain expression patterns between mouse and zebrafish appear to be well conserved, suggesting that the potential mechanisms underlying *Dlx* regulation might be also conserved over evolutionary time (Akimenko et al., 1994; Ellies et al., 1997; Panganiban and Rubenstein, 2002; MacDonald et al., 2010a). Indeed, orthologous *Dlx* CREs exhibit a high degree of sequence similarity among distantly related vertebrate species (Ghanem et al., 2003). In Chapter III, I demonstrated that in addition to the sequence conservation, zebrafish *dlx5a/dlx6a* regulatory elements display a well conserved activity by targeting GFP transgene expression to GABAergic neurons in the developing telencephalon and diencephalon, highly mimicking the regulatory activities of their mouse orthologs when tested in transgenic mice (Ghanem et al., 2007; Ghanem et al., 2008). In addition, zebrafish *dlx5a/dlx6a* regulatory elements may participate in regulating the differentiation of at least some subsets of GABAergic interneurons in certain specific regions in the developing forebrain. These data are interesting in terms of evolutionary biology since the teleost lineage has been proposed to undergo divergence from mammals about 430 million years ago (Powers, 1991). Considering the entire or partial genome of increasing number of vertebrates have been sequenced so far, one resultant question would be whether some of these CREs, if not all, are also present within the *Dlx* locus in these vertebrate species. If this is the case, it will be also important to investigate whether the activity of the CREs is functionally conserved as compared to their respective mouse and zebrafish orthologs. Our laboratory has identified a URE2-like regulatory element in the genome of the elephant shark (*Callorhinchus milii*), which is a cartilaginous fish and has a key phylogenetic position essential for studying the evolution of jawed vertebrates (Venkatesh et al. 2006; Venkatesh et al.

2007). The sequence of elephant shark URE2 shares remarkably high degree of identity with that of its mouse (~85% identity) and zebrafish orthologs (~73% identity) (MacDonald et al., 2010b). When tested in transgenic animals, the elephant shark URE2 targets reporter transgene to the developing forebrain in an expression pattern that is extensively overlapping with that of mouse and zebrafish URE2 CREs, even though the activities of the three orthologous enhancers slightly vary in the diencephalon (MacDonald et al., 2010b). Additionally, large-scale comparative studies done by Venkatesh and colleagues (Venkatesh et al. 2006; Venkatesh et al. 2007) has shown that the number of conserved non-coding regions shared between the human and elephant shark genomes is significantly higher than that between the human and zebrafish genomes, suggesting that the elephant shark may be a novel and appropriate model organism for studying the gene regulatory mechanism during vertebrate evolution. Along this line, it will be interesting to extend our current findings to other *Dlx* CREs (I12b, I56i and I56ii) to test if they are also present in the elephant shark and then compare their activity with that of their corresponding mouse and zebrafish orthologs. From an evolutionary perspective, understanding the function of *Dlx* CREs in a more diverse set of vertebrate species will hopefully shed more light on how the regulatory mechanisms responsible for *Dlx* forebrain expression are established and maintained as well as how distinct function of orthologous *Dlx* genes in forebrain development is acquired over evolutionary time since the divergence of modern vertebrates.

5.4. Investigation of molecular mechanisms underlying enhancer sharing

The bigene organization of *Dlx* genes has found to be a common feature in many vertebrate species (Zerucha and Ekker, 2000). In addition to vertebrates, this typical arrangement of the *Dlx* gene family has also been documented in the tunicate *Ciona intestinalis* as well as in the lampreys (Di Gregorio et al., 1995; Kuraku et al., 2009). Yet, the molecular basis underlying this conserved genomic arrangement through evolution has not been precisely explored so far. Our identification of multiple CREs in the intergenic sequences between *Dlx* bigene pairs may provide a plausible explanation for this phenomenon, as these regulatory sequences are spatially located in close proximity to both genes constituting a bigene pair and are thus probably shared between the two genes. The highly overlapping expression patterns of the two *Dlx* genes from the same bigene cluster lend supports to this hypothesis (Ellies et al., 1997a; Liu et al., 1997). Indeed, sharing an intergenic enhancer as well as competition between the promoter regions of adjacent genes for an intergenic regulatory element has been described for the regulation of the *Hoxb* genes. It was shown that *Hoxb3* and *Hoxb4* are able to equally share an intergenic r6/7 neural enhancer and *Hoxb4* and *Hoxb5* can share an intergenic mesodermal enhancer (Gould et al., 1997; Sharpe et al., 1998). On the contrary, another enhancer within *Hoxb4* and *Hoxb5* intergenic region that targets reporter gene expression to the anterior part of neural tube was found to be capable to activate both genes, but its preferential activity depends on the competition between these genes and also can vary with the different local genomic context (Sharpe et al., 1998). In Chapter IV, I showed evidence that the levels of *Dlx1* and *Dlx2* transcripts are significantly reduced by ~15-25% in I12b$^{-/-}$ mutants, suggesting that I12b enhancer may be shared by these two genes *in vivo*. To further investigate whether each intergenic enhancer or possibly other

enhancers at the locus (e.g. URE2) can activate and regulate expression of one or both genes from a *Dlx* bigene pair, a large transgene construct containing about 45kb of the mouse *Dlx1/Dlx2* loci (encompassing all the CREs) has been generated in the laboratory. Two fluorescent reporter genes, *GFP* and *mCherry*, have been fused independently in frame with the first exon of *Dlx1* and *Dlx2*, respectively. Currently, this construct is being injected for the generation of transgenic mice. Once stable transgenic lines are established, the corresponding regulatory element (I12b, URE2, or both) can be deleted and qualitative and quantitative impacts of each of these elements on the expression of *Dlx1/GFP* and *Dlx2/mCherry* will be systematically analyzed. Data obtained from these experiments may also supplement, at least in some aspects, our ongoing enhancer deletion projects. Moreover, a chromosome conformation capture technique has been shown to successfully characterize the physical interactions among different genomic sequences, including those between transcriptional regulatory elements and the sequences of their target genes (Splinter et al., 2004; Simonis et al., 2007). Thus, this methodology may be applied in the future to better understand the specific interplay between *Dlx* promoter regions and the various individual CREs.

5.5. Functional redundancy between different forebrain CREs and existence of additional CREs in the *Dlx* locus

As shown in Chapter IV, we have not identified any severe phenotypes in the I12b$^{-/-}$ mice, except that I12b deletion results in a decrease in *Dlx1/Dlx2* transcript levels, together with an elevated *Mash1* expression and may possibly reduce the proliferation of progenitor cells in the VZ/SVZ of the LGE and MGE in the mutants. These observations

are at least partially consistent with some forebrain phenotypes seen in *Dlx1*/*Dlx2* double knockouts, such as a significantly decreased cell proliferation and deficient neurogenesis in the progenitor domains of the LGE and MGE (Anderson et al., 1997b). Given that adult *Dlx1*$^{-/-}$ knockouts show a subtype-dependent loss of both cortical and hippocampus GABAergic interneurons and also harbor some behavioral alterations (e.g. epilepsy and reduced fear response) due to the loss of interneurons (Cobos et al., 2005a; Mao et al., 2009), we are presently testing these possibilities in our adult I12b$^{-/-}$ mutant mice by using immunohistochemistry and designing behavioral assays. We expect to see a subset of the phenotypes seen in the adult *Dlx1*$^{-/-}$ mutants. As mentioned in Chapter IV, sharing of CREs between the two *Dlx* genes on the same cluster may contribute to their overlapping expression patterns in the developing forebrain. In addition, the forebrain expression patterns of the reporter transgene under the control of various individual various *Dlx* CREs also largely overlap (Ghanem et al., 2007, 2008). Thus, functional redundancy among various individual *Dlx* forebrain CREs may be a possible reason for the lack of apparent forebrain phenotypes in I12b$^{-/-}$ mutants (at least at this moment). Since URE2 activity in the forebrain overlaps in part with that of I12b (Ghanem et al., 2007), it is highly possible that the remaining URE2 CRE may compensate for the loss of I12b function in the forebrain as a protective mechanism, at least in certain specific populations of cells. This scenario may be particularly true for the I56i CRE, as we have previously demonstrated that I12b and I56i CREs are active in almost identical subsets of GABAergic interneuron progenitors (adult interneurons as well) and could be possibly activated by the same or similar regulatory signals (Ghanem et al., 2007). In support of this idea, recent evidence has suggested that the various enhancers within the zebrafish

hoxb4a locus have overlapping activities to ensure the correct *hoxb4a* dosage and expression pattern in the hindbrain (Punnamoottil et al., 2010). Similarly, Rastegar et al. (2008) reported that the combined activities of two regulatory elements are required to maintain the proper notochord expression of *sox9a*. Therefore, generation of mice with simultaneous deletion of two *Dlx* CREs would be a good option to better address this question. In addition to the four forebrain-specific CREs identified thus far within the *Dlx1/Dlx2* and *Dlx5/Dlx6* loci, there is evidence that additional conserved regulatory elements controlling *Dlx* forebrain expression may exist and have not yet been fully characterized. Two such elements have been found in the 5' flanking region of mouse *Dlx6* gene (Jarinova and Ekker, unpublished observations) and in the 3' flanking sequence of zebrafish *dlx2b* gene (MacDonald et al., 2010c). Moreover, regulatory elements that have relatively shorter length (<100 bp) and contain fewer conserved motifs may have been missed in the original phylogenetic analysis (Ghanem et al., 2003). Recent development of more sophisticated and powerful bioinformatics tools may provide us a good opportunity to refine the original search with the purpose of identifying more potential regulatory elements in the *Dlx* loci.

5.6. Potential association of *Dlx* CRE alterations with human diseases

In the future, it will be of pivotal importance to extend our findings in mouse and zebrafish to the studies of the function of *Dlx* genes during human GABAergic neuron production. In fact, it has been previously documented that up to ~50% of GABAergic interneurons in the cerebral cortex of human brain originate from the supallium-derived progenitor cells (Letinic et al., 2002), which is very similar to the case in mice.

Considering that genetic aberrations and abnormal expression of *DLX* genes have been increasingly detected in many human neurological disorders (e.g. epilepsy, schizophrenia and autism) associated with the lack/reduction of inhibitory control over signaling activities (Liu et al., 2009; Nakashima et al., 2009), it would be meaningful to learn what exact functions *DLX* genes may exert in controlling human GABAergic interneuron generation as well as the implications of the highly conserved forebrain-specific CREs in human *DLX* gene regulation and in these diseases. Indeed, aberrant alterations in regulatory elements have been found as one of the potential causes for many human genetic diseases. For example, several single base mutations identified in a *SHH* enhancer and a duplication of an enhancer of *BMP2* gene have been showed to cause human limb malformations (Vandermeer and Ahituv, 2011). As noted in the introduction, we have recently found that abnormal I56i enhancer activity due to a SNP is able to induce *Dlx* dysfunction and then affect the development of several GABAergic interneuron subtypes (Poitras et al., 2010). Clearly, more comprehensive investigations are warranted to further reveal the potential links between altered activity of various *DLX* enhancers and *DLX* regulation as well as the occurrence of human neurological diseases.

In conclusion, understanding the regulation and function of *Dlx* genes via distinct individual CREs during vertebrate forebrain development will provide more clues about how diverse types of neuronal cells, particularly GABAergic neurons, in the developing forebrain are generated and differentiated from their common and/or respective progenitors and then migrate and populate different specific areas. In the long run, studying these underlying mechanisms will gradually bulid a pivotal molecular basis for a

better knowledge of many common human neurological disorders linked to abnormal production and specification of GABAergic interneurons. Comparison of orthologous *Dlx* function and the interaction with their CREs among distantly related vertebrates will provide more insight into how *Dlx* genes have evolved and gained differential functional properties through the evolutionary process and may also contribute to the establishement of the molecular mechanisms underlying differential morphologies of the forebrain structures between different vertebrate species.

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