ROLE OF DLX GENES IN FOREBRAIN DEVELOPMENT

By

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Abstract

Nervous system development is a highly regulated and complex process in which neurons and glial cells are generated and through migration arrive at their final destinations. *Dlx* genes are required for central neural development. They encode transcription factors that bind to TAAT/ATTA motifs of regulatory regions and regulate target gene expression. *Dlx1/2* genes are important factors involved in neuronal versus glial cell fate switch favoring GABAergic interneuron differentiation. DLX2 represses oligodendrocyte differentiation by negatively regulating the oligodendrocyte marker *Olig2*. Expression of another oligodendrocyte differentiation gene, *Nkx2.2* is increased in mice lacking *Dlx1/2*. Based on these observations, I hypothesized that DLX2 directly mediates transcriptional repression of oligodendroglial differentiation in determination of neuronal versus glial cell fates during forebrain development, in part through inhibition of *Nkx2.2.*

Differentiation and tangential migration of inhibitory interneurons from the ganglionic eminences (GE) to the neocortex is disrupted in the *Dlx1/Dlx2* double knockout (DKO) mouse. CXCR4/CXCL12 signalling also has an important role in the guidance of interneurons from the ganglionic eminences to the neocortex and mice lacking *Cxcr4* or *Cxcl12* have defects in positioning of interneurons in cortical layers. Moreover, in the absence of *Dlx1/2* function, expression of *Cxcr4* is significantly decreased in GABAergic interneurons in the medial and caudal GEs. However, the mechanism by which CXCR4/CXCL12 is activated is not well known. I therefore sought to understand the upstream activation of *Cxcr4* expression. I hypothesized that DLX2 directly mediates transcriptional activation of GABAergic interneuron migration, in part, through activation of *Cxcr4* during forebrain development.

Little is known about posttranslational modifications of the DLX transcription factors and whether these modifications affect DLX's subcellular localization or transcriptional activity. A regulatory subunit of DNA-dependent protein kinase interacts with DLX2, which has the potential to phosphorylate DLX2. Moreover, previous experiments in Eisenstat lab also showed the presence of posttranslational modifications in DLX2 extracted from E13.5 GE. Furthermore, DLX2 is both expressed in the nucleus and cytoplasm of the ventral thalamus revealing the capability of DLX2 to translocate between the nucleus and cytoplasm. Therefore, I proposed to identify the key amino acid residues that are phosphorylated in DLX2. I hypothesized that DLX2 functions as a phosphoprotein and is localized to the nucleus in determining progenitor fate.

ChIP-based PCR of embryonic mouse forebrain demonstrated that DLX2 occupies regions containing putative DLX2 binding sites upstream of the *Nkx2.2* and *Cxcr4* genes. DLX2 significantly affected luciferase reporter gene expression *in vitro* when co-expressed with the regulatory regions of *Cxcr4* and *Nkx2.2* occupied by DLX2 *in vivo*. Quantitative RT-PCR showed an increase in transcript levels of *Nkx2.2* and a decrease in transcript levels of *Cxcr4* in the *Dlx1/2* DKO tissues compared to the wild-type supporting a repressing and activating role of DLX2 on *Nkx2.2* and *Cxcr4* expression, respectively. Conducting Boyden Transwell assays on SK-N-BE (2) neuroblastoma cells revealed that there was a significant increase in cell migration in the presence of CXCL12; however, this migration was significantly decreased in *Dlx2*-siRNA treated cells compared to untreated cells, supporting the contribution of DLX2 to cell migration *in vitro*. These results were corroborated by pharmacological inhibition of CXCR4 signalling. Western Blotting on tissues extracted from E13.5 GE using a specific DLX2 antibody supported the presence of posttranslational modifications in the detected DLX2. Treating the samples with

lambda protein phosphatase eliminated the upper band suggesting that phosphorylation is a major posttranslational modification present in DLX2.

My results support the hypothesis that DLX2 regulates expression of *Cxcr4* and *Nkx2.2* in order to maintain proper differentiation and migration of interneurons and concurrently repress oligodendrocyte cell fate in the developing forebrain. This research will contribute to the emerging evidence supporting a role for the DLX transcription factors and their downstream target genes in maintaining the balance of excitation to inhibition during brain development by actively repressing oligodendrocyte differentiation while promoting interneuron differentiation and migration.

Preface

This thesis is an original work by Sara Japoni. No part of this thesis has been previously published.

All animal experiments received ethics approval from the University's Animal Care and Use Committees (ACUC), protocol number: AUP 0001115.

Western Blotting experiments on *Dlx2*-siRNA1 and *Dlx2*-siRNA2 treated SK-N-BE (2) cells was performed by Ms. Xiaohua Song to compare the knockdown efficiencies between the two siRNAs.

All *in silico* analysis for *Nkx2.2* in chapter 3 was performed by Ms. Kirby Ziegler and results were provided by Dr. Alan Underhill. Figure 3.2 and figure legends were provided by Dr. Alan Underhill and Ms. Kirby Ziegler.

In Figure 4.12 CXCR4 immunostaining of wild type sections was performed by Dr. Q Jiang. In Figure 4.13 and 4.14 *in situ* hybridizations were done with Dr. Q Jiang. Figure 5.1 was performed by Ms. S Zhang.

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Pursuing my doctoral degree in Medical Genetics has been a unique experience equipping me with diverse capabilities to overcome many challenges in life. This journey would not have been possible without the guidance and assistance of numerous people.

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List of Abbreviations

AEP	anterior entopeduncular region
AMPK	AMP-activated protein kinase
ANR	anterior neural ridge
APS	Ammonium Persulfate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BDNF	brain-derived neurotrophic factor
bHLH	basic helix loop helix
BLBP	brain lipid binding protein
BMP	bone morphogenic protein
bp	base pairs
CBP	CREB binding protein
ChIP	chromatin immunoprecipitation
ChIP-seq	ChIP-sequencing
CD184	cluster of differentiation 184
Cdk5	cyclin-dependent kinase 5
CGE	caudal ganglionic eminences
CNS	central nervous system
CREB	cyclic AMP response element-binding protein
Ct	cycle of threshold
D	Dalton
2-D	two-dimensional gel electrophoresis
DiI	1, 19-dihexadecyl-3, 3, 39-tetramethylindocarbocyanine perchlorate
DIG	digoxigenin
DKO	double knockout
Dll	distal-less
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DNase I	Deoxyribonuclease I
DNA-PK	DNA-dependent protein kinase
DV	dorsoventral
E	embryonic days

E. coli	Escherichia coli
EMSA	electrophoretic mobility shift assay
FBS	fetal bovine serum
FGF	fibroblast growth factor
GABA	gamma-aminobutyric-acid
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
GE	ganglionic eminences
gDNA	genomic DNA
GDNF	glial cell line-derived neurotrophic factor
GFRα1	GDNF family receptor α 1
HBSS	Hank's Balanced Salt Solution
IF	immunofluorescence
IPC	intermediate progenitor cell
ISH	in situ hybridization
IZ	intermediate zone
kb	kilobase pairs
LB	lysogeny broth
LGE	lateral ganglionic eminences
MABT	maleic acid buffer
MBP	myelin basic protein
MGE	medial ganglionic eminences
MZ	marginal zone
NBT	4-Nitro blue tetrazolium chloride
NEC	neuroepithial cells
Nrp	Neuropilin
NSC	neural stem cells
OCT	optimal cutting temperature compound
O/N	overnight
OPCs	oligodendrocyte progenitor cells
PBM	protein binding microarray
PBS	Phosphate buffered saline
Pdgfra	platelet-derived growth factor receptor alpha

PFA	paraformaldehyde
PIC	protease inhibitor cocktail
PIK3C3	class III phosphoinositide 3-kinase
PKC	protein kinase C
PLP	proteolipid protein
pMN	motor neuron progenitor domain
PTM	posttranslational modifications
PV	parvalbumin
PWM	position weight matrix
POA	preoptic area
RGCs	radial glial cells
RNAi	RNA interference
Robo1	Roundabout
RT	room temperature
S100b	S100 calcium binding protein B
SDF-1	stromal derived factor 1
SEM	standard error of the mean
Sema3A	Semaphorin 3A
SHH	Sonic Hedgehog
siRNA	small interfering RNA
Sirt2	Sirtuin 2
SST	somatostatin
SVZ	subventricular zone
TBE	Tris/Borate/EDTA
TEMED	Tetramethylethylenediamine
TSS	transcription start site
vnd	ventral nervous system defective
VZ	ventricular zone
WNT	Wingless-related integration site
WT	Wild type

1 Chapter 1: Introduction

1.1 Forebrain development in mice

1.1.1 Overview of central nervous system development

Nervous system development is very complex and to simplify how the central nervous system (CNS) is developed distinctive milestones are considered. At first the neural plate is induced to create the neural tube. Once the neural tube is formed, regionalization and patterning of the neural tube will form three specific sections in the developing brain. Then progenitor cells produce neurons and glial cells. After neural specification, neuronal and glial cells differentiate and migrate to different areas in the brain. Finally synapses form and neural circuits are established (Sanes, Reh et al. 2003, Wigle and Eisenstat 2008).

During vertebrate gastrulation, the neural tube forms from the neuroectoderm located dorsal to the notochord. Regionalization and patterning of the neural tube develops three regions in the developing brain known as the prosencephalon (which will form the forebrain), the mesencephalon (which will form the midbrain), and the rhombencephalon (which will form the hindbrain). Once the forebrain is developed it is further divided into rostral telencephalon and caudal diencephalon. The rostral telencephalon contains the dorsally located pallium and ventrally located subpallium while the caudal diencephalon contains the prethalamus, thalamus, and the pretectum (Rubenstein, Shimamura et al. 1998, Wilson and Houart 2004). A schematic overview of patterning in the neural tube during brain development is illustrated in Figure 1.1.

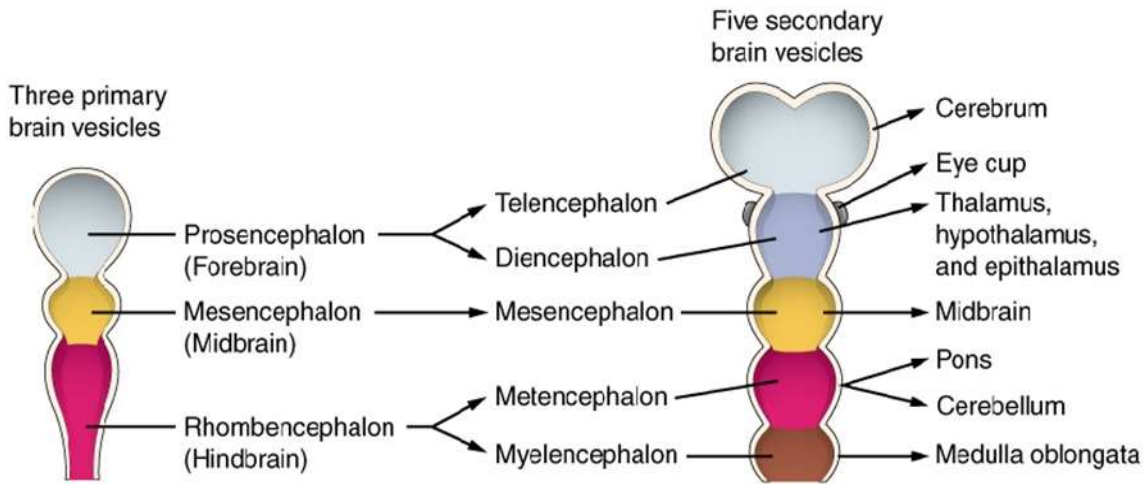


Figure 1.1. A schematic overview of patterning in the neural tube during brain development.

Before the anterior end of the neural tube closes, proliferation of neuroepithelial cells in the rostral end of tube forms three swellings known as the primary vesicles. The anterior vesicle is named the prosencephalon and will form the forebrain. The mesencephalon is in the middle and will develop into the midbrain and the posterior vesicle known as the rhombencephalon will form the hindbrain.

As the brain continues to develop, these primary vesicles differentiate into five secondary vesicles. The prosencephalon further develops into the telencephalon and the diencephalon. The telencephalon will be the future cerebrum. The diencephalon generates the thalamus, the hypothalamus and retina. The mesencephalon does not further differentiate. The rhombencephalon develops into the metencephalon and myelencephalon. The metencephalon will generate the pons and the cerebellum. The myelencephalon will differentiate into the medulla oblongata.

Figure adapted from OpenStax, Anatomy & Physiology. OpenStax CNX. Feb 26, 2016. Download for free http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@8.24 .

1.1.2 **Induction of the telencephalon**

Initially, cells of the anterior neural ridge (ANR), located between neuroectoderm and underlying ectoderm induce cells at the rostral tip of the neural tube to form the telencephalon (Eagleson, Ferreiro et al. 1995, Cobos, Shimamura et al. 2001). An intricate set of interactions between cell extrinsic and cell intrinsic factors in the neuroepithelium is involved to regulate cell proliferation and differentiation of neural progenitors in the developing telencephalon. Cell extrinsic factors are signalling pathways and include fibroblast growth factor (FGF), bone morphogenic protein (BMP), Wingless-related integration site (WNT), and Sonic Hedgehog (SHH). Cell intrinsic factors are mainly transcription factors and can include but are not limited to *Foxg1, Gli3, Emx2, Gsx2, Pax6, Lhx2, Nkx2.1*, *Dlx1* and *Dlx2*.

ANR cells activate the Forkhead transcriptional repressor, *Foxg1* and also induce expression of *Emx1* and *Dlx2* transcription factors (Shimamura and Rubenstein 1997, Houart, Westerfield et al. 1998). Furthermore, expression of *Fgf* genes in the ANR is involved in inducing the tissue by mediating organizer activity (Shimamura and Rubenstein 1997). Another factor involved in telencephalic induction is WNT signalling. Inhibition of WNT is necessary to induce the telencephalon. A frizzled related WNT antagonist known as TLC is expressed in the ANR cells and antagonises with WNT signalling to induce the telencephalon (Houart, Caneparo et al. 2002). Figure 1. 4 summarizes factors involved in telencephalic induction.

1.1.3 **Regionalization and patterning of the telencephalon**

Shortly before the neural tube closes, dorsal and ventral midlines form in the telencephalon. After the neural tube folds, expression of transcription factors and signalling molecules from the dorsal and ventral regions guide the regionalization of the telencephalon.

During regionalization, the telencephalon is further divided into the dorsal pallial and ventral subpallial domains. Each telencephalic domain is further divided into several regions. The dorsal pallium primarily gives rise to the cerebral cortex. The hippocampus is located caudomedially in the pallium. The olfactory bulb located at the anterior point of the telencephalon is also in the dorsal domain. The ventral subpallium is mainly separated into four domains. The medial region forms the medial ganglionic eminences (MGE), the posterior-lateral region organizes into lateral ganglionic eminences (LGE) and caudal ganglionic eminences (CGE), and the ventral region forms the telencephalic stalk (Wilson and Rubenstein 2000). Figure 1.2 illustrates different regions in a coronal section of the developing forebrain.

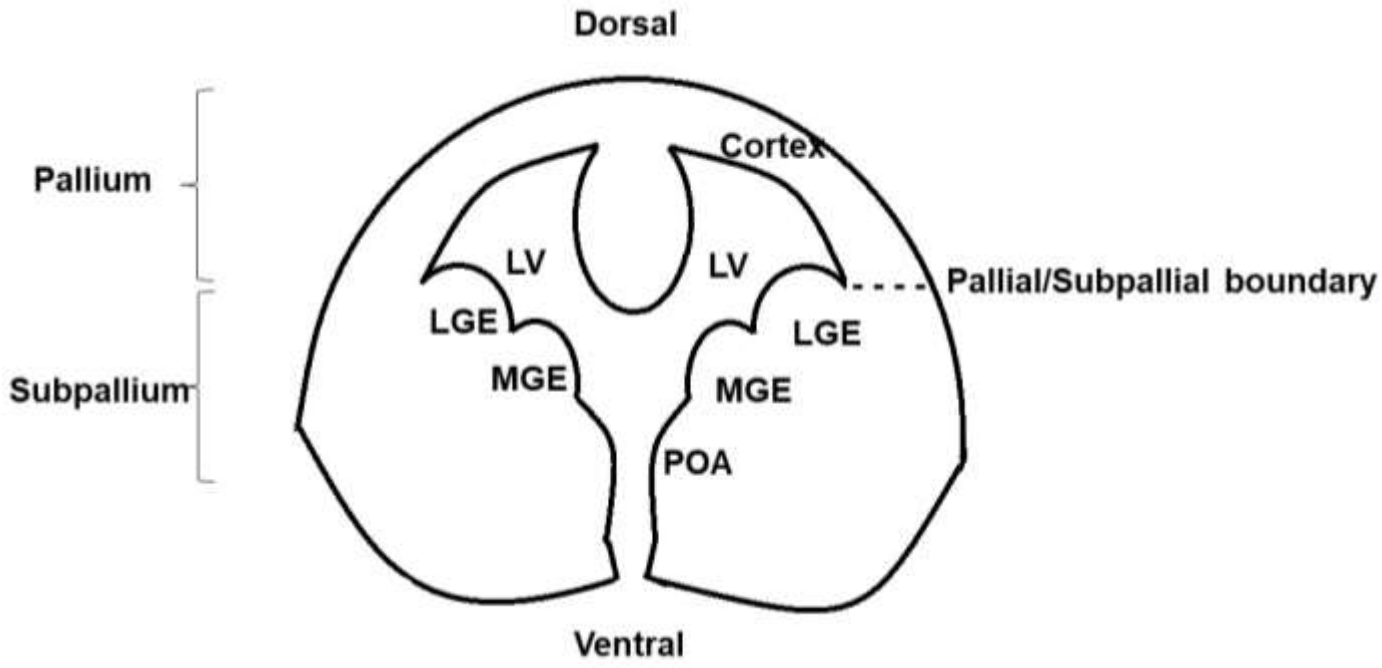


Figure 1.2. A schematic illustration of a coronal section of the developing forebrain.

The telencephalon is subdivided into the dorsal pallium and the ventral subpallium. Each telencephalic domain is further divided into several regions. The cerebral cortex is the main structure in the pallium. The ventral subpallium is mainly divided into ganglionic eminences and the telencephalic stalk. The ganglionic eminences are divided into lateral, medial and caudal sections called the LGE, MGE and CGE, respectively. The CGE is the posterior area of the ventral subpallium and is located caudally to the LGE and MGE and therefore is not shown in this figure. The border between the pallium and the LGE is known as the pallial/subpallial boundary. The telencephalic stalk is located next to the MGE and mainly includes the POA. MGE: Medial ganglionic eminences, LGE: lateral ganglionic eminences, CGE: caudal ganglionic eminences, POA: preoptic area.

Roof plate signaling molecules BMP and WNT are crucial for dorsal telencephalic development (Furuta, Piston et al. 1997, Monuki, Porter et al. 2001, Backman, Machon et al. 2005). SHH is secreted from the floor plate and induces ventral cell fates (Ericson, Muhr et al. 1995, Chiang, Litingtung et al. 1996). However, SHH signaling is not necessary for this process since mice lacking SHH do not exhibit severe telencephalic defects (Huh, Hatini et al. 1999, Sussel, Marin et al. 1999). Although the exact role of SHH in telencephalic patterning is yet to be determined, gain of function studies reveal SHH signaling is involved in inducing ventral telencephalic identity (Gaiano, Kohtz et al. 1999). Furthermore, ectopic expression of SHH in dorsal telencephalic cells of mice induces ventral telencephalic genes such as *Dlx2, Nkx2.1*, and *Gsx2* (formally known as *Gsh2*) (Gaiano, Kohtz et al. 1999, Corbin, Gaiano et al. 2000). *Gli3*, a Zinc Finger transcription factor with suppressor functions in SHH signalling, is expressed in the dorsal telencephalon and is involved in promoting dorsal cell types and repressing ventral fates. Mice lacking *Gli3* have defects in developing dorsal telencephalic structures as well as a loss of expression of many dorsal telencephalic markers (Theil, Alvarez-Bolado et al. 1999, Tole, Ragsdale et al. 2000). Interestingly, mice lacking both SHH and *Gli3* have nearly normal dorsoventral patterning indicating that SHH and *Gli3* antagonism is not the only mechanism involved in dorsoventral patterning in the telencephalon (Rallu, Machold et al. 2002). *Foxg1* plays an important role in generating ventral telencephalic progenitors since mice lacking *Foxg1* display an increase in the expression of dorsal telencephalic markers and a loss of ventral progenitor cells (Xuan, Baptista et al. 1995, Martynoga, Morrison et al. 2005). Furthermore, RNA interference in organoid cultures from individuals with autism spectrum disorders show that overexpression of *FOXG1* is partially responsible for the increase in the production of GABAergic neurons and is a developmental precursor of autism spectrum disorders, indicating

the important role of *FOXG1* in ventral telencephalic development (Mariani, Coppola et al. 2015). Figure 1.4 summarizes factors involved in dorsoventral regionalization of the telencephalon.

1.2 Forebrain germinal zones

The neural tube consists of a monolayer of neuroepithelial cells known as the neuroepithelium (Gotz and Huttner 2005). During neurogenesis, these neuroepithelial cells differentiate into radial glial cells (RGCs). Furthermore, the single layered neuroepithelium transforms into multiple layers forming the germinal zones where neuroepithelial cells and RGCs reside (Gotz and Huttner 2005, Kriegstein and Alvarez-Buylla 2009). The ventricular zone (VZ), located adjacent to the ventricle wall is the first germinal zone to emerge. The subventricular zone (SVZ) is then generated from the VZ (Wichterle, Garcia-Verdugo et al. 1999). The majority of neuronal subtypes are generated from the VZ and SVZ during CNS development (Gotz and Huttner 2005, Kriegstein and Alvarez-Buylla 2009). The SVZ also contains cells that proliferate in the SVZ at the expense of the VZ later in neural development. Expansion of the SVZ continues postnatally, resulting in the disappearance of the VZ in the adult mouse forebrain (Eisenstat, Liu et al. 1999, Tramontin, Garcia-Verdugo et al. 2003).

1.3 Neurogenesis

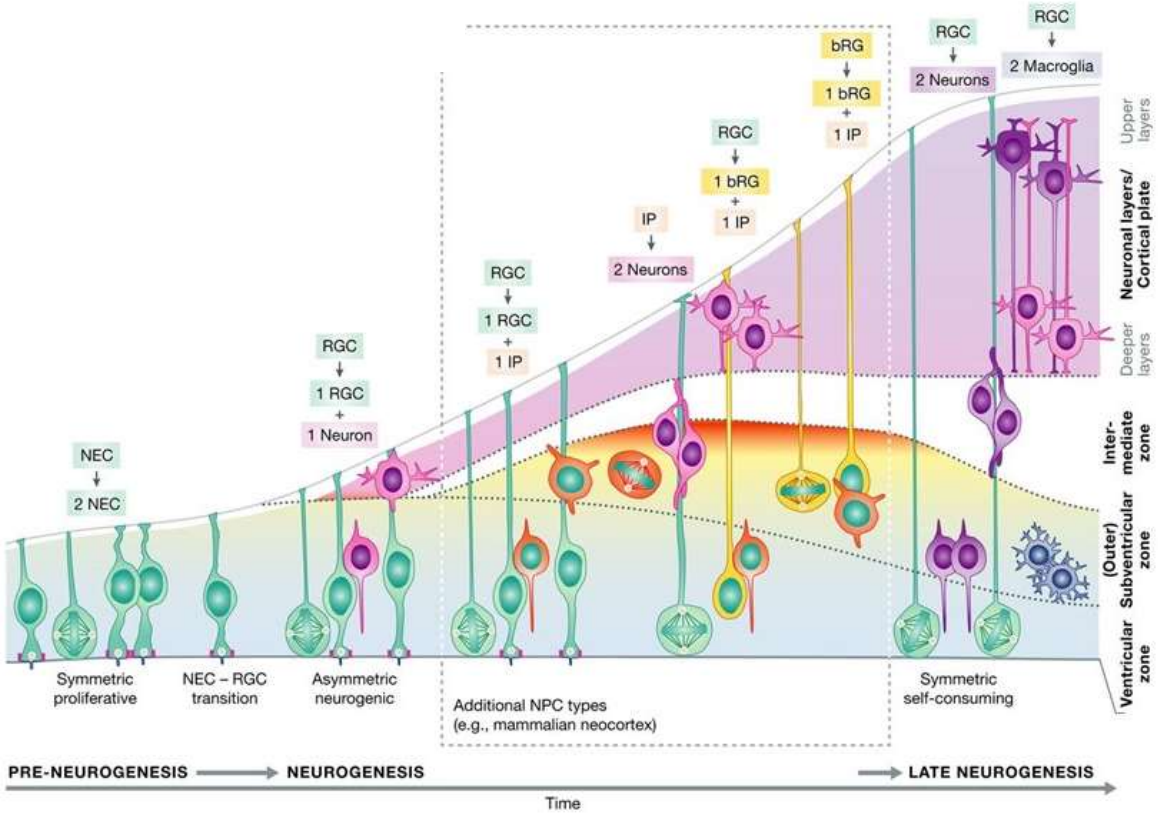
1.3.1 Symmetric and asymmetric cell divisions in progenitor cells of the forebrain

Early in brain development, neuroepithelial cells divide symmetrically to populate the VZ. At the onset of neurogenesis and as the neuroepithelium expands, neuroepithelial cells transition into RGCs which are elongated cells with their basal processes extending into the pial surface and their apical cilium reaching into the ventricular lumen (Tramontin, Garcia-Verdugo et al. 2003). RGCs generate neurons by dividing asymmetrically. Asymmetrical divisions in RGCs can also generate intermediate progenitor cells (IPC, also called basal progenitors) (Kriegstein and Alvarez-Buylla 2009). The IPCs are not polarized and lack an apical basal axis. IPC divisions are mainly symmetrical, producing IPCs that populate the SVZ (Haubensak, Attardo et al. 2004, Noctor, Martinez-Cerdeno et al. 2004). Compared to RGCs, the IPCs have a more restricted cell fate and generate neuronal or glial progenitors (Kriegstein and Alvarez-Buylla 2009). While the VZ consists mainly of neuroepithelial cells, RGCs and IPCs are predominantly in the SVZ (Kriegstein and Alvarez-Buylla 2009). Figure 1.3 A illustrates an overview of neurogenesis in progenitor cells of the forebrain.

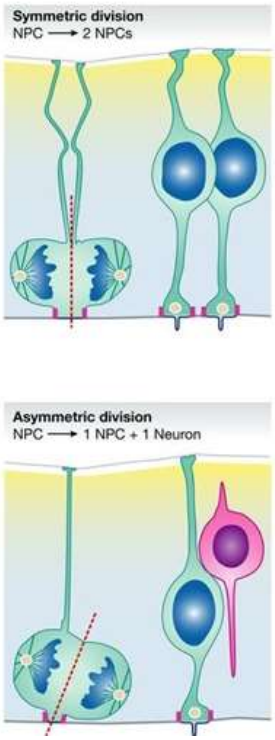
Cell divisions, especially those that occur during differentiation of neuronal progenitors found in the ventricular zone, can either be symmetrical or asymmetrical. Regulation of the cleavage plane of the cell is complex and includes intrinsic cell fate determinants as well as factors that control the orientation of the mitotic spindle (Dewey, Taylor et al. 2015, Matsuzaki and Shitamukai 2015). During symmetric cell division, the plasma membrane is cleaved on the vertical plane perpendicular to the ventricular surface in the apical-to-basal direction resulting in

an equal distribution of cell fate components within daughter cells (Gotz and Huttner 2005, Huttner and Kosodo 2005, Fietz and Huttner 2011). On the other hand, in asymmetric cell divisions, cells are cleaved horizontally parallel to the ventricular surface in invertebrates or oblique in vertebrates. This oblique or horizontal cleavage plane causes an unequal distribution of cell fate components between two daughter cells (Gotz and Huttner 2005, Huttner and Kosodo 2005, Fietz and Huttner 2011). Figure 1.3 B illustrates symmetric and asymmetric cell divisions.

A.


NEC
2 NEC
RGC
1 RGC
+
1 Neuron
RGC
1 RGC
+
1 IP
IP
2 Neurons
RGC
1 bRG
+
1 IP
bRG
1 bRG
+
1 IP
RGC
2 Neurons
RGC
2 Macroglia
Upper layers
Neuronal layers/ Cortical plate
Deeper layers
Inter- mediate zone
(Outer) Subventricular zone
Ventricular zone
Symmetric proliferative
NEC – RGC transition
Asymmetric neurogenic
Additional NPC types (e.g., mammalian neocortex)
Symmetric self-consuming
PRE-NEUROGENESIS
NEUROGENESIS
LATE NEUROGENESIS
Time

B.


Symmetric division
NPC → 2 NPCs
Asymmetric division
NPC → 1 NPC + 1 Neuron

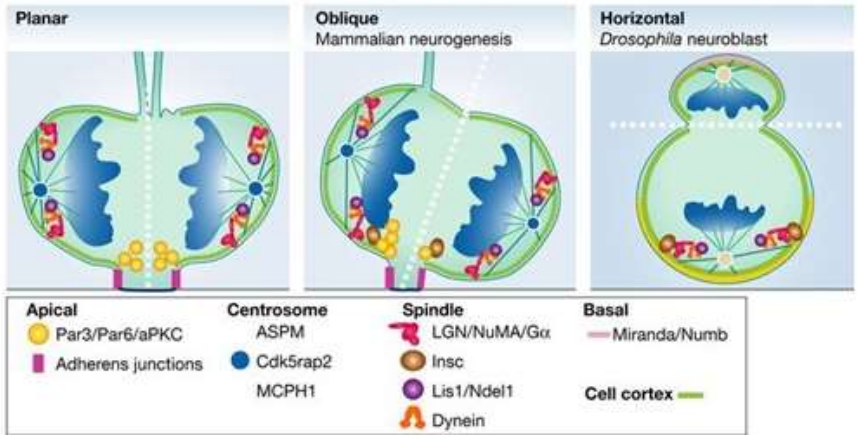

Planar
Oblique
Mammalian neurogenesis
Horizontal
Drosophila neuroblast
Apical
Par3/Par6/aPKC
Adherens junctions
Centrosome
ASPM
Cdk5rap2
MCPH1
Spindle
LGN/NuMA/Gα
Insc
Lis1/Ndel1
Dynein
Basal
Miranda/Numb
Cell cortex

Figure 1.3. Overview of neurogenesis and symmetric versus asymmetric cell division in progenitor cells of the forebrain.

A. An overview of neurogenesis in vertebrate brain development.

During early development of the vertebrate embryo, neuroepithial cells (NEC) divide symmetrically to populate the VZ. At the onset of neurogenesis, NEC transition into radial glial cells (RGCs) that will generate neuronal subtypes and glial cells. RGCs can produce neurons directly or via intermediate progenitor cells (IP).

B. Symmetric versus asymmetric cell division in progenitor cells of the forebrain.

During symmetrical cell division, cells are cleaved on the vertical plane perpendicular to the ventricular surface; therefore, cell fate determinants divide equally between the daughter cells, giving rise to two identical cells. During asymmetric division, cells are cleaved on the horizontal (in invertebrates) or oblique plane (in vertebrates), giving rise to a differentiating cell and a stem cell, respectively.

NEC: neural epithelial cell, RGC: radial glial cell; IP: intermediate progenitor cell, bRG: basal radial glial cell; NPC: neural progenitor cell.

Figure adapted from Paridaen et al. EMBO Rep. 2014. License Number 4390490435044.

1.3.2 **Specification of pallium/neocortex**

The pallium of the telencephalon contains the six layered neocortex, which develops in an "inside out manner" with neurons generated at later stages migrating to the most external region of the neocortex. Consequently, the earliest neurons to differentiate will migrate to layer VI (Gilmore and Herrup 1997).

Cortical neurogenesis in the mouse starts at embryonic days (E) 8-9, when the developing neocortex is one layer of proliferating neuroepithelial cells. At around E10 to E12, these neuroepithelial cells gradually convert into RGCs, which will generate neuronal subtypes that populate layer II to layer IV of the cortex (Gilmore and Herrup 1997). As mentioned previously, early in neurogenesis RGCs divide symmetrically to proliferate but further in neurogenesis, asymmetrical divisions generate IPCs (Noctor, Martinez-Cerdeno et al. 2004). The intermediate progenitors then divide to give rise to neurons. During gliogenesis, the majority of RGCs give rise to glial cells (Noctor, Martinez-Cerdeno et al. 2004). However, a number of RGCs undergo terminal differentiation to generate neurons (Haydar, Ang et al. 2003) and some are maintained as adult forebrain stem cells (Bonfanti and Peretto 2007) (Figure 1.3 B).

Several extrinsic and intrinsic factors are required during the transition of neuroepithelial stem cells to RGCs. First, Notch and FGF signaling play a crucial role in the transition from neuroepithelial stem cells to RGC. Expression of radial glial cell markers RC2, Nestin and brain-lipid-binding protein (BLBP) is upregulated in telencephalic vesicles overexpressing Notch (Gaiano, Nye et al. 2000). *Fgf10* is expressed between E9.5 and E11.5 and regulates the timely differentiation of RGCs from neuroepithelial stem cells. By regulating RGC differentiation, *Fgf10* controls the timing of neurogenesis, influences the number of neuronal cells produced in the germinal zones and ultimately determines the laminar patterning and thickness of the cortex

(Sahara and O'Leary 2009). Furthermore, *Sox1* is involved in maintaining neuroepithelial stem cell fate by repressing *Prox1* (Elkouris, Balaskas et al. 2011). Overexpression of *Sox1* promotes the neuroepithelial stem cell fate stage (Suter, Tirefort et al. 2009). Finally, *Pax6* is expressed in neuroepithelial cells and is involved in the differentiation of neuroepithelial cells into RGCs. Overexpression of *Pax6* in neuroepithelial cells promotes their transition to RGCs and down regulation of *Pax6* decreases the formation of RGCs (Suter, Tirefort et al. 2009). Other transcription factors involved in the specification and differentiation of RGCs and IPCs into cortical neurons include *Lhx2, Foxg1, Sp8, Emx2,* and *Couptf1* (Molyneaux, Arlotta et al. 2007, Borello and Pierani 2010). Figure 1.4 summarizes factors involved in specification of the pallium.

1.3.3 **Specification of the pallial-subpallial boundary**

The border between the pallium and the LGE is known as the pallial-subpallial boundary. Two transcription factors, *Pax6* and *Gsx2*, are important for the specification of the pallial-subpallial boundary (Corbin, Gaiano et al. 2000, Toresson, Potter et al. 2000, Cocas, Georgala et al. 2011). *Pax6* is expressed dorsally and *Gsx2* is expressed ventrally. *Gsx2* negatively regulates *Pax6* and at the same time activates *Mash1* (also known as *Ascl1*) and *Dlx* (Toresson, Potter et al. 2000, Wang, Waclaw et al. 2009). In mice lacking *Gsx2*, genes involved in dorsal specification including *Pax6, Ngn1*, and *Ngn2* are ventrally expanded and there is a signification loss of *Mash1* and *Dlx* in the lateral ganglionic eminences (Toresson, Potter et al. 2000, Stenman, Yu et al. 2003).

1.3.4 Specification of subpallium/ganglionic eminences

1.3.4.1 Lateral ganglionic eminence

The LGE is the area in the subpallium between the pallial-subpallial boundary and MGE. *Dlx1, Dlx2*, *Dlx5,* and *Dlx6* genes are expressed in the LGE and *Nkx2.1* is not expressed in this region (Szucsik, Witte et al. 1997, Sussel, Marin et al. 1999). *Gsx* genes are involved in specification of LGE progenitors (Pei, Wang et al. 2011). *Gsx2* regulates the expression of *Mash1*, *Dlx*, and *Sp8* (Corbin, Gaiano et al. 2000, Toresson, Potter et al. 2000, Waclaw, Wang et al. 2009, Wang, Waclaw et al. 2009). The LGE is involved in the development of telencephalic regions including striatum and olfactory bulb. *Dlx1, Dlx2, Dlx5, Dlx6* and *Isl1* are ventrally expressed transcription factors involved in the production of projection neurons that will populate the striatum. Dorsal progenitors express *Dlx1, 2, 5, 6* as well as *Er81* to generate interneurons that will reside in the olfactory bulb (Stenman, Yu et al. 2003). The LGE is also involved in the production of cortical and hippocampal interneurons (Pleasure, Anderson et al. 2000). Figure 1. 4 summarizes factors involved in specification of the LGE.

1.3.4.2 Medial ganglionic eminence

The MGE is located between the LGE and the telencephalic stalk. *Nkx2.1* is highly expressed in the MGE (Sussel, Marin et al. 1999, Flames, Pla et al. 2007). *Nkx2.1* is a crucial marker for MGE specification and represses LGE and CGE identity (Sussel, Marin et al. 1999, Butt, Sousa et al. 2008). Primarily FGF and SHH signaling induce *Nkx2.1* expression in the MGE progenitors (Fuccillo, Rallu et al. 2004, Xu, Wonders et al. 2005, Gutin, Fernandes et al. 2006). *Nkx2.1* regulates the expression of the LIM homeobox genes, *Lhx6* and *Lhx7*. Furthermore, loss of *Nkx2.1* in mice alters the cell fate of MGE progenitors towards an LGE/CGE identity (Sussel, Marin et al. 1999, Butt, Sousa et al. 2008). Similar to LGE, the MGE

is also involved in neuronal development of the striatum, septum, olfactory bulb, and hippocampal neurons (Letinic and Kostovic 1997, Pleasure, Anderson et al. 2000). Furthermore, the MGE is the main source of GABAergic interneurons (Butt, Fuccillo et al. 2005, Wonders and Anderson 2006). Figure 1.4 summarizes factors involved in specification of the MGE.

1.3.4.3 Caudal ganglionic eminence

The CGE is the posterior area of the ventral subpallium and is located caudally to the LGE and MGE (Nery, Fishell et al. 2002). Gene expression profiles revealed that CGE progenitors do not express specific genes, the anterior CGE domain expresses LGE and MGE genes and the posterior CGE domain expresses pallial genes (Flames, Pla et al. 2007). The CGE is involved in the production of neurons that populate the cortex, amygdala, and hippocampus (Nery, Fishell et al. 2002, Yozu, Tabata et al. 2005).

1.3.4.4 Telencephalic stalk

The telencephalic stalk is located next to the MGE and consists of the anterior entopeduncular region (AEP) and the preoptic area (POA). *Nkx2.1* is expressed in both the AEP and POA regions and is important for the specification of these two regions (Sussel, Marin et al. 1999). However, AEP and POA progenitors express different genes than the progenitors of the MGE (Flames, Pla et al. 2007). This region is involved in the production of GABAergic interneurons that populate the cortical layers (Xu, Cobos et al. 2004, Butt, Sousa et al. 2008).

1.3.5 Generation of cortical interneurons

The cortex mainly contains excitatory projection neurons that secrete glutamate and inhibitory interneurons that secrete gamma-aminobutyric acid (GABA). Interneurons comprise approximately 20% of murine neurons in the cortex (Tamamaki, Yanagawa et al. 2003) and play

a crucial role in functional balance between excitatory and inhibitory networks in the neuronal circuitry (Isaacson and Scanziani 2011).

Contrary to cortical glutamatergic projection neurons, the dorsal pallium is not the source of GABAergic interneurons (Fogarty, Grist et al. 2007). Studies have shown that interneurons are born in the subpallium and migrate past the pallial-subpallial boundary to populate the cortex through a specific migratory stream known as tangential migration (Anderson, Eisenstat et al. 1997, Marin and Rubenstein 2003). The majority of interneurons are generated from the MGE; however the LGE/CGE and the telencephalic stalk are also sources for interneuron production (Fogarty, Grist et al. 2007). Generation of interneurons in mice starts at E9.5 in the MGE and at E12.5 in LGE/CGE (Miyoshi, Butt et al. 2007, Miyoshi, Hjerling-Leffler et al. 2010). Another study has shown that approximately 10% of the interneurons arise from the embryonic POA (Gelman, Griveau et al. 2011). Regardless of the source of generation, GABAergic interneurons will then migrate tangentially to the neocortex and also radially to the striatum (Marin and Rubenstein 2003).

1.3.5.1 Specification of cortical GABAergic interneurons

The transcriptional network involved in the specification of GABAergic interneurons and their developmental regulation and migration is complex. To better understand candidate genes and transcription factors involved in the process, the specification of each GABAergic interneuron subtype is discussed separately.

1.3.5.1.1 Specification of MGE-derived GABAergic interneurons

Most of the GABAergic interneurons originate from the MGE. These interneurons express parvalbumin (PV) or somatostatin (SST). *Nkx2.1* is the main transcription factor for specification of both PV and SST-expressing progenitors in the MGE (Xu, Tam et al. 2008).

Deletion of the *Nkx2.1* homeobox transcription factor alters the fate of progenitor subtypes that arise from the MGE so that the cortical interneurons are re-specified and LGE/CGE-derived interneurons originate instead of MGE-derived interneurons (Butt, Sousa et al. 2008). The maintenance of *Nkx2.1* expression is regulated by SHH. Furthermore, the level of SHH signaling in the MGE also influences the expression of PV and SST. Increased SHH expression promotes the production of SST-expressing interneurons (Xu, Guo et al. 2010).

Lhx6 is the downstream target of *Nkx2.1* (Du, Xu et al. 2008). Studies have shown that in mice lacking *Lhx6* function, interneurons do not express PV or SST demonstrating Lhx6's role in determining the fate of PV and SST-expressing interneurons (Liodis, Denaxa et al. 2007). *Lhx8* is another transcription factor that is regulated by *Nkx2.1* and works together with *Lhx6*. Mutation in *Lhx8* does not affect MGE-derived interneuron production but mutation in both *Lhx6* and *Lhx8* results in deficiency in MGE-derived interneuron production and SHH expression (Flandin, Zhao et al. 2011). Of the six known *Dlx* transcription factors, *Dlx1, Dlx2, Dlx5* and *Dlx6* are involved in the specification of the GABAergic interneurons. Using RNA expression arrays, Long and his colleagues showed *Dlx1* and *Dlx2* regulate expression of various transcription factors including *Dlx1, Dlx2, Dlx5, Dlx6, Cux2, Arx, ER81, Pbx1, Sox4, Sox1, Lhx6, Lhx7,* and *Vax1* in progenitors of the MGE (Long, Cobos et al. 2009). Figure 1.4 summarizes factors involved in specification of the MGE.

1.3.5.1.2 Specification of LGE/CGE-derived GABAergic interneurons

The LGE/CGE regions contribute to 30-40% of interneuron generation in the cortex (Gelman et al. 2012). *Gsx1*/*Gsx2* are involved in specifying interneurons derived from the LGE/CGE region. Loss and gain of function studies of *Gsx2* demonstrate the role of *Gsx2* in the specification of calretinin-expressing GABAergic interneurons from the CGE region (Xu, Guo et

al. 2010). *Dlx* genes (*Dlx1, Dlx2, Dlx5* and *Dlx6*) are involved in the specification of interneurons generated from the LGE/CGE as well as the MGE. Studies on the *Dlx1/2* double knockout mouse using microarray analysis have identified many different transcription factors as downstream targets of *Dlx1/2* in the CGE including but not limited to *Arx, Brn4, Dlx5, Dlx6, Gbx1, Gsx1, Gsx2, Pbx1, Pbx3, Sp8, Sox4, Sox11, Mash1*, and *Olig2* (Long, Cobos et al. 2009). The transcription factors *Couptf1* and *Couptf2* are expressed in the CGE, and conditional loss of function studies in *Couptf1* have shown the change of marker expression without an alteration in the number of cortical interneurons, indicating its role in CGE interneuron specification and migration (Lodato, Tomassy et al. 2011). Figure 1. 4 summarizes factors involved in specification of the LGE.

1.4 Forebrain gliogenesis

Glial cells are a subtype of cells in the nervous system that provide support to neurons. Glial cells are involved in neuron myelination, protecting neurons, removing dead cells, isolating neurons from each other and maintaining homeostasis in the nervous system. In the CNS, glial cells consist of oligodendrocytes, astrocytes, microglia and ependymal cells. Glial cells are generated from the progenitor cells that were previously involved in neurogenesis during gliogenesis (Kessaris, Fogarty et al. 2006, Rowitch and Kriegstein 2010). Several extrinsic and intrinsic factors are involved in the cell fate switch from neurogenesis to gliogenesis. Proneural basic helix-loop-helix (bHLH) transcription factors involved in neurogenesis are downregulated in neuronal progenitors during gliogenesis (Bertrand, Castro et al. 2002, Rowitch and Kriegstein 2010). Notch signaling promotes gliogenesis by suppressing proneural bHLH factors and activating genes involved in the differentiation of astrocytes by activating the JAK–STAT signaling pathway (Kamakura, Oishi et al. 2004, Rowitch and Kriegstein 2010). On the other

hand, SHH signalling and *Olig2* expression promotes gliogenesis (Rowitch and Kriegstein 2010). Furthermore, BMP and SHH signaling have opposite effects during gliogenesis. While BMP induces astrocyte production and negatively regulate specification of oligodendrocytes, SHH promotes oligodendrocyte specification and inhibits astrogenesis (Kessaris, Pringle et al. 2008, Rowitch and Kriegstein 2010).

1.4.1 **Origin and specification of astrocytes**

Astrocytes are the most common glial cells in the nervous system and are mainly involved in maintaining synaptic homeostasis (Blackburn, Sargsyan et al. 2009). Astrocytes originate from the same RGCs that were initially involved in neurogenesis (Noctor, Martinez-Cerdeno et al. 2008). During early forebrain development, gliogenesis is inhibited by bHLH transcription factors *Ngn1, Ngn2 and Ascl1* (Sun, Nadal-Vicens et al. 2001, Parras, Hunt et al. 2007). These proneural bHLH transcription factors negatively regulate the activity of the JAK-STAT signalling pathway that is involved in activating astrocyte specific genes (Kessaris, Pringle et al. 2008). However, as *Ngn1/2* expression downregulates during neurogenesis, STAT1/3 suppression is released resulting in STAT-mediated activation of astrogenesis (Kessaris, Pringle et al. 2008). After specification, astrocytes migrate tangentially along the white matter tracts to populate the CNS (Rowitch and Kriegstein 2010).

1.4.2 **Origin and specification of oligodendrocytes**

Oligodendrocytes are a subtype of glial cells that are involved in producing myelin, a lipid-rich sheath that covers axons to protect and insulate neurons. There are three sources for oligodendrocyte generation with a different temporal sequence (Kessaris, Fogarty et al. 2006). Primarily, oligodendrocyte progenitor cells (OPCs) originate from the MGE and AEP in the

ventral subpallium (Kessaris, Fogarty et al. 2006, Kessaris, Pringle et al. 2008). VZ progenitor cells expressing *Nkx2.1* are the source of these OPCs. MGE and AEP generated OPCs then migrate to the cortex (Spassky, Goujet-Zalc et al. 1998, Tekki-Kessaris, Woodruff et al. 2001). Another source of OPCs, generated after MGE/AEP OPCs, are from the LGE/CGE and express *Gsx2*. The final source of OPCs is the cortex and these OPCs express *Emx1* (Kessaris, Fogarty et al. 2006).

Several transcription factors including helix-loop-helix, homeodomain, and high-mobility-group family members are involved in oligodendrocyte progenitor specification and different levels of oligodendrocyte differentiation and migration (Nicolay, Doucette et al. 2007, Emery 2010). Most of the knowledge about oligodendrocyte differentiation comes from studies in the embryonic spinal cord. OPCs initially arise from a ventral region on the neural tube called the motor neuron progenitor domain (pMN) (Fu, Qi et al. 2002). The pMN domain is specified by *Olig2* that in turn is induced by SHH. SHH controls the patterning of the ventral neural tube progenitor domains p2, pMN, and p3. pMN originally gives rise to motor neurons during neurogenesis but subsequently, by expressing *Olig2*, generates oligodendrocytes. SHH also induces *Nkx2.2* and *Irx3* in the ventral p3 and dorsal p2 progenitor domains, respectively (Briscoe, Sussel et al. 1999, Briscoe, Pierani et al. 2000). Both these transcription factors are involved in repressing *Olig2* to define the pMN domain.

Early in forebrain gliogenesis, SHH is important for the production of MGE/AEP OPCs (Spassky, Goujet-Zalc et al. 1998). SHH activates *Olig1* and *Olig2,* bHLH transcription factors involved in oligodendrocyte differentiation (Nery, Wichterle et al. 2001, Kessaris, Pringle et al. 2008). Mice lacking both *Olig1* and *Olig2* have a complete loss of oligodendrocytes (Zhou and Anderson 2002, Kessaris, Pringle et al. 2008). BMP signalling on the other hand, is involved in

suppressing oligodendrocyte differentiation. *Dlx1* and *Dlx2* are important for differentiation of GABAergic interneurons and act upstream of *Olig2* to inhibit oligodendrocyte differentiation. In mice lacking *Dlx1/2*, the number of *Olig2* expressing cells has increased significantly in the SVZ of the AEP and MGE as well as the neocortex and the CGE compared with WT. Furthermore, *Dlx1/2* DKO mice have higher expression of oligodendrocyte progenitor genes *platelet-derived growth factor receptor alpha* (*Pdgfra*) and *Sox10*, and also an increase in oligodendrocyte differentiating genes *Nkx2.2*, *Proteolipid protein* (*Plp)*, and *Myelin basic protein* (*Mbp)*. Transfection of slice cultures from E13.5 DKO's with DLX1 and DLX2 expression vectors resulted in a reduction in *Olig2* expression, indicating that DLX1/2 can negatively regulate *Olig2* expression (Petryniak, Potter et al. 2007). Figure 1.4 summarizes factors involved in gliogenesis.

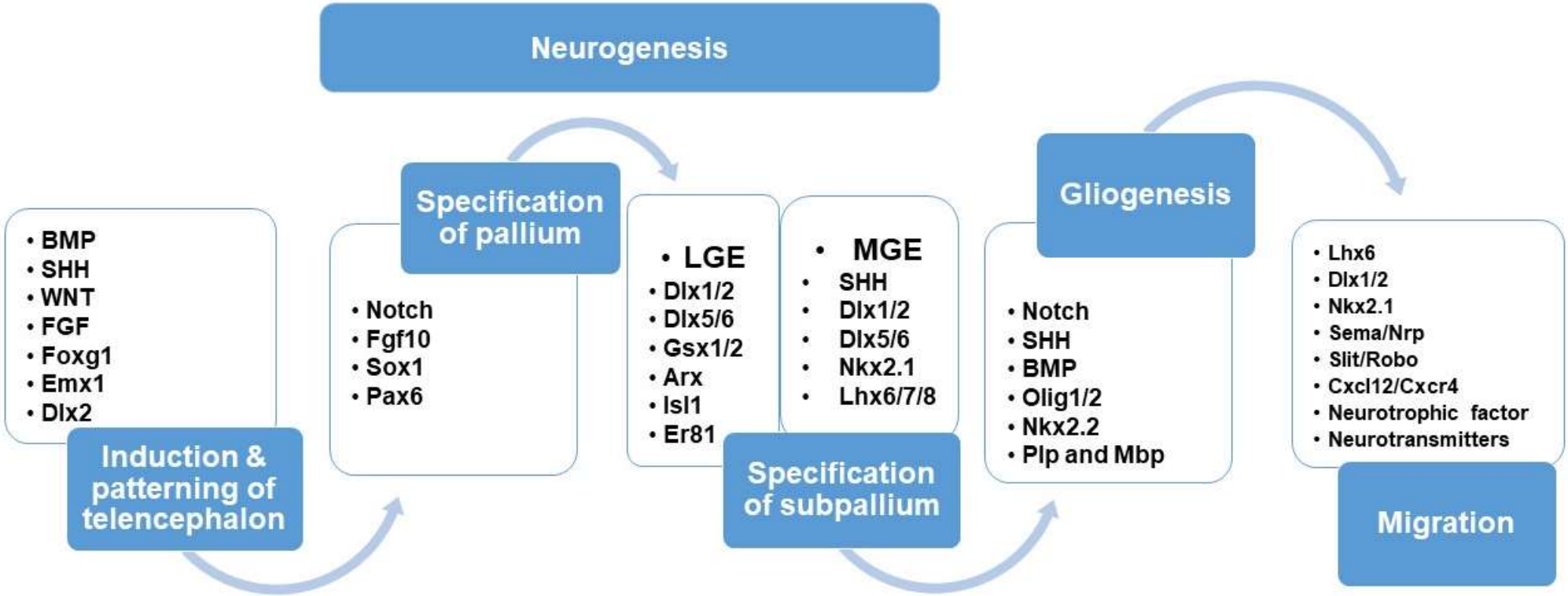


Figure 1.4. Summary of factors involved in brain development from telenchephalic induction to neural migration.

1.5 Neuronal migration

After originating from their sources, neurons and glial cells migrate to their final destination. There are two modes of migration in the CNS: radial migration and tangential migration.

1.5.1 Radial migration

Radial migration is mainly observed in the cortex where early generated neurons populate the deep layers of the six layered neocortex, while neurons born at later stages reside in more external layers (Angevine and Sidman 1961, Gilmore and Herrup 1997). Radial migration of cortical neurons is accomplished in two different ways: somal translocation and locomotion (Nadarajah, Brunstrom et al. 2001). Somal translocation is the main method of migration during early development (Nadarajah, Brunstrom et al. 2001). During somal translocation, neurons use their long radial process to move their cell body to the pial surface (Nadarajah, Brunstrom et al. 2001). Later in development, neurons utilize another method of migration known as locomotion. During locomotion, neurons attach to radial processes of RGCs and use them as a scaffold to reach their appropriate locations (Nadarajah and Parnavelas 2002). Radial migration is regulated by different factors. An extracellular matrix protein produced by Cajal-Retzius neurons in cortical layers, Reelin, is crucial for radial migration. In homozygous Reeler null mice, neurons are positioned reversibly and populate the cortical layers in the order they are generated; therefore earlier born neurons constitute the more superficial layers (D'Arcangelo, Miao et al. 1995, Frotscher 1998).

1.5.2 **Tangential migration**

Cortical interneurons are generated from the subpallium and migrate tangentially into the neocortex. Once interneurons arrive into the cortex they migrate radially to populate proper laminar positions (Anderson, Eisenstat et al. 1997, Marin and Rubenstein 2003, Metin, Baudoin et al. 2006, Huang 2009). There are two main tangential migration routes that lead interneuron progenitors to the neocortex. At E11.5, MGE-derived interneurons can migrate within the cortical marginal zone (MZ). Later in development a deep pathway in the intermediate zone (IZ) and SVZ is the migratory route (Anderson, Marin et al. 2001).

Figure 1.5 illustrates the radial and tangential migration pathways of developing neurons.

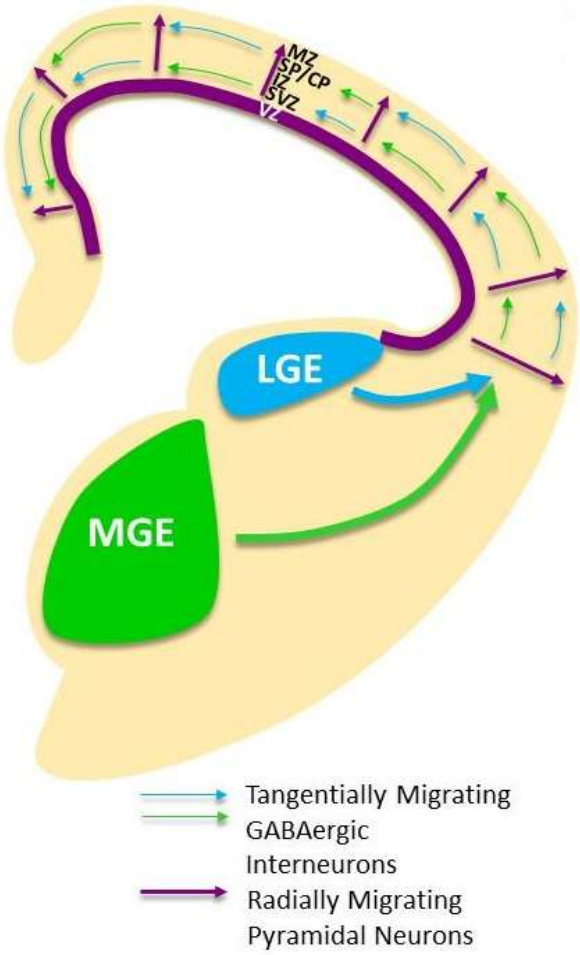


Figure 1.5. Schematic diagram illustrating migration pathways of the majority of glutamatergic neurons and GABAergic neurons.

Glutamatergic neurons originate from the VZ of the pallium and migrate radially into the developing cerebral cortex (purple arrows). The majority of GABAergic interneurons migrate tangentially into the cortical plate from the MGE (green arrow) and LGE (blue arrow) in the ventral telencephalon.

Figure adapted from Delatour and Yeh, OBM Neurobiology. 2017. No permission required.

1.5.2.1 Factors involved in tangential migration of GABAergic interneurons

Tangential migration of GABAergic interneurons is complex and involves the activity of several factors including transcription factors, motogens and chemotactic factors. Loss of function studies for *Lhx6* using RNA interference (RNAi) showed a role for this gene in migration as well as specification in GABAergic interneurons (Alifragis, Liapi et al. 2004). *Nkx2.1* is also involved in migration of GABAergic interneurons. Negative regulation of *Nkx2.1* is essential for the migration of GABAergic interneurons, and the ectopic expression of *Nkx2.1* in migrating interneurons interferes with the repulsive cues of Semaphorin 3A and 3F (Nobrega-Pereira, Kessaris et al. 2008). The DLX transcription factors, especially DLX1 and DLX2 are also involved in GABAergic interneuron migration. There is a loss of tangential migration of GABAergic interneurons from the GE to the neocortex in mice lacking *Dlx1/2* (Anderson, Eisenstat et al. 1997, Anderson, Qiu et al. 1997, Marin and Rubenstein 2001).

Motogens are substances that stimulate migration. Neurotrophic factors are crucial for tangential migration of MGE originated interneurons. Brain-derived neurotrophic factor (BDNF) and neurotrophin 4 both contribute to tangential migration using TrkB signalling (Polleux, Whitford et al. 2002). Furthermore, the glial cell line-derived neurotrophic factor (GDNF) uses GFRα1 (GDNF family receptor α 1) receptor to promote tangential migration of interneurons (Pozas and Ibanez 2005). Tyrosine kinase receptors, TrkB and TrkC, are known motogens for GABAergic interneurons (Powell, Mars et al. 2001). Neurotransmitters can also influence tangential migration. Recent studies indicate that the neurotransmitters GABA and dopamine are involved in the migration of GABAergic interneurons to the cortex (Cuzon Carlson and Yeh 2011). Migrating cortical interneurons express the dopamine receptors D1 and D2, and knockout

studies revealed an opposite functions for these receptors. While D1 promotes migration of these interneurons, D2 has an inhibitory role in migration (Crandall, McCarthy et al. 2007).

Chemotactic factors provide cells with information regarding the direction of migration. There are two types of chemotactic factors, chemorepellants and chemoattractants, involved in guiding interneurons through migratory routes to enter the neocortex. Chemorepellants are located in the ventral telencephalon and guide cortical interneurons away from the subpallium. Semaphorins are chemorepellants that are expressed in the LGE and enforce their repulsive cues by interacting with Neuropilins (NRP1 and NRP2,) receptors expressed on the interneurons, guiding them away from the LGE to the cortex (Marin, Yaron et al. 2001, Le, Du et al. 2007). Slit1 is a second chemorepellant which guides the interneurons away from the VZ and SVZ of the GE where it is expressed, by binding to its receptor Roundabout (Robo1), expressed on cortical interneurons (Andrews, Barber et al. 2007).

On the other hand, chemoattractants are expressed in the dorsal pallium, where they guide the interneurons towards the cortical layers. The most characterized chemoattractive molecule is the chemokine CXCL12 that interacts with its receptors CXCR4 and CXCR7, expressed on MGE-derived interneurons (Sanchez-Alcaniz, Haege et al. 2011). These chemotactic factors are essential for shaping the tangential migratory routes. In the absence of *Cxcl12*, there is an irregular migration of interneurons where interneurons are located in the MZ but absent from the SVZ and IZ. Furthermore, similar results are reported in *Cxcr4* null mice where interneurons lack proper migratory streams and are dispersed throughout the neocortex. Therefore, CXCL12/ CXCR4 signalling is important for interneuron SVZ/IZ migratory routes (Tiveron, Rossel et al. 2006). Figure 1. 4 summarizes factors involved in neural migration.

1.6 Molecular basis of tissue-specific gene regulation

Cell fates are established through transcription factors that bind to DNA and regulate patterns of gene expression (Fulton, Sundararajan et al. 2009). Transcription factors bind to one or more specific binding sequences with over 1000 fold preference known as transcription factor binding sites. Transcription factor binding sites are normally between 6 to 12 base pairs, consequently, there will be several binding sites for transcription factors on each gene (Wunderlich and Mirny 2009, Geertz, Rockel et al. 2012).

1.6.1 Transcription factor binding and regulatory regions

Transcription factors regulate gene expression by interacting with *cis* regulatory regions of target genes. *Cis* regulatory regions are located throughout the genome. Proximal promoter regions contain *cis* regulatory elements located near the transcription start sites (TSSs). Other *cis* regulatory elements such as enhancers, silencers and insulators act more distally from TSSs. A promoter is defined as a region upstream of the TSS that is involved in the initiation of transcription (Lenhard, Sandelin et al. 2012). A core promoter is the minimal component of the promoter required to initiate transcription. Core promoters include the TSS, RNA polymerase binding site and general transcription factor binding sites (Juven-Gershon, Hsu et al. 2008). The proximal promoter includes the core promoter and is located upstream and proximal to the TSS. The proximal promoter binds to tissues-specific transcription factors and further assists binding of RNA polymerase to initiate transcription (Lenhard, Sandelin et al. 2012).

Enhancers and silencers are regulatory regions that can be located thousands of base pairs (bp) upstream or downstream away from the TSS. Upon binding of transcription factors these enhancer and silencer elements can activate or repress tissue-specific gene expression (Spitz and Furlong 2012).

1.7 DLX transcription factors

My thesis is focused on the role of *Dlx* genes in regulating forebrain development. DLX proteins are homeodomain transcription factors with important roles in the development of the CNS, retina, craniofacial structures and limbs (Panganiban and Rubenstein 2002). *Dlx* genes encode a 60 amino acid homeodomain transcription factor that binds to the promoter region of target genes through the ATTA/TAAT tetra-nucleotide motifs and promotes transcriptional repression or activation of target gene expression (Wigle and Eisenstat 2008). The *Distal-less (Dll)* gene is the orthologue of *Dlx* genes in Drosophila and is required for distal limb development. *Dll* is crucial for Drosophila development since *Dll* null flies lack specific embryonic peripheral sense organs and do not survive (Cohen and Jurgens 1989). *Dll* is also important in development of the proximodistal axis and antenna identity (Cohen and Jurgens 1989). Although *Dll* is expressed in the optic lobe of Drosophila (Kaphingst and Kunes 1994), its role in CNS development is not well understood. However, *Dll* is required for peripheral nervous system development (Cohen and Jurgens 1989).

1.7.1 Sequence and structure of *Dlx* genes in vertebrates

In both mice and humans, six *Dlx* genes are present; *Dlx1, Dlx2, Dlx3, Dlx4, Dlx5* and *Dlx6*. These genes are arranged in bigenic clusters with *Dlx1* and *Dlx2*, *Dlx3* and *Dlx4*, *Dlx5* and *Dlx6* being organized in pairs. Each cluster is transcribed divergently and is linked to a *Hox* locus (Figure 1.6) (Panganiban and Rubenstein 2002). *Dlx1* and *Dlx2* are associated with *HoxD*, *Dlx3* and *Dlx4* are associated with *HoxB*, and *Dlx5* and *Dlx6* are associated with *HoxA* (McGuinness, Porteus et al. 1996, Nakamura, Stock et al. 1996, Stock, Ellies et al. 1996). Studies indicate that the present structure of the mammalian *Dlx* genes is a result of duplication of a

lineal *Dlx* gene and a loss of a *Dlx* pair that was linked to *HoxC* (Ellies, Stock et al. 1997, Neidert, Virupannavar et al. 2001).

Each vertebrate *Dlx* gene has three exons and two introns (McGuinness, Porteus et al. 1996, Ellies, Stock et al. 1997). The homeobox is encoded in exons 2 and 3 (Figure 1.6). The intergenic regions located between each pair contain *cis* regulatory elements. In Drosophila *Dll* there are 7 exons and the homeodomain is encoded centrally between exons 3 and 4 (Vachon, Cohen et al. 1992, Ellies, Stock et al. 1997).

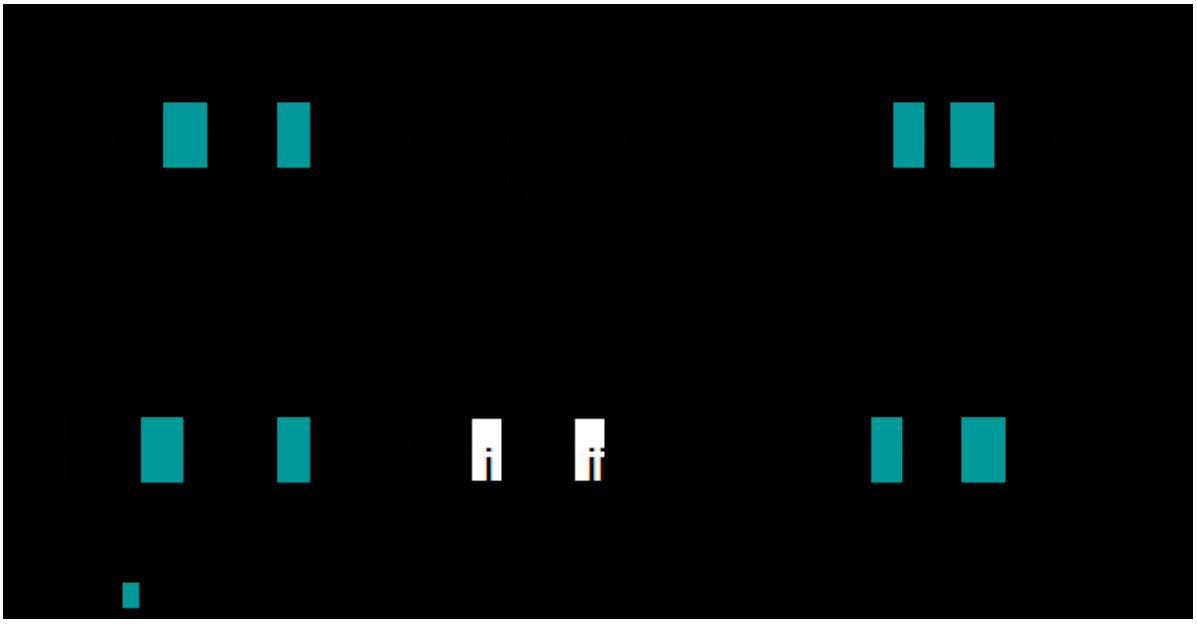


Figure 1.6. *Dlx1/Dlx2* and *Dlx5/Dlx6* bigenic clusters in mice.

Dlx1/Dlx2 are located on chromosome 2 and *Dlx5/Dlx6* are located on chromosome 6. *Dlx* genes are positioned in bigenic clusters with each *Dlx* pair facing one another and transcribed divergently. The homeodomain (blue boxes) is encoded in exons 2 and 3. There are enhancer elements located in the *Dlx1/Dlx2* and *Dlx5/Dlx6* intergenic regions.

Figure adapted from Zhou et al. NAR. 2004. License Number 4283890575885.

1.7.2 **Regulation of *Dlx* genes**

Several transcription factors, signaling pathways, and *cis* regulatory elements that regulate *Dlx* expression have been elucidated. SHH signalling can induce *Dlx* expression in forebrain, since expression of *Dlx2* in the forebrain remarkably decreases in SHH mutant mice (Gaiano, Kohtz et al. 1999, Rallu, Machold et al. 2002). BMPs are reported as inducers of *Dlx2* in various developing tissues (Bei and Maas 1998, Miyama, Yamada et al. 1999, Xu, Harris et al. 2001). FGFs are also positive regulators of *Dlx* expression. FGF2 can induce *DLX5* expression in the developing limb in chick (Ferrari, Harrington et al. 1999) and FGF8 activates *Dlx1* and *Dlx2* expression in dental mesenchyme in mice (Bei and Maas 1998). Retinoids are negative regulators of *Dlx* expression. In the presence of excess retinoic acid, zebrafish embryos have reduced *dlx* gene expression in ectomesenchymal cells (Ellies, Langille et al. 1997).

Cis-acting elements are present in each pair of *Dlx* genes. There is an enhancer regulatory region between the intergenic region of the *Dlx5* and *Dlx6* that is highly conserved between zebrafish and mouse (Zerucha, Stuhmer et al. 2000). *Dlx1* and *Dlx2* are regulators of this enhancer (Zerucha, Stuhmer et al. 2000, Stuhmer, Anderson et al. 2002, Zhou, Le et al. 2004). Similarly, intergenic regions of mouse *Dlx1* and *Dlx2* as well as *Dlx3* and *Dlx4* also have *cis* regulatory elements (Sumiyama, Irvine et al. 2002).

1.7.3 **Patterns of expression of *Dlx* genes**

Early in development, all six mouse *Dlx* genes are expressed in the ectoderm and the nervous system arising from the ectoderm. During brain development *Dlx1, Dlx2, Dlx5* and *Dlx6* are expressed (Price, Lemaistre et al. 1991, Bulfone, Kim et al. 1993, Simeone, Acampora et al. 1994, Eisenstat, Liu et al. 1999). In CNS development these genes are highly expressed in the

diencephalon and telencephalon (Fernandez, Pieau et al. 1998, Puelles, Kuwana et al. 2000, Zerucha and Ekker 2000, Neidert, Virupannavar et al. 2001).

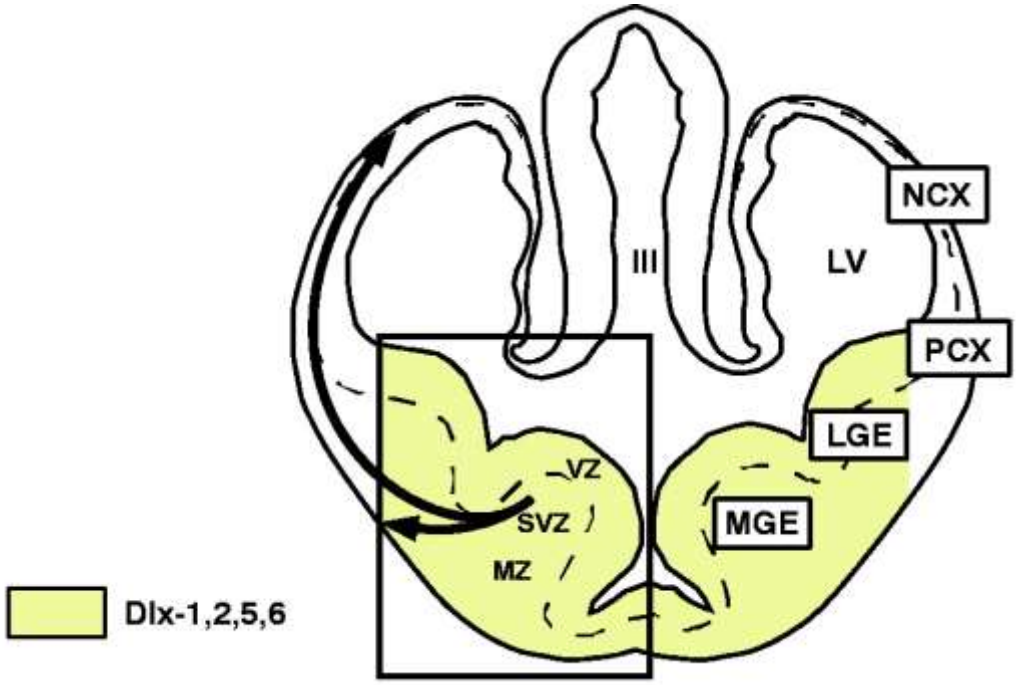
Ectomesenchymal cells express all six *Dlx* genes (Robinson, Wray et al. 1991, Bulfone, Kim et al. 1993, Neidert, Virupannavar et al. 2001). The ectomesenchymal cells are generated from the cranial neural crest and populate the branchial arches, to form facial skeleton and connective tissue (Depew, Lufkin et al. 2002). *Dlx* genes are expressed in a temporal/spatial sequence in the branchial arches. *Dlx1* and *Dlx2* are expressed in proximal regions, *Dlx1, Dlx2, Dlx5* and *Dlx6* are expressed in intermediate regions, and all six *Dlx* genes are expressed in distal regions (Qiu, Bulfone et al. 1997, Acampora, Merlo et al. 1999). *Dlx* genes are also expressed in peripheral and enteric nervous systems that are also derived from neural crest cells (Qiu, Bulfone et al. 1995, Depew, Liu et al. 1999). *Dlx1* and *Dlx2* are also expressed in neuronal progenitor cells of the retina (Eisenstat, Liu et al. 1999, de Melo, Qiu et al. 2003).

At later developmental stages, *Dlx* genes are expressed in differentiating skeletal tissues and ectodermal and mesenchymal segments of developing teeth (Depew, Lufkin et al. 2002). Specifically, *Dlx5* and *Dlx6* are highly expressed in skeletal tissues derived from the mesoderm as well as neural crest (Zhao, Zhao et al. 1994, Yang, Zhang et al. 1998, Acampora, Merlo et al. 1999). *Dlx4* is expressed in hematopoietic cells and is involved in proliferation and survival of these cells (Shimamoto, Ohyashiki et al. 2000).

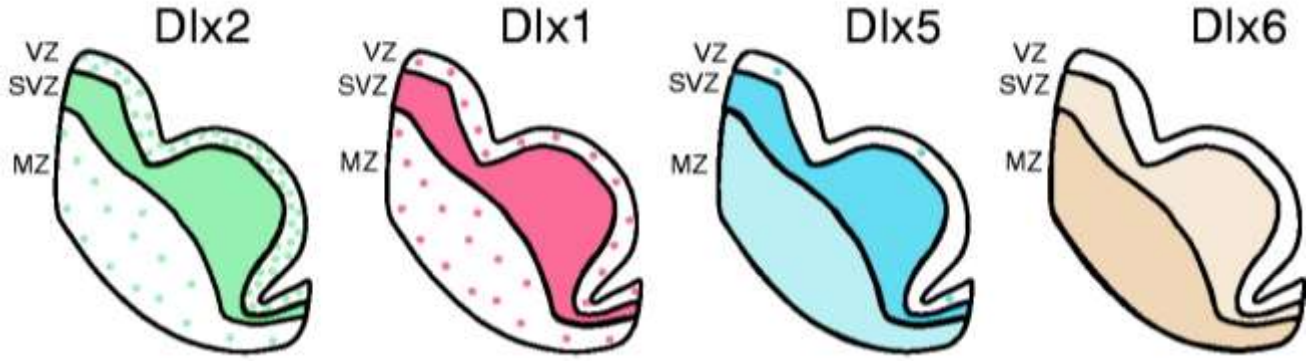
During brain development, expression of *Dlx1*, *Dlx2, Dlx5* and *Dlx6* follows a temporal sequence. *Dlx2* is expressed first, *Dlx1* is expressed after *Dlx2, Dlx5* follows *Dlx1* and *Dlx6* is expressed the latest (Eisenstat, Liu et al. 1999, Zerucha and Ekker 2000). *Dlx1/Dlx2* are expressed in the VZ and SVZ, *Dlx5* is localized in the SVZ and MZ and *Dlx6* is mainly

expressed in the MZ (Wigle and Eisenstat 2008). The patterns of expression of *Dlx* genes during brain development are illustrated in Figure 1.7.

A.



B.



C.

Model:

Dlx2 → Dlx1 → Dlx5 → Dlx6

Figure 1.7. Patterns of expression of *Dlx* genes during murine brain development.

A. Schematic illustration of a coronal section of the E12.5 mouse telencephalon showing the collective expression of *Dlx* genes (yellow region). The majority of progenitors in the subpallium express *Dlx1, Dlx2, Dlx5* or *Dlx6* during differentiation. The arrows show the migration of neurons from the subpallium to the neocortex.

B. Patterns of expression of *Dlx1, Dlx2, Dlx5* or *Dlx6* within the germinal zones. *Dlx2* is mainly expressed in the SVZ (uniform green) and VZ (green dots), and also in scattered cells in the MZ (green dots). *Dlx1* is mainly expressed in the SVZ (uniform pink) and also in scattered cells in the MZ and VZ (pink dots). *Dlx5* is mainly expressed in the SVZ and MZ (uniform blue). *Dlx6* is mainly expressed in the MZ (uniform peach).

C. Temporal expression of *Dlx* genes in brain development. There is a sequential expression of *Dlx2, Dlx1, Dlx5* and *Dlx6* during development.

NCX: neocortex, PCX: palliocortex, LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence, VZ ventricular zone, SVZ: subventricular zone, MZ: marginal zone, LV: lateral ventricle, III: third ventricle.

Figure adapted from Panganiban and Rubenstein, Development 2002, Order License Id: 4284390122027.

1.7.4 *Dlx* gene function during development

Loss of function studies mainly conducted in mice have elucidated the roles of *Dlx* genes during vertebrate development. Homozygous mutations for each individual *Dlx* are embryonically lethal revealing the importance of these genes during development. Each of the *Dlx* genes have both unique and redundant functions. Mice with single mutations of *Dlx* genes show very minor anomalies in phenotypes due to the fact that other *Dlx* genes can compensate for the loss of function to some extent (Anderson, Eisenstat et al. 1997, Qiu, Bulfone et al. 1997, Acampora, Merlo et al. 1999). Severe phenotypes are mostly observed when at least two *Dlx* genes are deleted (Qiu, Bulfone et al. 1997, Robledo, Rajan et al. 2002). *Dlx1/Dlx2* mutant mice do not survive after birth and have a loss of tangential migration of GABAergic interneurons from the ganglionic eminences to the neocortex as well as inhibition in striatal neurogenesis (Anderson, Eisenstat et al. 1997, Anderson, Qiu et al. 1997, Liu, Ghattas et al. 1997).

Dlx genes are involved in development of placodes in the dorsal midline of the neural tube and the neural crest (Depew, Liu et al. 1999). *Dlx3* is expressed in the surface ectoderm where it is involved in development of hair, teeth and the craniofacial skeleton. Deletion in the coding region of *Dlx3* just downstream of the *Dlx3* homeobox causes tricho-dento-osseous (TDO) syndrome (Price, Bowden et al. 1998) that affects morphogenesis of hair, teeth and the craniofacial skeleton (Lichtenstein, Warson et al. 1972). *Dlx* genes have an important role in craniofacial development since deletion of *Dlx5/6* genes results in severe deformities in jawbones, palate, and middle ear (Depew, Lufkin et al. 2002). Furthermore, *Dlx* genes are expressed in the ectomesenchyme and surface ectoderm where they are involved in tooth development (Thomas, Porteus et al. 1995). *Dlx1/2* double knockout mice lack upper molars (Qiu, Bulfone et al. 1997, Thomas, Tucker et al. 1997).

Dlx5 and *Dlx6* are involved in cartilage formation and bone development. Although *Dlx5* and *Dlx6* single mutants present mild anomalies in the limb skeleton (Acampora, Merlo et al. 1999, Depew, Liu et al. 1999), DKO mice for *Dlx5* and *Dlx6* have a severe axial skeleton malformation with kinked tail (Robledo, Rajan et al. 2002). Collagen 1A1 and osteocalcin are downstream targets of *Dlx* genes that are important for skeletal development (Dodig, Kronenberg et al. 1996, Ryoo, Hoffmann et al. 1997, Xu, Harris et al. 2001). Furthermore, *Dlx5* and *Dlx6* are expressed in the developing inner ear. In single *Dlx5* mutant mice, inner ear formation is close to normal and only anomalies in the vestibule are observed (Depew, Liu et al. 1999). Double knock out mice for *Dlx5* and *Dlx6* on the other hand, have severe malformations throughout the ear (Robledo, Rajan et al. 2002).

During vertebrate development, all *Dlx* genes are expressed in the limb bud. Single *Dlx1, Dlx2*, *Dlx5* and *Dlx6* mutations and double *Dlx1/Dlx2* mutations does not alter limb development (Qiu, Bulfone et al. 1997, Acampora, Merlo et al. 1999, Depew, Liu et al. 1999). On the other hand*, Dlx5/Dlx6* mutants have severe defects in the distal limb (Robledo, Rajan et al. 2002). Furthermore, *Dlx5/Dlx6* mutants have split hand and foot defects, which is similarly observed in ectodactyly syndromes in humans. Mutations in the *SHFM1* locus in humans is closely linked to *Dlx5* and *Dlx6* genes on the long arm of chromosome 7 (7q) and can cause Split Hand/Split Foot Malformation (Crackower, Scherer et al. 1996).

1.7.5 *Dlx* genes in brain development

Dlx1, Dlx2, Dlx5 and Dlx6 are expressed in overlapping patterns in the developing ventral forebrain (Liu, Ghattas et al. 1997, Eisenstat, Liu et al. 1999). *Dlx1* and *Dlx2* are expressed in the VZ and SVZ, *Dlx5* is expressed in the SVZ and MZ and *Dlx6* is mainly expressed in post mitotic cells of the MZ (Figure 1.7 B) (Liu, Ghattas et al. 1997).

Single KO of *Dlx1*, *Dlx2* and *Dlx5* and DKO of *Dlx1/Dlx2* and *Dlx5/Dlx6* have been produced and phenotypically analyzed. While all the heterozygotes are normal and viable, both the homozygous single KO and DKO mice die just after birth. *Dlx* single mutation in mice results in minor defects in forebrain development (Acampora, Merlo et al. 1999, Depew, Liu et al. 1999, Eisenstat, Liu et al. 1999), whereas double mutants exhibit more serious anomalies. The *Dlx1/Dlx2* double mutants show severe defects in neurogenesis of the SVZ in the telencephalon (Anderson, Qiu et al. 1997, Marin, Anderson et al. 2000). In mice lacking *Dlx1/ Dlx2*, primary proliferative population cells generated from the VZ around E10-E12 are normal but differentiation of the secondary proliferative population in the SVZ is lost (Anderson, Eisenstat et al. 1997, Anderson, Qiu et al. 1997). The blockage of neurogenesis caused by the lack of *Dlx1* and *Dlx2* reduces the production of dopaminergic and cholinergic interneurons and late born basal ganglia GABAergic neurons (Anderson, Eisenstat et al. 1997, Marin, Anderson et al. 2000, Pleasure, Anderson et al. 2000, Anderson, Marin et al. 2001).

The expression of *Dlx* is observed in the majority of interneurons secreting GABA as their neurotransmitter revealing the critical role of these genes in GABAergic interneuron differentiation (Anderson, Eisenstat et al. 1997, Anderson, Qiu et al. 1997, Stuhmer, Anderson et al. 2002). Most GABAergic interneurons are generated from the subpallium and migrate tangentially to populate the cortex. Double knockout mice for *Dlx1/Dlx2* have a complete loss of tangential migration of GABAergic interneurons from the subpallium into the cortex resulting in a decrease in the number of inhibitory interneurons in the cortex, hippocampus and olfactory bulb (Anderson, Eisenstat et al. 1997, Anderson, Qiu et al. 1997, Pleasure, Anderson et al. 2000, Marin and Rubenstein 2001). As mentioned previously, two main subcortical telencephalic sources of the GABAergic interneurons are the MGE and LGE/CGE. MGE derived interneurons

migrate laterodorsally to the cerebral cortex and striatum while LGE originated interneurons migrate mediodorsally to the olfactory bulb and the cerebral cortex (de Carlos, Lopez-Mascaraque et al. 1996, Anderson, Mione et al. 1999). Both migratory routes are impaired in the *Dlx1/Dlx2* double mutants resulting in a reduction in the numbers of interneurons in the striatum, olfactory bulb and cortex (Anderson, Mione et al. 1999, Marin, Anderson et al. 2000). The loss of tangential migration in the DKO may be the result of the loss of DLX mediated repression of Semaphorin receptor, Neuropilin 2. In the mutants the increased amount of *Nrp2* results in responsiveness of interneurons to Semaphorin signaling and partial blockage of tangential migration to the neocortex (Marin, Yaron et al. 2001, Le, Du et al. 2007).

Dlx genes also have an important role in GABA synthesis in the developing forebrain. The *Gad1 and Gad2* genes encode GAD67 and GAD65 enzymes, respectively. GAD67 and GAD65 are responsible for converting L-glutamic acid to GABA (Stuhmer, Anderson et al. 2002). In the Eisenstat lab, it has been shown that *Dlx1* and *Dlx2* bind to the promoter regions of *Gad1* and *Gad2*, and loss of *Dlx1* and *Dlx2* results in a decrease in the expression of GAD65 and GAD67 in the subpallium (Le, Zhou et al. 2017).

Dlx genes have potential roles in neuropsychiatric disorders. Reduction of GABAergic interneurons can cause seizures (Cobos, Calcagnotto et al. 2005). Furthermore, GABAergic abnormalities in the telencephalon can lead to learning impairment (Rubenstein and Merzenich 2003). Moreover, lack of *Dlx2* in the diencephalon results in abnormal activity in the thalamus and the hypothalamic-pituitary circuit (Rubenstein and Rakic 1999).

1.7.6 **Downstream targets of *Dlx* genes**

Towards understanding *Dlx* gene function, several *Dlx* downstream targets have been identified. During telencephalic development, DLX2 regulates *Wnt1* by directly interacting with

a homeodomain binding sites known as HBS-1 within the enhancer region of *Wnt1* (Iler, Rowitch et al. 1995). *Dlx1* and *Dlx2* are upstream regulators of the *Arx* homeobox gene during brain development (Cobos, Broccoli et al. 2005). During retinal development, *TrkB* is a DLX2 transcriptional target, partially explaining retinal ganglion cell apoptosis in the *Dlx1/2* DKO (de Melo, Zhou et al. 2008). Also in the Eisenstat laboratory, *Dlx1* and *Dlx2* have been identified as direct activators of *Gad1* and *Gad2* (Le, Zhou et al. 2017). Furthermore, *Dlx1* and *Dlx2* both bind directly to the *Nrp2* promoter and are involved in facilitating the tangential migration of interneurons from the basal forebrain (Le, Du et al. 2007). *Olig2*, an oligodendrocyte differentiation marker, is also repressed by *Dlx2* but the mechanism has not been determined (Petryniak, Potter et al. 2007). *Dlx* genes can also regulate other members of their own family. The intergenic enhancer region between *Dlx5* and *Dlx6* is regulated by *Dlx1*, *Dlx2* and *Dlx5* (Zerucha, Stuhmer et al. 2000, Stuhmer, Anderson et al. 2002, Zhou, Le et al. 2004). Several downstream targets of *Dlx5* have been identified during bone development. Osteocalcin is repressed by *Dlx5* (Ryoo, Hoffmann et al. 1997), and collagen 1A1 (Dodig, Kronenberg et al. 1996) and bone sialoprotein (Benson, Bargeon et al. 2000) are both activated by *Dlx5* during bone development.

1.8 *Nkx2.2* gene as a downstream target of DLX2

Dlx1/2 genes are important factors involved in neuronal vs glial cell fate switch favoring GABAergic interneuron differentiation. DLX2 represses oligodendrocyte differentiation by negatively regulating *Olig2* (Petryniak, Potter et al. 2007). On the other hand, by activating *Gad1* and *Gad2*, DLX2 promotes GABAergic interneuron differentiation (Le, Zhou et al. 2017). *Nkx2.2* is involved in the differentiation of oligodendrocyte progenitor cells (Qi, Cai et al. 2001,

Zhu, Zhao et al. 2014) and the expression of *Nkx2.2* increases in mice lacking *Dlx1/2* (Petryniak, Potter et al. 2007). These results reveal that *Nkx2.2* has a potential to be regulated by DLX2.

1.8.1 NKX2.2 transcription factor

NKX2.2 is a member of the NK2 family of homeobox transcription factors. It is orthologous to the *ventral nervous system defective (vnd)* gene in Drosophila (Zhang, Syu et al. 2008). The murine *Nkx2.2* gene has five exons and is located on chromosome 2. It encodes a 273 amino acid protein with a molecular mass of 30,133 Dalton (D) (NCBI 2018). The NKX2.2 protein consists of four functional domains: a transcriptional repressor domain, a homeodomain, a NK2 specific domain, and a transcriptional activation domain (Lessnick and Owen 2008). The homeodomain interacts with target genes and is involved in repressing or activating gene expression with the transcriptional repressor domain and the transcriptional activation domain, respectively. The transcriptional repression domain, DNA binding homeodomain, and NK2 specific domain regions have the highest homology with other members of the NK2 family of transcription factors (Lessnick and Owen 2008).

Nkx2.2 has been studied extensively in development of beta cells in pancreatic islets. In pancreatic development, *Nkx2.2* expression is observed in pancreatic progenitors from E 9.5 (Wilson, Scheel et al. 2003). In *Nkx2.2* mutants, beta cells and most alpha cells do not differentiate and are substituted with ghrelin-producing cells (Sussel, Kalamaras et al. 1998, Prado, Pugh-Bernard et al. 2004). Expression of NKX2.2 is retained in beta cells in adult mice where it is involved in forming islet structures and maintaining mature beta cell function within the pancreas (Doyle and Sussel 2007).

1.8.2 **Role of *Nkx2.2* genes in forebrain development**

During early stages of CNS development, several transcription factors of the NKX family including NKX2.1, NKX2.2, NKX2.9, NKX6.1, NKX6.2 and NKX6.3 are expressed in the ventral neural tube and are involved in patterning the neural tube (Qiu, Shimamura et al. 1998). *Nkx2.2* is activated by SHH signaling in the ventral region of the neural tube near the floor plate (Briscoe, Sussel et al. 1999). *Nkx2.2* is expressed in the p3 progenitor region of the neural tube located most ventrally near the floor plate where it promotes the differentiation of *Olig*-expressing OPCs to oligodendrocytes and suppresses the differentiation of pMN progenitors into motor neurons (Briscoe, Sussel et al. 1999). Postnatal expression of *Nkx2.2* facilitates myelination of matured oligodendrocytes (Kucenas, Snell et al. 2008). *Nkx2.*2 is also required for the establishment and maintenance of V3 excitatory interneurons generated from the p3 progenitor domain (Briscoe, Sussel et al. 1999). *Nkx2.2* is involved in oligodendrocyte progenitor cell differentiation by negatively regulating *Pdgfra* (Zhu, Zhao et al. 2014). During CNS development, platelet-derived growth factor (Pdgf) is involved in OPC proliferation and inhibits oligodendrocyte differentiation (van Heyningen, Calver et al. 2001). *Pdgfra* is expressed in immature OPCs, but as OPCs start to differentiate *Pdgfra* is silenced in OPCs (Pringle, Collarini et al. 1989). Direct repression of *Pdgfra* by NKX2.2 can promote the differentiation of OPC (Zhu, Zhao et al. 2014). Later in oligodendrocyte maturation, *Nkx2.2* is activated by OLIG2 and SOX10, where it functions as a transcriptional regulator of the myelin producing genes *Plp* and *Mbp* (Qi, Cai et al. 2001).

1.9 *Cxcr4* gene as a downstream target of DLX2

There are other candidate *Dlx* targets to consider during vertebrate forebrain development. CXCL12/CXCR4 signalling has an important role in cell migration in the developing CNS.

During cortical development, CXCL12 is expressed in the meninges (Tham, Lazarini et al. 2001) and in the cerebral cortex between E11.5 and E18.5 (Daniel, Rossel et al. 2005). CXCR4, the receptor for CXCL12, is identified on Cajal-Retzius projection neurons and GABAergic interneurons. While CXCL12/CXCR4 does not seem to play an important role in migration of preplate generated Cajal-Retzius cells, the guidance of cortical interneurons from the GE to the neocortex is dependent on CXCL12/CXCR4 signalling. Indeed, mice deficient in *Cxcl12* or *Cxcr4* have defects in interneuron migration and show altered lamination of GABAergic interneurons in cortical layers (Stumm, Zhou et al. 2003, Tiveron, Rossel et al. 2006, Li, Adesnik et al. 2008, Lopez-Bendito, Sanchez-Alcaniz et al. 2008). CXCR4/CXCL12 also plays an important role in the guidance of interneurons from the GE to the neocortex (Stumm, Zhou et al. 2003, Tiveron, Rossel et al. 2006, Li, Adesnik et al. 2008, Lopez-Bendito, Sanchez-Alcaniz et al. 2008). Furthermore, in the absence of *Dlx1/2,* expression of CXCR4 is significantly decreased in GABAergic interneurons in the MGE and CGE (Long, Cobos et al. 2009). These results suggest that *Cxcr4* could be a potential transcriptional target of DLX2.

1.9.1 **CXCR4 receptor**

The CXC chemokine receptor type 4 (CXCR4) gene contains 2 exons and is located on chromosome 2 in human and on chromosome 1 in mice (Caruz, Samsom et al. 1998). CXCR4, also known as Fusin or CD184 (cluster of differentiation 184), encodes a 7-transmembrane G-protein coupled receptor that binds to the chemokine, CXCL12. CXCR4 is highly expressed in hematopoietic stem cells as well as in T and B lymphocytes, macrophages, monocytes, neutrophils, endothelial progenitors, dendritic cells, Langerhans and vascular endothelial cells (Bleul, Fuhlbrigge et al. 1996, Gupta, Lysko et al. 1998, Zabel, Agace et al. 1999). CXCR4 is

also expressed in neurons, neuronal stem cells, microglia and astrocytes (He, Chen et al. 1997, Hesselgesser, Halks-Miller et al. 1997).

1.9.2 **CXCL12 chemokine**

Chemokines are small proteins with a molecular weight of 8 to 10 kD that induce gradient-dependent directional chemotaxis (Howard, Ben-Baruch et al. 1996). Chemokines have conserved cysteine amino acids and are arranged into four groups based on the number and position of the cysteine amino acids into CXC, CX3C, CC, and C (Le, Zhou et al. 2004). Chemokines, secreted by various stromal and epithelial cells, are involved in chemotaxis through interaction with chemokine receptors on cells (Baggiolini 1998).

CXCL12, also known as stromal derived factor 1 (SDF-1), is from the CXC family. It is highly expressed in heart, liver, pancreas, spleen and brain of both embryonic and adult tissue (Yu, Cecil et al. 2006). During development, CXCL12 has a role in the proliferation and differentiation of immature progenitors (Karin 2010). CXCL12 and CXCR4 deficiency in mice is fatal confirming the important role for both factors during development (Zou, Kottmann et al. 1998). In the adult, CXCL12 plays a key role in maintaining tissue homeostasis and survival of immune cells (Lataillade, Domenech et al. 2004). CXCL12 is also reported to be important in spreading of cancer metastases (Juarez and Bendall 2004).

CXCL12 is the specific ligand for CXCR4 (Bleul, Fuhlbrigge et al. 1996). CXCL12 is the only known chemokine for CXCR4; however, it can also bind to another chemokine receptor, CXCR7 (Burns, Summers et al. 2006). CXCR7 can bind to CXCL12 with high affinity and control CXCL12 gradients through fast degradation (Hoffmann, Muller et al. 2012).

1.9.3 **CXCR4/CXCL12 signalling in neurogenesis**

Other than its well established role as a co-receptor for human immunodeficiency virus (HIV) entry (Feng, Broder et al. 1996), CXCR4 has diverse physiological functions which include but are not limited to hematopoiesis, immune responses, germ cell development, and neurogenesis (Zou, Kottmann et al. 1998, Moser and Loetscher 2001, Agarwal, Ghalayini et al. 2010, Richardson and Lehmann 2010, Nagasawa 2014).

CXCL12/CXCR4 signalling has an important role in cell migration in the cerebellum and the cortex of the developing CNS. CXCR4 is expressed on the granule cells of the cerebellum and migrate toward CXCL12-producing meninges to form the external granule cell layer (Sotelo 2004). During cortical development, CXCL12 is constantly expressed in the meninges (Tham, Lazarini et al. 2001) and in the intermediate zone of embryonic mouse cerebral cortex between E11.5 and E18.5 (Daniel, Rossel et al. 2005). Cajal-Retzius cells and GABAergic inhibitory interneurons are two cell populations expressing CXCR4 during corticogenesis. CXCR4 signalling is important for proper migration of Cajal-Retzius cells since CXCL12 promotes the migration of Cajal-Retzius neurons from cortical hem explants extracted from the dorsal pallium. Furthermore, injection of CXCR4 antagonist, AMD3100 (also known as Plerixafor), in E12.5 embryos results in a dispersion of Cajal-Retzius from the MZ to the cortical plate (Borrell and Marin 2006).

CXCL12/CXCR4 plays an important role in the guidance of cortical interneurons from the GE to the neocortex. Null mice for *Cxcl12* or *Cxcr4* fail to maintain migrating streams of interneurons and show altered expression of GABAergic interneuron precursors in the SVZ and in the MZ (Stumm, Zhou et al. 2003, Tiveron, Rossel et al. 2006, Li, Adesnik et al. 2008, Lopez-Bendito, Sanchez-Alcaniz et al. 2008).

1.10 Posttranslational modifications of transcription factors

In addition to understanding transcription factor function through the identification of their gene targets, posttranslational modifications (PTM) are important to consider. Posttranslational modification of proteins are chemical changes in proteins after they are translated from RNA. PTM are mainly catalyzed by enzymes that alter specific binding sites on proteins. There are many different types of PTM including cleavage of proteins, formation of disulfide bonds from cysteine residues, and adding or eliminating functional groups which can lead to modifications such acetylation, hydroxylation, methylation, glycosylation, ubiquitination, oxidation, and phosphorylation (Karve and Cheema 2011).

Knowledge of posttranslational modifications of transcription factors and how these modifications are important for gene expression has improved dramatically over the past 10 years (Planey, Kumar et al. 2013, Carr, Poppy Roworth et al. 2015). Phosphorylation is now established as an important PTM regulating the activity of many transcription factors (Hunter 2000). Phosphorylation is a common PTM in eukaryotes and is the addition of phosphate groups to serine, threonine or tyrosine residues by a protein kinase. Phosphorylation of proteins affects their activity. Addition of a phosphate group introduces a negative charge that induces allosteric conformational changes, leading to changes in the activity of the protein (Sprang, Acharya et al. 1988, Hurley, Dean et al. 1990). For example, phosphorylation of the cyclic AMP response element-binding protein (CREB) activates it by recruiting the transcriptional co-activator CREB binding protein (CBP) (Mayr and Montminy 2001).

1.10.1 Role of Posttranslational modifications on transcription factor function

There are several ways that phosphorylation can alter the activity of transcription factors. Phosphorylation can positively and negatively affect the stability of the transcription factor.

Phosphorylation of p53 and activating transcription factor 2 stabilizes these two proteins by protecting them against degradation (Appella and Anderson 2000, Fuchs, Tappin et al. 2000). On the other hand, phosphorylation of MyoD and E2F-1 facilitates their degradation (Song, Wang et al. 1998, Vandel and Kouzarides 1999).

Phosphorylation can also regulate a transcription factor's nuclear translocation. Phosphorylation of transcription factors can sequester the transcription factor in the cytoplasm and prevent its binding to target sequences resulting in transcription factor inactivation. In *Saccharomyces cerevisiae*, SW15 is localized to the nucleus and activates transcription in the G1 phase of the cell cycle. During other stages of the cell cycle, SW15 is phosphorylated and is retained in the cytoplasm (Moll, Tebb et al. 1991). On the other hand, phosphorylation of a transcription factor can translocate it into the nucleus, where it activates target genes. For instance, phosphorylation of the IKB regulatory subunit of NF-KB triggers its nuclear import (Schmid, Perkins et al. 1991).

Phosphorylation can facilitate protein-protein interactions and oligomerization. Examples of protein-protein interaction are seen with the CREB transcription factor where it interacts with CBP. Furthermore, phosphorylation is involved in STAT dimerization (Whitmarsh and Davis 2000). Phosphorylation can also positively or negatively affect the DNA binding activity of the protein. Phosphorylation promotes the DNA binding activity of STAT (Whitmarsh and Davis 2000). In addition, phosphorylation of HSF1 regulates its transcriptional activity (Knauf, Newton et al. 1996, Holmberg, Hietakangas et al. 2001). Phosphorylation can result in transcription factor inactivation. In *Saccharomyces cerevisiae*, phosphorylation of Gcn4 by TFIIH marks it to be degraded by the ubiquitin proteasome pathway (Chi, Huddleston et al. 2001). Similarly, phosphorylation of E2F-1 by TFIIH in humans results in its degradation (Vandel and Kouzarides

1999). Phosphorylation can affect DNA binding by several mechanisms. Phosphorylation of a transcription factor can cause electrostatic repulsion between phosphates on the protein and DNA and subsequently interfere with DNA binding. Furthermore, phosphorylation can change the conformation and its DNA binding. Phosphorylation can also regulate the interplay between the transcription factor and transcriptional machinery. In many cases transcription factors have transcriptional activation domains that are distinct from DNA-binding domains, and phosphorylation of transcription factors can influence transcription factor transactivation. For example, phosphorylation of CREB by PKA activates cAMP-inducible genes (Gonzalez and Montminy 1989).

1.10.2 **DLX2 phosphorylation**

Little is known about the posttranslational modifications of DLX proteins. In one study it was shown that a regulatory subunit of DNA-dependent protein kinase (DNA-PK) known as Ku antigen interacts with homeodomain proteins HOXC4, OCT1, OCT2 and DLX. Ku antigen interaction with OCT1 increased the phosphorylation of OCT1 by DNA-PK, suggesting that Ku may function as a scaffold to recruit homeodomain proteins for phosphorylation by DNA-PK. However, phosphorylation of DLX2 was not investigated in this study (Schild-Poulter, Pope et al. 2001). Furthermore, while DLX1 is only expressed in the nucleus of the ventral thalamus, DLX2 is both expressed in the nucleus and cytoplasm of the ventral thalamus (Eisenstat, Liu et al. 1999). Perhaps DLX2 is bound to different proteins in the cytoplasm and nucleus of this neuroanatomic region or alternatively, PTM of DLX2, such as phosphorylation, affects DLX2's subcellular localization.

1.11 Project outline

To date, only a few DLX2 transcriptional targets have been validated *in vivo*. Furthermore, knowledge regarding posttranslational modifications of DLX2 and whether it affect's DLX2 localization and function is limited. I therefore proposed to assess regulation of DLX2 mediated oligodendroglial differentiation in forebrain development through inhibition of *Nkx2.2* expression (Aim 1). I also proposed to explore DLX2 transcriptional regulation of GABAergic interneuron migration during forebrain development through *Cxcr4* expression (Aim 2). I also wanted to identify the key amino acid residues that are phosphorylated in DLX2 during CNS development (Aim 3).

I hypothesized that:

1. DLX2 directly mediates transcriptional repression of oligodendroglial differentiation in determination of neuronal versus glial cell fates during forebrain development through inhibition of *Nkx2.2*.

2. DLX2 directly mediates transcriptional activation of GABAergic interneuron migration through promotion of CXCR4 expression through the interaction of DLX2 with the *Cxcr4* promoter during forebrain development.

3. DLX2 functions as a phosphoprotein and is localized to the nucleus in determining progenitor fate.

To test the transcriptional regulation of *Nkx2.2* and *Cxcr4* by DLX2, I assessed the interaction of DLX2 with the promoter region of *Nkx2.2* and *Cxcr4* using chromatin immunoprecipitation (ChIP) experiments and further characterized this interaction using electrophoretic mobility shift assays (EMSA) and luciferase reporter assays. I also determined

Nkx2.2 and *Cxcr4* mRNA expression levels in forebrain tissue, SK-N-BE (2) cells and E13.5 GE primary cultures in the presence and absence of *Dlx1/2* using quantitative real-time PCR (qRT-PCR). Furthermore, I interrogated NKX2.2 and CXCR4 protein levels *in vivo* using immunofluorescence (IF) staining on E13.5 wildtype (WT) and DKO cryopreserved sections.

To determine the role of DLX2 in migration of interneurons *in vitro*, I performed the Boyden migration assay on SK-N-BE (2) cells as well as E13.5 primary cultures in the presence and absence of CXCL12. Furthermore, cell migration capabilities were assessed in *Dlx2*-siRNA treated SK-N-BE (2) cells as well as Plerixafor (AMD3100) treated SK-N-BE (2) cells in the presence and absence of CXCL12.

For my last aim, I conducted Western Blotting on E13.5 WT ganglionic eminences to confirm phosphorylation of DLX2 and dephosphorylation by lambda protein phosphatase. To identify sites of phosphorylation, I immunoprecipitated DLX2 from E13.5 WT GE and sent the precipitated samples for analysis by mass spectrometry.

2 Chapter 2: Materials and Methods

2.1 Animals

Tissue from E13.5 wild-type CD-1 strain and E13.5 *Dlx1/Dlx2* DKO transgenic mice were utilized to assess *Dlx2* transcriptional regulation. CD-1 Swiss mice were obtained from the Charles River Laboratory. The *Dlx1/Dlx2* DKO mice were initially produced by Dr. John Rubenstein (UCSF, CA, USA) and were maintained in a CD-1 background. Since the *Dlx1/Dlx2* DKO dies at birth, heterozygous mice, which are phenotypically normal and fertile, were crossed to generate a *Dlx1/Dlx2* DKO colony (Anderson, Qiu et al. 1997, Qiu, Bulfone et al. 1997). For genotyping DKO mice, ear biopsy samples were collected and genomic DNA (gDNA) was extracted using phenol and chloroform. PCR amplification using Hotstar Plus DNA polymerase (Qiagen, catalog #: 203601) with *Dlx2* and *Neo*-specific primers was then carried out. Primer sequences are provided in Table 2.2.

To obtain embryonic tissue, the presentation of a vaginal plug in the female mouse was marked day 0.5 of pregnancy. Consequently, mothers were sacrificed by cervical dislocation. Embryos were then removed, added to ice cold phosphate buffered saline (PBS) and euthanized through decapitation.

All experiments using CD-1 or *Dlx1/2* DKO mice were performed with the guidelines of the University of Alberta Animal Care and Use Committees. For the *Dlx1/Dlx2* DKO colony, daily breeding, biopsy collection and colony maintenance tasks, were performed by Health Sciences Laboratory Animal Services, HSLAS, at the University of Alberta.

2.2 Tissue preparation and cryopreservation

E13.5 embryos were decapitated and tissues were cross-linked with 4% paraformaldehyde (PFA) (Sigma, catalog #: 158127) at 4°C for three hours with rotation. After fixation, tissue was

cryopreserved using a sucrose gradient of 15% to 30% at 4°C for at least an hour or until the tissue sunk to the bottom of the tube. Tissue was then embedded into blocks using optimal cutting temperature compound (OCT) (VWR, catalog #: 361603E) on dry ice and stored at -80°C.

Cryosections of 12µm were collected using a Cryostat Leica CM 3000 and mounted on Superfrost Plus slides (Fischer Scientific, catalog #: 12-550-15). After sectioning, slides were stored at -80°C in the dark for later use in immunostaining.

2.3 Tissue immunofluorescence

Cryosections were blocked for 2 hours with blocking buffer consisting of 0.1% bovine serum albumin (BSA, Fisher Scientific, catalog #: BP1600-100), 0.2% Triton-X 100 (Biorad, catalog #: 1610407), 5% Serum in PBS at room temperature (RT). For the next step, blocking buffer was removed and primary antibodies were diluted and added to the cryosections and incubated overnight (O/N) at 4°C. Primary antibodies were diluted in the blocking buffer. For negative control to ensure specificity of antibody binding, sections were incubated without primary antibody in blocking buffer O/N. After O/N incubation, slides were washed three times for 5 minutes with wash solution containing PBS with 0.05% Triton X-100. The secondary antibodies were placed on the sections and incubated for two hours in the dark room. Secondary antibodies were also diluted in blocking solution. A list of primary and secondary antibodies and dilutions used is given in Table 2.1.

After incubation with the secondary antibody, the slides were washed three times for 5 minutes with wash buffer and mounted using Vectashield mounting medium containing DAPI (Vector Labs, catalog #: H-1200). Coverslips were then added and sealed using clear nail polish. Fluorescent images were taken using Eclipse TE2000U (Nikon) and NIS Elements software.

2.4 *In situ* hybridization (ISH)

In situ hybridization was used to detect the location of the *Cxcr4* mRNA using a digoxigenin (DIG) labeled RNA. Plasmids containing cDNA of murine *Cxcr4* were kindly provided by Dr. John Rubenstein (UCSF, CA, USA). Plasmids were linearized using *Pst1* restriction enzyme (NEB, catalog #: R0140S) and purified using standard phenol/chloroform extraction. After purification, SP6/T7 Transcription Kit (Roche, catalog #: 10999644001) using T7 primers was used to generate antisense RNA probes and DIG-RNA Labelling Mix (Roche, catalog #: 11277073910) was used to DIG-label the RNA. RNA probes were then purified by running through a SigmaSpin Post-Reaction Clean-Up Column (Sigma, catalog #: S5059) and stored at -80°C for further experiments.

In situ hybridization was carried out on 12µm E13.5 brain sections obtained both from WT and DKO tissues. Slides were initially fixed in 4% PFA at RT for 10 minutes. Then the slides were acetylated in freshly prepared triethanolamine (750µl) (Sigma, catalog#: T1377) and acetic anhydride (125µl) (Sigma, catalog #: 320102) in 50ml H_2O containing 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC). After acetylation, slides were washed in PBS with DMPC three times for 5 minutes. RNA probe was diluted 1:10 in hybridization solution (50% formamide, 5X SCC,10% Dextran Sulfate, 1X Denhardt's solution, 250µg/ml Yeast tRNA, 0.1% Tween, in H_2O/DMPC) and incubated at 65°C for 5 minutes. 50µl of probe was then added to each slide, covered with glass coverslips and incubated at 65°C overnight in a hybridization chamber (Boekel Scientific). Then slides were washed three times with a previously warmed (65ºC) wash buffer containing 50% formamide solution, 1X SCC and 0.1% Tween for 15 minutes each at 65°C. After cooling down the slides, they were washed in MABT buffer (maleic acid buffer containing 0.1 % Tween) three times for 10 minutes. Following washes, slides were

blocked in 2% blocking buffer (Roche, catalog #: 11096176001) and 20% goat serum for an hour at RT. 1/5000 dilution of anti-DIG antibody in hybridization solution was added to the samples at 4ºC O/N. The slides were washed twice with NTMT buffer (100 mM NaCl, 100 mM Tris-HCl (pH 9.5), 50 mM $MgCl_2$, 1% Tween 20) and stained with a solution of 4-Nitro blue tetrazolium chloride (NBT, Roche, catalog #: 11383213001) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP, Roche, catalog #: 11383221001). After achieving optimal colour, slides were fixed with 4% PFA, washed with PBS and mounted with Permount mounting media (Fisher Scientific, catalog #: SP15-500).

2.5 Production and affinity purification of recombinant DLX2

The production and affinity purification of recombinant DLX2 was done previously as described (Porteus et al. 1994). The C-terminal domain of DLX2 which contains the homeodomain was cloned into pET11d expression vector (Novagen) and transformed into BL21DE3pLysS *Escherichia coli* (*E. coli*). Isopropyl-β-thiogalactopyranoside (IPTG) was used to induce expression of rDLX2 and HisTrap FF columns (GE Healthcare) were used to affinity purify the recombinant DLX2 protein. The recombinant protein was used in electrophoretic mobility shift assays.

2.6 Chromatin Immunoprecipitation

To conduct chromatin immunoprecipitation experiments, GE and hindbrain tissue were dissected from E13.5 WT CD-1 mice. *Dlx* genes are not expressed in the hindbrain; therefore, embryonic hindbrains were used as a negative tissue expression control. After tissue collection, samples were washed twice with PBS and the cells were dissociated by pipetting several times. Tissues were then cross-linked with 1% PFA with 1X protease inhibitor cocktail (PIC, Sigma,

catalog #: P8340) for 90 minutes at 4°C. Cells were then washed with PBS and lysed in 400µl lysis buffer (1% SDS, 10mM Tris-HCl pH 8.1, 10mM EDTA). The DNA was then sheared into 300 to 500 bp fragments by sonication on ice using a 60 Sonic Dismembrator (25-30 repeats of 15 seconds sonication and 30 seconds rest). Sheared DNA was size separated on a 1% agarose gel to confirm DNA complexes were between 300-500 bp in size.

Pierce UltraLink Protein A/G beads (Thermo Fisher, Catalog #: 53132) were used to remove non-specific background IgG from the tissue. Initially, the beads needed to be primed. For this step, beads were washed twice with 1ml of dilution buffer containing 0.01% SDS, 1% Triton X-100, 1.2mM EDTA, 167mM NaCl, 16.7mM Tris-HCl pH8.1, and 1X PIC. 60µl of the primed beads were added to each sample and incubated at 4°C with rotation for an hour. Samples were then centrifuged and 500µg/ml of BSA and tRNA (Sigma, catalog #: R5636) and 2µl of concentrated DLX2 antibody was added to the supernatant and incubated at 4°C O/N with rotation. A negative antibody control for both the GE and the hindbrain was also performed where no primary antibody was added to the supernatant.

A/G beads were also primed with 500µg/ml tRNA and BSA overnight at 4°C and added to the samples the following day. The beads were rotated with the samples at 4°C O/N to precipitate the DNA-DLX2 complexes. Beads containing complexes were separated from the supernatant that contain unbound complexes and were saved as Total Input.

After precipitating DNA-protein complexes, the beads were washed initially with a low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8.1 and 150mM NaCl) for 5 minutes with rotation at 4°C. Second, a high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8.1 and 500mM NaCl) was used for 30 minutes. Third, a LiCl buffer wash containing 0.25M LiCl 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl and

1% NP-40 was performed. Finally, washes with TE buffer were carried out. After performing the washes, the DNA-protein complexes were eluted from the beads with a 65°C warmed elution buffer containing 1% SDS and 0.1M $NaHCO_3$ for 15 minutes with agitation. The elution step was repeated once more.

To reverse the cross-linking between DNA- protein complexes, 25µl 5M NaCl was added to the eluted samples and incubated at 65°C. RNase A (Thermo Fisher Scientific, Catalog #: 12091021) was also used to remove RNA contamination. Next, samples were incubated for two hours at 65°C in a mixture containing 20µl of 1M Tris-HCl (pH 6.5), 10µl of 0.5M EDTA, and 2µl of Proteinase K. Finally, DNA was purified using QiaQuick PCR purification kit (Qiagen, catalog #: 28104). PCR amplification with primers designed to flank TAAT/ATTA motifs in the regulatory region of *Nkx2.2* and *Cxcr4* was used to identify DLX2 occupancy within these regions. Primers used for ChIP are listed in Table 2.2.

2.7 Molecular cloning

Regulatory regions identified with ChIP were cloned into the pGL3-Basic Vector reporter plasmid (Promega, catalog #: E1751) for subsequent EMSA and reporter gene assays. To clone the regulatory sites into the vector, each regulatory region was PCR amplified with Phusion High-Fidelity DNA Polymerase (NEB, catalog #: M0530L) using primers that had restriction sites to the 5' and 3' ends to create overlapping ends and purified with a QIAquick PCR Purification Kit. For digestion, 500ng of DNA, 1unit of restriction enzyme, 2µl of NEB Buffer, and 2µl of 10X BSA to a total reaction volume of 20µl was incubated at 37°C for two hours. To create overhanging ends on empty pGL3 plasmid, the plasmid was digested with the same restriction enzymes as before. Digested plasmid and regulatory regions were separating on a 1% gel electrophoresis, cut from the gel and cleaned with QIAquick Gel Extraction Kits (Qiagen,

catalog #: 28704). Finally, T4 DNA ligase (NEB, catalog #: M0202S) was used to insert the regulatory region DNA into the matching pGL3 vector.

After ligation, the plasmid was used to transform competent DH5α *E. coli* cells for 30 minutes on ice. The bacteria were then heat shocked at 42°C for 1 minute and placed on ice for 5 minutes. These cells were added to Lysogeny Broth (LB) for 2 hours at 37°C and then plated onto LB agar plates containing 50mg/ml carbenicillin. Plates were incubated O/N at 37°C and single colonies resistant to carbenicillin were picked and grown in LB broth at 37°C O/N. Finally, QIAprep Spin Miniprep Kit (Qiagen, catalog #: 27106) was used to isolate the plasmids. Insertions of regulatory regions into the pGL3 Plasmids were first checked with restriction digestion and gel separation and then sequenced (TAGC, University of Alberta).

2.8 Electrophoretic mobility shift assay

ChIP experiments reveal the presence of DLX2 at the regulatory region. EMSAs were used to identify specific binding of DLX2 at DNA regulatory elements. After cloning ChIP positive regulatory regions into pGL3 reporter vectors, the Qiagen Plasmid Maxi Kit (Qiagen, catalog #: 12163) was used to make large amounts of plasmid containing the regulatory regions. To create 5' overhangs on each regulatory region, the plasmid was digested using an appropriate restriction enzyme, isolated on a 1% agarose gel and purified using a QIAquick gel extraction kit (Qiagen, catalog #: 28704). Restriction enzyme used for each region is provided in Table 2.3.

These 5' overhanging DNA pieces were radiolabelled using [α-^{32}P]-dGTP (Perkin Elmer, catalog #: BLU006H250UC) and DNA Polymerase I (Klenow) (Invitrogen, catalog #: 18012021) for 15 minutes at RT. Probes were purified with Illustra Micro-Spin G-25 columns

(GE Healthcare, catalog #: 27532501). Radioactivity levels were measured using a LS 6500 scintillation counter (Beckman Coulter) and diluted to 80,000 counts per million/µl.

For the *Cxcr4* regulatory region, 25-30 bp oligonucleotides for each TAAT/ATTA motif were designed. Sequences of each oligonucleotide are provided in Table 2.6. Each oligo was then labelled at the 5' end using a polynucleotide kinase and [γ-^{32}P]-dGTP (Perkin Elmer, catalog #: BLU004Z250UC). For the labelling reaction, 5pmol of oligonucleotide was incubated with T4 polynucleotide kinase (Thermo Fisher Scientific, catalog #: EK0031), 5X exchange buffer (Thermo Fisher Scientific, catalog #: EK0031), and [γ-^{32}P]-dGTP for 30 minutes at RT and then radiolabelling was terminated with 1µl of 0.5M EDTA. Labelled probes were purified with Illustra Micro-Spin G-25 columns.

Samples were then incubated in 5X Binding Buffer (Promega, catalog#: E3581), 1mg/ml Poly (dI-dC) (Thermo Fisher Scientific, catalog #: 20148E) and 200ng of recombinant DLX2 protein for 30 minutes followed by incubation with labelled probe for another 30 minutes. Controls for this experiment included: free probe where no rDLX2 was added to the binding solution, "supershift" where anti-DLX2 antibody was added, and "cold competition" with excess unlabelled probes added to the binding solution.

Binding reactions were then run on a 4% acrylamide gel containing 40.5ml H_2O, 2.5ml 10X Tris/Borate/EDTA buffer (TBE), 1.875ml 40% Acrylamide, 3.125ml 40% (37.5:1) Acrylamide/Bisacrylamide, 375µl 10% Ammonium Persulfate (APS, Biorad, catalog #: 1610700), 1.56ml 80% Glycerol (Thermo fisher Scientific, catalog #: 15514011 , and 25µl Tetramethylethylenediamine (TEMED, Sigma, catalogue #: T9281) at 300V for two hours in 0.5X TBE buffer. Once the samples were size separated through the gel, the gels were dried using a gel dryer and HydraTech vacuum pump (Biorad) for 2 hours at 80°C. Once the gels

dried, they were placed in a cassette (Biorad) then exposed to X-ray film (Kodak) for an hour at -80°C. Films were then developed in the dark using a Mini-Medical 90 film developer (AFP Imaging).

2.9 Site-directed mutagenesis

To further validate the specific binding site within regulatory regions containing multiple candidate binding sites, site-directed mutagenesis was performed. The overlap extension PCR method was used to delete TAAT/ATTA motifs within the regulatory region (Lee, Shin et al. 2010). For this technique, two sets of primers were used and three polymerase chain reactions were conducted. One primer set was designed to amplify the region of interest and the second sets of primers were designed to introduce the desired mutation. In the first and second PCR reactions, the mutation was inserted to the 5' and 3' ends of the region, respectively. The third PCR is then conducted in order to amplify these two overlapping 5' and 3' end regions and produce a final product that contains the whole region with the desired deletions. After successfully amplifying this mutated region, the product is cloned into an empty pGL3-Basic Vector (Promega, catalogue #: E1751), sequenced (TAGC, University of Alberta) and radiolabelled as previously described. The radiolabelled mutated regions were then used in an EMSA assay. Primers used to generate site directed mutagenesis are in Table 2.4.

2.10 Transfection and gene reporter assays

Luciferase reporter assays were conducted on regulatory regions that showed specific binding to DLX2 *in vitro*. Regulatory DNA regions were sub-cloned into pGL3-Basic Vectors upstream of the luciferase gene as described in the Molecular Cloning section. *Dlx2* expression

vectors were kindly provided by Dr. John Rubenstein (UCSF, CA, USA) and were generated by sub-cloning *Dlx2* cDNA into the pCDNA3 expression vector.

HEK293 cells were used for the reporter gene assays. HEK293 cells were grown at 37°C in DMEM media (Gibco, catalog #: 11965084) with 10% Fetal Bovine Serum (FBS, Gibco, catalog #: 26400044). Once the cells reached 90% confluence, they were washed with PBS and trypsinized for 5 minutes to detach from the flask. After counting with a haemocytometer, $2x10^5$ cells/well were seeded in a 12 well plate and incubated O/N before transfecting. 1µg pCDNA3-DLX2 expression vectors, 0.5µg of pGL3 plasmid containing specific regulatory region and 4ng β-gal vector (Promega, catalog #: E1081) were then transfected into each well using Lipofectamine 2000 (Invitrogen, catalog #: 11668030). For controls, empty pGL3 and empty pCDNA3 were added. β-gal vector was also added to each well as a control for transfection efficiency.

Cells were incubated for 48 hours after transfection in DMEM and 10% FBS at 37°C, 5% CO_2. After 48 hours, cells were washed in ice-cold PBS and lysed using a reporter lysis buffer (Promega, catalog #: E4030) for 15 minutes on ice. Cell lysates were then collected and plated in two 96 well plates. The Firefly substrate (Promega, catalog #: E1500) was added to one 96 well plate to measure expression levels in the presence or absence of DLX2. The firefly luminescence was measured for 1 second per well in the 1420 Victor Multilabel Counter. The β-gal assay buffer (Promega, catalog #: E2000) was added to the second 96 well plate to measure the plasmid transfection efficiency and the photometric measurement of the β-galactosidase was measured at 570 nm absorbance after one hour incubation at 37ºC. Experiments were performed in three technical and three biological replicates. The Student's unpaired t-Test was used to determine statistical significance.

2.11 *Dlx2* knockdown using small interfering RNA (siRNA)

To investigate the expression levels of *Nkx2.2* and *Cxcr4* after *Dlx2* knockdown, SK-N-BE (2) cells were used. SK-N-BE (2) are a neuroblastoma cell line and were chosen because of their biological relevance to developing brain tissue and endogenous expression of *Dlx2*. SK-N-BE (2) cells were cultured with DMEM/F-12 Media (Gibco, catalog #: 11320033) supplemented with 10% FBS. $1x10^6$ cells were plated and incubated for 24 hours to reach 90% confluence before transfection.

Cells were transfected with duplex *Dlx2*-siRNA and scrambled-siRNA complexes ordered from Ambion using Lipofectamine 2000. Two different double strand siRNAs were tested. The sequences for each of the double stranded siRNA are: *Dlx2*-siRNA1: sense 5'GGAAGACCUUGAGCCUGAATT3', antisense 5'UUCAGGCUCAAGGUCUUCCTT3'; *Dlx2*-siRNA2: sense 5'CCUGAAUCCGAAUAGUGATT3', antisense 5'UCACUAUUCGGAUUUCAGGCT3'. Western Blotting experiments revealed that both *Dlx2*-siRNA1 and *Dlx2*-siRNA2 had similar knockdown efficiencies (90% knockdown efficiency, X. Song). The cells were then incubated for 48 hours. Consequently cells were collected, RNA was extracted using an RNA isolation kit (Qiagen, catalog #: 74104) and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, catalog #: 18080093) with Oligo (dT) primers. qRT-PCR was then performed to quantify expression levels of *Nkx2.2* and *Cxcr4* using cDNA-specific primers. For internal control, glyceraldehyde 3-phosphate dehydrogenase (Gapdh) primers were used. Primers used in qPCR experiments are listed in Table 2.2.

2.12 Quantitative real-time PCR

qRT-PCR was performed on E13.5 *Dlx1/2* DKO GE tissue, *Dlx2*-siRNA treated primary cultures prepared from E13.5 GE tissue, and *Dlx2*-siRNA treated SK-N-BE (2) cells to assess the

expression levels of *Nkx2.2* and *Cxcr4* after the loss of *Dlx*2 both *in vivo* and *in vitro*. To extract RNA from WT and DKO *Dlx1/2* tissue, TRIzol (Invitrogen, catalog #:15596026) was used. Chloroform was then added to separate the phases. The upper phase was then collected and precipitated in 75% ethanol.

To synthesize cDNA, the Superscript III Reverse Transcriptase (Invitrogen, catalog #: 18080-044) was used. At first, 500ng of isolated RNA was added to 1µl oligo (dT) and 1µl dNTP mix and heated at 65°C for 5 minutes. After cooling this mixture on cold ice for one minute, 4µl 5X First-Strand Buffer, 1µl RNaseOUT, 1µl DTT, and 1µl Superscript III RT were added to the mixture and incubated at 50°C for an hour, followed by 70°C for 15 minutes. qRT-PCR was performed on a LightCycler 96 System (Roche) using the FastStart SYBR Green Master System (Roche, catalog #: 03003230001). The qPCR primers were designed for exon-exon junctions. Gapdh was used as an internal control. Experiments were performed in three technical and three biological replicates. For data analysis, the delta delta Ct method was performed, where difference between the cycle of threshold (Ct) of DKO and WT was analysed when both conditions were normalized to Gapdh. The Student's unpaired t-Test was used to determine statistical significance. The sequences of primers for qRT-PCR are provided in Table 2.5.

2.13 Primary culture preparation

The expression levels of *Nkx2.2* and *Cxcr4* were also assessed in E13.5 GE primary cultures. Furthermore, E13.5 GE primary cultures were used to assess the role of *Dlx2* in migration of neuronal cells *in vitro.* To prepare primary cultures, GE's were dissected from E13.5 embryos and placed in Hank's Balanced Salt Solution (HBSS) (Gibco, catalog #: 14025092) on ice. After gently grinding the tissue, 0.25% trypsin was added to the cells and incubated for 15 minutes. The cells were centrifuged at 1500 for 5 minutes and resuspended and

triturated to a single cell suspension in HBSS with 100µg/ml DNaseI (Sigma, catalog #: D4527). The cells were transferred to Neurobasal media containing 2% B-27 (Gibco, catalog #: 17504044) and 1% pen-strep (Gibco, catalog #: 15070063). Finally, $1x10^6$ cells were plated in poly D-lysine coated plates and incubated at 37°C with 5% CO_2.

2.14 Transwell migration assay

To determine the role of DLX2 in migration of neurons *in vitro*, the Boyden migration assay, also known as the Transwell migration assay, was performed. In this assay, Transwell plates containing 8µm polycarbonate plate inserts (VWR, catalog #: 10769-212) are used. Cells are placed on the upper layer of the permeable membrane and chemotactic solution is added to the lower layer beneath the membrane. The cells can then migrate through the pores of the membrane after incubation. Finally, the cells that have migrated through the pores to the other side of the inserts are fixed, stained and counted (Boyden 1962, Chen 2005).

SK-N-BE (2) cells as well as E13.5 primary cultures were used to assess the role of *Dlx2* knockdown in cell migration. For the SK-N-BE (2) cells, the cells were starved the night before the experiment by exchanging DMEM/F-12 medium containing 10% FBS with serum free medium. Serum deprivation synchronizes the cell cycle and improves response to the chemoattractant. The day of the experiment, the first step was to hydrate the base membrane of the Transwell chambers by adding 1ml of DMEM/F-12 medium containing 1% BSA to each membrane in the wells. 200µl of DMEM/F-12 medium with 1% BSA were also added to the top of the membrane. Then the plate was incubated at 37°C with 5% CO_2 for an hour.

While the membranes were soaking in the incubator, the SK-N-BE (2) cells were washed twice with PBS (Gibco, catalog #: 14190250) and detached using 0.25% trypsin (Gibco, catalog #: 25200056) for 5 minutes. Trypsin was then deactivated by using an equal amount of

DMEM/F-12 with 10% FBS. The cells were collected and washed twice with free FBS DMEM/F-12 medium to remove traces of trypsin. The cells were resuspended in DMEM/F-12 medium with 1% BSA and counted using a haemocytometer. The Transwell chamber was taken out of the incubator and the membrane inserts were transferred to 12 well plates containing DMEM/F12 medium with 20% FBS using clean forceps. To the upper compartment of the membranes, $1x10^5$ cells were added very gently to prevent bubbles.

Half of the chambers received 100ng/ml CXCL12/SDF1α (R&D Systems, catalog#: 460-SD) in the lower side while the rest only received 1% BSA. Cells were then incubated at 37ºC with 5% CO_2 for 24 hours to migrate to the lower side of the membrane. After the incubation period, the membranes were washed with PBS. The cells that did not migrate through the membrane were removed with a sterile cotton swab from the top of the membrane. The cells on the bottom side of the membranes were then fixed with 4% PFA for 20 minutes. Finally, the cells on the lower side of the membrane were stained with 1% crystal violet for 20 minutes and 2% ethanol for an hour. Cells were then imaged using Nikon Eclipse TE2000U microscope and counted with Image J software.

MDA-MB-231 and MCF-7 breast cancer cell lines (kindly provided by Dr. R. Leng, University of Alberta) were used as positive and negative controls, respectively. MDA-MB-231 cells have a strong migration capability and are used as positive control, while MCF-7 cells have much weaker migration capability and are used as a negative control (Hooshmand, Ghaderi et al. 2013).

To assess the effects of the loss of *Dlx2* expression on cell migration, *Dlx2* expression in SK-N-BE (2) cells was knocked down using siRNA for 48 hours (explained above) prior to performing the migration assay. The role of Plerixafor (AMD3100, Genzyme Corporation) on

cell migration was also assessed. Plerixafor is a CXCR4 antagonist that inhibits the binding of CXCL12 to its receptor, CXCR4. To determine the role of Plerixafor on cell migration, SK-N-BE (2) cells were treated with 100mM Plerixafor for an hour, prior to conducting the Transwell migration assay.

I also assessed cell migration in E13.5 GE primary cultures. After preparing E13.5 primary cultures as explained above, $1x10^5$ cells were added to the upper chamber of the Transwell plates. The medium used for the primary cultures was Neurobasal medium containing 2% B-27, and no FBS was added to any part of the experiments. The cells were incubated at 37ºC with 5% CO_2 for different times (from 6 to 48 hours), fixed, stained and assessed for migration. The Student's unpaired t-Test was used to determine statistical significance.

2.15 Stripe assay

The stripe assay is an *in vitro* test system to study the migration capabilities of cells in response to guidance molecules. The stripe assay is also known as the Bonhoeffer assay since it was initially designed by Professor F. Bonhoeffer to investigate axonal guidance mechanisms in the chick retino-tectal system in 1987 (Walter, Henke-Fahle et al. 1987, Walter, Kern-Veits et al. 1987). Briefly, a silicon matrix containing 90µm gaps with an inlet and outlet channel is used to generate stripes of the chemoattractant molecule. The matrix is then removed and cells are then cultured onto the striped area. After incubation, migration of cells away and towards the chemoattractant-containing stripes is assessed (Knoll, Weinl et al. 2007).

The silicone matrices (purchased from Professor Martin Bastmeyer, Germany) were boiled in a microwave for 10 minutes and dried under laminar flow for an hour. Transparent sticky tape was then used to remove any dust from the striped side of the matrices. For the next step, the matrices were placed with the stripe side down onto plastic petri dishes, pressed down to remove

any bubbles and marked on the bottom side of the culture dish. 25µl FC-tagged BSA and 25µl FC-tagged CXCL12 were mixed and incubated at RT for 30 minutes. This mixture was then injected very carefully onto the control and CXCL12 matrix using a 22-G syringe (Hamilton, catalog #: 11552425) and incubated for 30 minutes at 37°C in the incubator.

After incubation, the dishes were removed from the incubator and unattached FC-mixture was removed by three PBS washes. For a PBS wash, 300µl of PBS was placed on the top of the matrix where the outlet channel was located and aspirated from the small inlet hole located on the side of the matrix. The matrix was then removed from the dishes, and $5x10^5$ cells were plated on the striped area. The Petri dish was once again placed in the incubator and incubated O/N at 37ºC. 24 hours after incubation, the plate was removed from the incubator and the cells were fixed with 4% PFA and stained using SOX2 antibody (Santa Cruz, catalog #: sc-365964) and DAPI. SOX2 is expressed in SK-N-BE (2) cells and was used as a proneural marker. Cells were then imaged using Nikon Eclipse TE2000U microscope and counted with Image J software. For quantification, the number of cells on the stripes to the total number of cells was identified as migrating cells. The Student's unpaired t-Test was used to determine statistical significance.

2.16 Immunoprecipitation (IP)

To identify posttranslational modifications on DLX2 using mass spectrometry, more than 100ng of the pure form of DLX2 protein was needed. Therefore, I conducted an immunoprecipitation assay using concentrated DLX2 antibody (DDE) to isolate DLX2 from E13.5 GE tissue. First, tissue was flash frozen in nitrogen and lysed with lysis buffer containing 1ml RIPA buffer, 40ul 25X cocktail, 50ul 1M NAF, 10ul 100mM vanadate and 10ul 100mM PMSF for 30 minutes on ice. Then, the sample was centrifuged and the supernatant was kept for further experiments. The amount of protein in lysates was quantified using a BCA Protein Assay

Kit (Thermo Scientific, catalog #: 23225) on a GeneQuant Spectrophotometer by preparing a standard curve made from 2mg/ml BSA and reading the absorbance at 562nm.

Following cell lysis, Protein G Sepharose beads (GE Healthcare, catalog #: 45000116) were washed with PBS and RIPA buffer. The next step was to pre-clear the samples by adding 50ul washed beads to 1000ug protein (for two IP samples) and rotating for 1 hour at 4ºC. The samples were centrifuged and the supernatant was kept for further use. Five percent of the supernatant was set aside as the sample pre-IP. The rest of the sample was divided into two tubes: 4ul concentrated DLX2 antibody was added to one tube and 10ul of Rabbit IgG (Santa Cruz, catalog #: sc-2027) was added to the second tube and centrifuged O/N with rotation at 4ºC. An IgG antibody was used as a negative control. On the next day, 100ul of washed Protein G Sepharose beads was added to each tube and rotated at 4ºC for 2.5 hours. Following the last incubation, the samples were centrifuged and the pellet was washed three times with 1ml RIPA buffer and once with washing buffer (50mM Tris, PH 8), carefully without disturbing the beads. Finally, the pellet was resuspended in 30ul 4X SDS buffer and heated at 100ºC for 5 minutes to linearize the isolated protein for Western Blot.

2.17 Western Blot

To identify posttranslational modifications using mass spectrometry, more than 100ng of protein is needed. Therefore, I explored DLX2 expression levels in the following cell lines by performing Western Blots: SK-N-SH, SH-SY5Y (neuroblastoma cell lines); Y79, WERI-RB1 (retinoblastoma cell lines); D341, D283 (medulloblastoma cell lines); PC-12 (cell-line from rat pheochromocytoma), RS4; 11 (leukemia cell line); and COLO 320 (colon carcinoma cell line). I also dissected E13.5 GE from embryos and performed Western Blots on tissue.

To collect protein, cells were trypsinized and collected. Cells were lysed in lysis buffer for 15 minutes on ice. The lysates were then collected and the amount of protein was quantified using a BCA Protein Assay Kit on a GeneQuant Spectrophotometer. 4X SDS buffer was then added to each sample and heated at 100^{o}C for 5 minutes to linearize the isolated protein and ensure sample denaturation.

Samples were then separated on a discontinuous SDS- PAGE (Polyacrylamide Gel Electrophoresis) gel with a 10% running gel containing 1.5mM Tris-HCL pH8.8, 40% acrylamide, 10% SDS, 10% APS, TEMED and a 5% stacking gel with 40% acrylamide, 0.5 M Tris, pH 6.8, 10% SDS, 10% APS, and TEMED. Precision Plus Protein Dual Color Standards (Biorad, catalog #: 1610374) were also loaded as a molecular weight marker. The samples were separated for 55 minutes at 180V in 1X running buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS. Proteins were transferred to nitrocellulose membranes in 1X transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) for 3 hours in the cold room. Membranes were blocked in 3% milk in TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) for an hour. Primary antibody was then diluted in 3% milk in TBST and added to the membranes O/N. Blots were washed three times in TBST for 5 minutes and incubated in secondary antibody (1:10000 goat anti rabbit horseradish peroxidase, Table 2.1) for an hour at RT. Subsequently the membranes were washed three times in TBST for 5 minutes. Chemiluminescence was detected using an ECL kit (GE, catalog #: RPN2108) and developed on X-ray film (Fuji) in a film cassette for 1 to 10 minutes. Each membrane was re-probed and detected for β-actin as a loading control.

2.18 Organotypic slice cultures

Organotypic slice cultures of embryonic mouse forebrain were modified from Anderson et al. 1997 (Anderson, Eisenstat et al. 1997). E13.5 and E14.5 timed pregnant mice were sacrificed

and the embryos were collected and placed on Krebs buffer containing 126 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 11 mM glucose, 1.2 mM NaH2PO4, and 5 mM NaH03 on ice. The brains were removed and embedded in 4% ultrapure low melting point agarose (Invitrogen, catalog #: 16520100). Sections of 400μm were cut and placed in post slicing Krebs buffer (10 mM HEPES, penicillin, streptomycin, and gentamicin, filter sterilized, pH 7.4) on ice for 15 minutes. The sections were then carefully placed on polycarbonate membranes (diameters 13mm; pore size 8μm) in 12 well tissue culture dishes. 1ml brain slices medium containing MEM with glutamine (Gibco, catalog #: 11095072), 10% FBS, 1% penicillin /streptomycin antibiotics were added to each well and incubated at 37°C for 1 hour. After an hour of incubation the media was exchanged with Neurobasal media containing 2% B-27. DiI crystals (1, 19-dihexadecyl-3, 3, 39-tetramethylindocarbocyanine perchlorate) (Invitrogen, catalog #: D282) were then placed on the GE using a fine pin under a dissection microscope under the laminar flow hood. The cultures were then incubated at 37ºC for various times (24, 30, 48, 72 hours) in the incubator. Slices were then fixed using 4% PFA at 4°C for at least 1 hour. Finally, slices were mounted on glass slides and visualized using Nikon Eclipse TE2000U platform microscope with rhodamine fluorescence filters.

2.19 Statistical analysis

Results are shown as mean ± standard error of the mean (SEM). All the qPCR experiments, luciferase reporter assays and migration assays were performed in three biological and three technical replicates. Statistical analyses were done by Student's t-Test and results were statistically significant when $p < 0.05$.

Table 2.1: List of antibodies used in the study

Antibody	Source	Dilution	Primary/ Secondary	Catalog #
Dlx2	Dr. D. Eisenstat	1:200	Primary	N/A
Dlx2 (E-7)	Santa Cruz	1:1000	Primary	sc-390468
Nkx2.2	Dr. T. Jessell	1:4000	Primary	N/A
Cxcr4	Abcam	1:300	Primary	Ab1670
Sox2	Santa Cruz	1:200	Primary	sc-17320
Alexa Fluor 488	Invitrogen	1:200	Secondary	A21206, A11055
Alexa Fluor 594	Invitrogen	1:200	Secondary	A11058, A21207
Biotinylated anti-mouse	Vector Labs	1:200	Primary	BA-2000
Streptavidin 488	Invitrogen	1:200	Secondary	S32354
Streptavidin 594	Invitrogen	1:200	Secondary	S32356
goat anti-rabbit HPR	Invitrogen	1: 10000	Secondary	65-6120
goat anti-mouse HPR	Invitrogen	1: 10000	Secondary	31430

Table 2.2: List of primers used in the study for ChIP

Primer name	Primer sequence (5' to 3')
Nkx2.2 R1-F	CTTGTGCTACCTCAGTGAAC
Nkx2.2 R1-R	CCATTTCCTTGTTAGACTACC
Nkx2.2 R2-F	GGCAACAGGCTCTAACGC
Nkx2.2 R2-R	CGATTGCTAAGCTGCGGAC
Nkx2.2 R3-F	AGCTCTGGCATGTCCAAGC
Nkx2.2 R3-R	GGTTTCTACCTCTCCACGC
Nkx2.2 R4-F	TTCAAGTGTGTGGACTCGAG
Nkx2.2 R4-R	CCATATAAGGCTGGGCTCC
Nkx2.2 R5-F	CCAGTCACAGCCTACATTTC
Nkx2.2 R5-R	AGTTGTAGCCTCACTTGGTC
Cxcr4 R1-F	GGAGCTACGCTACTAGTCC
Cxcr4 R1-R	GCAGTCARGCTACAAAGCTC
Cxcr4 R2-F	GAGCTTTGTAGCATGACTGC
Cxcr4 R2-R	CTTTAGCTCCTGTGAAGTCTC
Cxcr4 R3-F	GAGACTTCACAGGAGCTAAAG
Cxcr4 R3-R	GTGAGCTGTGTCTAGCCTC
Cxcr4 R4-F	GAGGCTAGACACAGCTCAC
Cxcr4 R4-R	CTTTCGGTTGGAAGTGCTGG
Cxcr4 R5-F	CCAGCACTTCCAACCGAAAG
Cxcr4 R5-R	GCGCCCTCAACTCCATCC
Cxcr4 R6-F	CTGCTCCATTTGGTCTCTTG

Cxcr4 R6-R	CAGAAAGCACTTGCGGACC
Cxcr4 R7-F	GGTCCGCAAGTGCTTTCTG
Cxcr4 R7-R	CCAAGAGCCACTGCTGTTG

Table 2.3: List of primers used in the study for cloning

Primer name	**Primer sequence (5' to 3')**
Nkx2.2 R1-F-*KpnI*	ATCAGGTACCGTCCCAAAGCACCCAA
Nkx2.2 R1-R-*SacI*	GACTGAGCTCCCATTTCCTTGTTAGAC
Nkx2.2 R2-F-*XhoI*	GATCCTCGAGGGCAACAGGCTCTAAC
Nkx2.2 R2-R-*BglII*	CATGAGATCTCGATTGCTAAGCTGCGG
Nkx2.2 R3-F-*KpnI*	CATAGGTACCAGCTCTGGCATGTCCAA
Nkx2.2 R3-R-*SacI*	ACACGAGCTCGGTTTCTACCTCTCCAC
Nkx2.2 R4-F-*KpnI*	GATCGGTACCTTCAAGTGTGTGGACTC
Nkx2.2 R4-R-*NheI*	ATGAGCTAGCGAGGAAAAGAGGGAG
Nkx2.2 R5-F-*KpnI*	TCAGAGGTACCCCAGTCACAGCCTAC
Nkx2.2 R5-R-*SacI*	GATCGAGCTCAGTTGTAGCCTCACTTG
Cxcr4 R1-F-*KpnI*	GATCGGTACCGGAGCTACGCTACTAG
Cxcr4 R1-R-*MluI*	GATCACGCGTGCAGTCATGCTACAAA
Cxcr4 R2-F-*KpnI*	GATCGGTACCGAGCTTTGTAGCATGAC
Cxcr4 R2-R-*BglII*	GATCAGATCTCTTTAGCTCCTGTGAAG
Cxcr4 R3-F-*KpnI*	ACAAGGTACCGAGACTTCACAGGAGCTA
Cxcr4 R3-R-*SacI*	ACAAGAGCTCGTGAGCTGTGTCTAGCCT
Cxcr4 R4-F-*KpnI*	ACAAGGTACCGAGGCTAGACACAGCTCA
Cxcr4 R4-R-*SacI*	ACAAGAGCTCCTTTCGGTTGGAAGTGCT
Cxcr4 R5-F-*KpnI*	TACGGGTACCGAGACTTCACAGGAGCTA
Cxcr4 R5-R-*MluI*	TACGACGCGTGCGCCCTCAACTCCATCC
Cxcr4 R6-F-*KpnI*	GATCGGTACCCTGCTCCATTTGGTCT

Cxcr4 R6-R-*BglII*	CGATAGATCTCAGAAAGCACTTGCGG
Cxcr4 R7-F-*KpnI*	ACAAGGTACCGGTCCGCAAGTGCTTTCT
Cxcr4 R7-R-*SacI*	ACAAGAGCTCCCAAGAGCCACTGCTGTT

Table 2.4: List of primers used in the study for site directed mutagenesis

Primer name	**Primer sequence (5' to 3')**
Nkx2.2 R3-1M-F	CTAGAATCAATTGCTTGACTTGTC
Nkx2.2 R3-1M-R	GACAAGTCAAGCAATTGATTCTAG
Nkx2.2 R3-2M-F	CAAATCGCTAAGTTTCTGGC
Nkx2.2 R3-2M-R	GCCAGAAACTTAGCGATTTG
Nkx2.2 R4-1M-F	ATCTTTGCAATTAATTGACACGTTACA
Nkx2.2 R4-1M-R	TGTAACGTGTCAATTAATTGCAAAGAT
Nkx2.2 R4-2M-F	GCCAACCTTTGCGGTTTGTCTCTTCCCC
Nkx2.2 R4-2M-R	GGGGAAGAGACAAACCGCAAAGGTTGG

Table 2.5: List of primers used in the study for qRT-PCR

Primer name	**Primer sequence (5' to 3')**
Dlx2-F	GCCTCACCCAAACTCAGGT
Dlx2-R	AGGCACAAGGAGGAGAAGC
Nkx2.2 cDNA-F	GCGGAGAAAGCATTTCAAAACC
Nkx2.2 cDNA-R	TCGCCCGACCTGAAATTGTT
Cxcr4 cDNA-F	CCGATCAGTGTGAGTATATAC
Cxcr4 cDNA-F	CATAAGTGTTAGCTGGAGTG
Gapdh-F	ACCATCCGGGTTCCTATAAAT
Gapdh-R	CAATACGGCCAAATCCGTT

Table 2.6: Sequence of oligos in the study

Oligo name	Oligo sequence (5' to 3')
CXCR4 R1.1 F	CCCCTTAGTAATAACAACTCTTCACAA
CXCR4 R1.1 R	TTGTGAAGAGTTGTTATTACTAAGGGG
CXCR4 R1.2 F	GGCTACTCAGCTATTACTAACTATT
CXCR4 R1.2 R	AATAGTTAGTAATAGCTGAGTAGCC
CXCR4 R1.3.4 F	CTATTCTATTATTAACACTATTGTCA
CXCR4 R1.3.4 R	TGACAATAGTGTTAATAATAGAATAG
CXCR4 R2.1.2 F	AGGTAGAATTACTAATCTCAAAAGGT
CXCR4 R2.1.2 R	ACCTTTTGAGATTAGTAATTCTACCT
CXCR4 R2.3 F	AATCCAGAAGCCAAATATTATCTTTGAAA
CXCR4 R2.3 R	CTTTCAAAGATAATATTTGGCTTCTGGAT
CXCR4 R2.4 F	TTTTTTTTTCTCTAATTCTATAGTCAC
CXCR4 R2.4 R	GTGACTATAGAATTAGAGAAAAAAAAA
CXCR4 R3.1 F	CTTTTGTCCACCAATAATCAACAA
CXCR4 R3.1 R	TTGTTGATTATTGGTGGACAAAAG
CXCR4 R3.2.3.4 F	AAATATTAATTATGCAAATCATTTTACTC
CXCR4 R3.2.3.4 R	GAGTAAAATGATTTGCATAATTAATATTT
CXCR4 R4.1 F	ACCGATCTTGAACACCAGCGCTCTTTTAA
CXCR4 R4.1 R	TGGACCGGAATATCAGACCCATTAAAAG
CXCR4 R4.2 F	TCTGTCCTAACTAGTTTATGGAAATATGA
CXCR4 R4.2 R	CAATCATTACTAAAACATAGGCTCATATT
CXCR4 R4.3.4 F	GATATAATTAAAAGTGCTTGTGGAAGTTC

CXCR4 R4.3.4 R	AAACTGCTAAGTTAAAAGCCAAGAACTT
CXCR4 R4.5 F	AGGGTCGCACTTCCTAATTTCATCCTTAG
CXCR4 R4.5 R	CACTTATGTGATCCAGAAAAGCTAAGGA
CXCR4 R5.1 F	GTGGCACACAACTGTAATCCCAGTAC
CXCR4 R5.1 R	GTACTGGGATTACAGTTGTGTGCCAC
CXCR4 R6.1 F	TGGGACTCTGTAATTATCTGTTAACTAG
CXCR4 R6.1 R	CTAGTTAACAGATAATTACAGAGTCCCA
CXCR4 R6.2 F	GTGCGGATTTCTTAGGATGTGATTACACA
CXCR4 R6.2 R	AATTGAGCGGCGAGGACTTTGTGTAATC
CXCR4 R6.3 F	GCTCAATTCTTTATAATAAAGCACATTTC
CXCR4 R6.3 R	TGTGTGGTGATGTAAAATGATTGAAATGT
CXCR4 R7.1 F	TTCTGCCACACCTGACCCTAATG
CXCR4 R7.1 R	CATTAGGGTCAGGTGTGGCAGAA
CXCR4 R7.2 F	TCATTATATGAATATGTAGTAACACACAC
CXCR4 R7.2 R	CTCTTCCAAGCAAGTGTGTGTTACTACAT
CXCR4 R7.3 F	TTTTGGTAGTAATCACTCCTGACAGCTCA
CXCR4 R7.3 R	TCCGCCAGGGTTCTATACTCTGAGCTGTC
CXCR4 R7.4 F	GCTTTCCCCAACTCTGAGAACATTATCCG
CXCR4 R7.4 R	CAGTATGGGTCCAGAGCCTGGCCGGATA

3 Chapter 3: Transcriptional regulation of *Nkx2.2* by DLX2 during forebrain development

3.1 Introduction

DLX transcription factors play key roles in the development of the forebrain, retina, craniofacial structures and limbs (Eisenstat, Liu et al. 1999, Panganiban and Rubenstein 2002). During CNS development, *Dlx1* and *Dlx2* are involved in the tangential migration of GABAergic interneurons from the basal telencephalon to the cortex (Anderson, Qiu et al. 1997, Eisenstat, Liu et al. 1999). The receptor for brain-derived neurotrophic factor, TrkB, is regulated by DLX2 during retinal development (de Melo, Zhou et al. 2008). Down regulation of *Nrp2* by *Dlx* genes may promote the tangential migration of interneurons from the subpallium (Le, Du et al. 2007). Furthermore, *Dlx1* and *Dlx2* genes are key transcription factors involved in neuronal versus glial cell fate switch where they promote interneuron differentiation and repressor oligodendrocyte differentiation. Previous studies in the Eisenstat lab have shown that *Gad2/Gad1*, which are involved in the synthesis of GABA from the excitatory neurotransmitter glutamate, are directly regulated by DLX1 and DLX2 (Le, Zhou et al. 2017). Furthermore, *Dlx1/2* act upstream of *Olig2* to inhibit oligodendrocyte production (Petryniak, Potter et al. 2007). Although several DLX1/2 downstream targets have been identified, our understanding of *Dlx* gene function in brain development and its role in GABAergic interneuron differentiation remains limited.

NKX2.2 is a homeodomain containing transcription factor involved in the differentiation of oligodendrocyte progenitor cells (Qi, Cai et al. 2001, Zhu, Zhao et al. 2014). In the *Dlx1/2* mutant mice, expression of *Nkx2.2* increases compared to the WT (Petryniak, Potter et al. 2007). Since *Dlx1/2* are key regulators of neuronal versus glial cell fate determination and in the absence of *Dlx1/2,* expression of the oligodendrocyte differentiating gene *Nkx2.2* increases, I investigated the role of DLX2 in *Nkx2.2* expression during murine forebrain development. I hypothesized that DLX2 directly mediates transcriptional repression of oligodendroglial

differentiation in determination of neural versus glial cell fates during forebrain development, in part through inhibition of *Nkx2.2*.

3.2 Results

3.2.1 The *Nkx2.2* proximal regulatory region contains several putative DLX2 binding sites

DLX homeodomain proteins regulate transcription through binding to conserved TAAT/ATTA motifs. I labelled each TAAT/ATTA motif present in the proximal regulatory region as a putative DLX2 binding site. There are 11 putative DLX2 binding sites located approximately 3 kilo base pairs (kb) upstream of the *Nkx2.2* gene. In order to make experiments manageable, this regulatory sequence was arbitrarily divided into 5 regions, R1 to R5. Figure 3.1 shows a schematic of the *Nkx2.2* proximal regulatory sequence containing putative DLX2 binding sites.

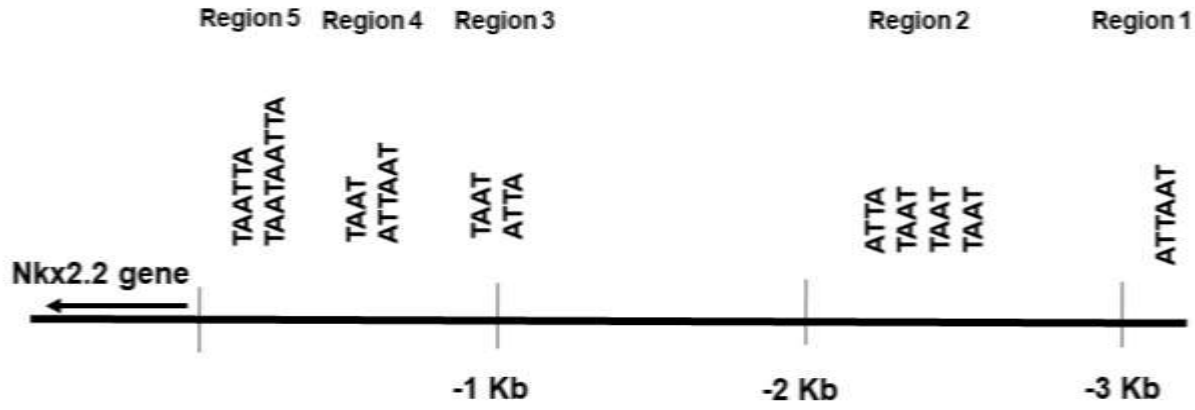


Figure 3.1. Schematic diagram of the *Nkx2.2* regulatory element highlighting putative DLX2 binding sites. All of the TAAT/ATTA motifs present in the proximal regulatory region approximately 3kb upstream of the *Nkx2.2* gene were assigned as putative DLX2 binding sites. There are 11 putative DLX2 binding sites located in the proximal regulatory region of *Nkx2.2*.

3.2.2 *In silico* prediction of DLX2 binding sites in *Nkx2.2* promoter region

To better investigate potential binding sites for DLX2, *in silico* analysis for DLX2 DNA-binding profiles from multiple databases were performed 5kb upstream of the mouse *Nkx2.2* gene from the mm10 assembly. *In silico* analysis revealed 23 significant motif occurrences with 7 highly conserved non-redundant putative DLX2 binding sites (Figure 3.2 A). Predicted DLX2 binding sites were observed in regions 2-5 (Figure 3.2 A).

3.2.2.1 *Dlx2* and *Nkx2.2* display opposing activity during murine forebrain and midbrain development

During CNS development in mice *Dlx2* is highly expressed in the forebrain and minimal levels of *Dlx2* expression are observed in the midbrain. Conversely, *Nkx2.2* expression is higher in the midbrain compared to the forebrain during CNS development in mice (Figure 3.2 B). When comparing histone modifications and DNA accessibility across the *Dlx2* and *Nkx2.2* loci in the forebrain and midbrain of embryonic tissues, there is a reciprocal deposition of active and inactive posttranslational modifications and DNA accessibility in forebrain and midbrain embryonic tissues (Figure 3.2 B). Together with the motif analyses, these findings support a model of DLX2 repression of *Nkx2.2* in forebrain, but not in midbrain where *Dlx2* is expressed at a lower level. All *in silico* analysis was performed by Ms. Kirby Ziegler and results were provided by Dr. Alan Underhill.

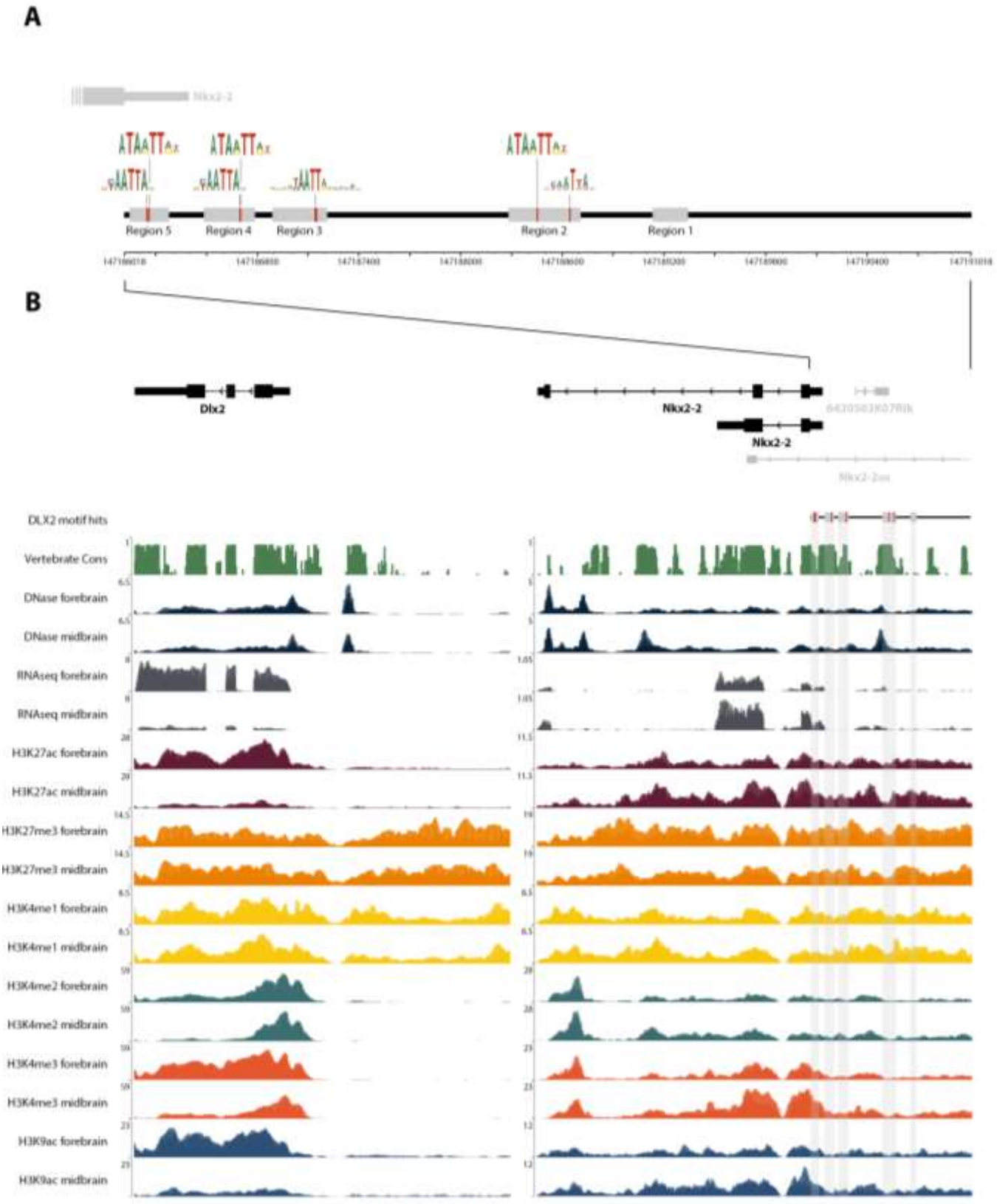

A
Region 5
Region 4
Region 3
Region 2
Region 1
B
Dlx2
Nkx2-2
Nkx2-2
DLX2 motif hits
Vertebrate Cons
DNase forebrain
DNase midbrain
RNAseq forebrain
RNAseq midbrain
H3K27ac forebrain
H3K27ac midbrain
H3K27me3 forebrain
H3K27me3 midbrain
H3K4me1 forebrain
H3K4me1 midbrain
H3K4me2 forebrain
H3K4me2 midbrain
H3K4me3 forebrain
H3K4me3 midbrain
H3K9ac forebrain
H3K9ac midbrain

Figure 3.2. Reciprocal epigenomic features of *Dlx2* and *Nkx2.2* loci during development (E10.5 to P0) with predicted *in silico* DLX2 binding sites.

A. Putative DLX2 binding sites (*red*) within biochemically assessed Regions 1-5 (*grey*) are depicted in the context of the FIMO query sequence (*black*), representing genomic coordinates (chromosome 2: 147,186,018 - 147,191,018; mm10). A portion of the *Nkx2.2* gene is shown for reference (*top, grey*). Motif occurrences with the highest PWM score are illustrated as sequence logos for each predicted DLX2 binding site.

B. *Top, left*, *Dlx2* locus with 5000bp promoter region (chromosome 2: 71,543,360 - 71, 551,754; mm10). *Right*, *Nkx2.2* locus with 5000bp FIMO query region containing potential DLX2 binding sites (*red*) within experimentally assessed genomic sequences (*vertical grey highlight*) (chromosome 2: 147,177,490 - 147,191,404; mm10). Genomic features; *Top, green*, evolutionary conservation across 60 vertebrate species (phastCons60way); Epigenomic trends in forebrain and midbrain embryonic tissues displayed as overlay of samples from embryonic days 10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5 and post-natal day 0 (P0); DNA accessibility, characterized by DNase-seq peaks **(***navy*); gene expression delineated by RNA-seq signal (*dark grey*); ChIP-seq of posttranslational histone modifications associated with transcriptional activation: histone 3 lysine 27 acetylation (H3K27ac, *burgundy*); histone 3 lysine 4 mono-methylation (H3K4me1; *yellow*); histone 3 lysine 4 di-methylation (H3K4me2; *teal*); histone 3 lysine 4 tri-methylation (H3K4me3; *coral*); histone 3 lysine 9 acetylation (H3K9ac; *blue*); and transcriptional repression: histone 3 lysine 27 tri-methylation (H3K27me3; *orange*).

All *in silico* analysis was performed by Ms. Kirby Ziegler and figure and figure legends were provided by Dr. Alan Underhill and Ms. Kirby Ziegler.

3.2.3 *Nkx2.2* is a transcriptional target of DLX2 *in vivo*

In order to identify DLX2 transcriptional targets important for forebrain development *in vivo*, ChIP experiments on E13.5 wild-type GEs were carried out. DLX2 is highly expressed between E12.5 to E14.5 during mouse forebrain development; therefore, E13.5 was chosen as a time point where DLX2 is abundantly expressed. Protein were crosslinked with DNA using formaldehyde and concentrated DLX2 antibody was added to precipitate the protein-DNA complexes. PCR was then performed with primers flanking putative DLX2 binding sites to amplify DLX2 bound regulatory regions of *Nkx2.2* Enrichment by DLX2 antibody of *Nkx2.2* regulatory elements at sites designated region 1 to 4 (R1- R4) was observed *in vivo* (Figure 3.3).

These four regions were used for subsequent regulatory region characterization and R5 was set aside as a non-binding region since no enrichment at R5 was observed *in vivo* (Figure 3.3 E). For controls, ChIP samples without antibody as well as samples from hindbrain tissue were used. Hindbrain was used as a tissue control since DLX2 is not expressed in this tissue (Zhou, Le et al. 2004). ChIP experiments without antibody or from the hindbrain tissue were negative. The positive amplicons from ChIP were sub-cloned into pGL3 Basic Vector for subsequent biochemical analysis.

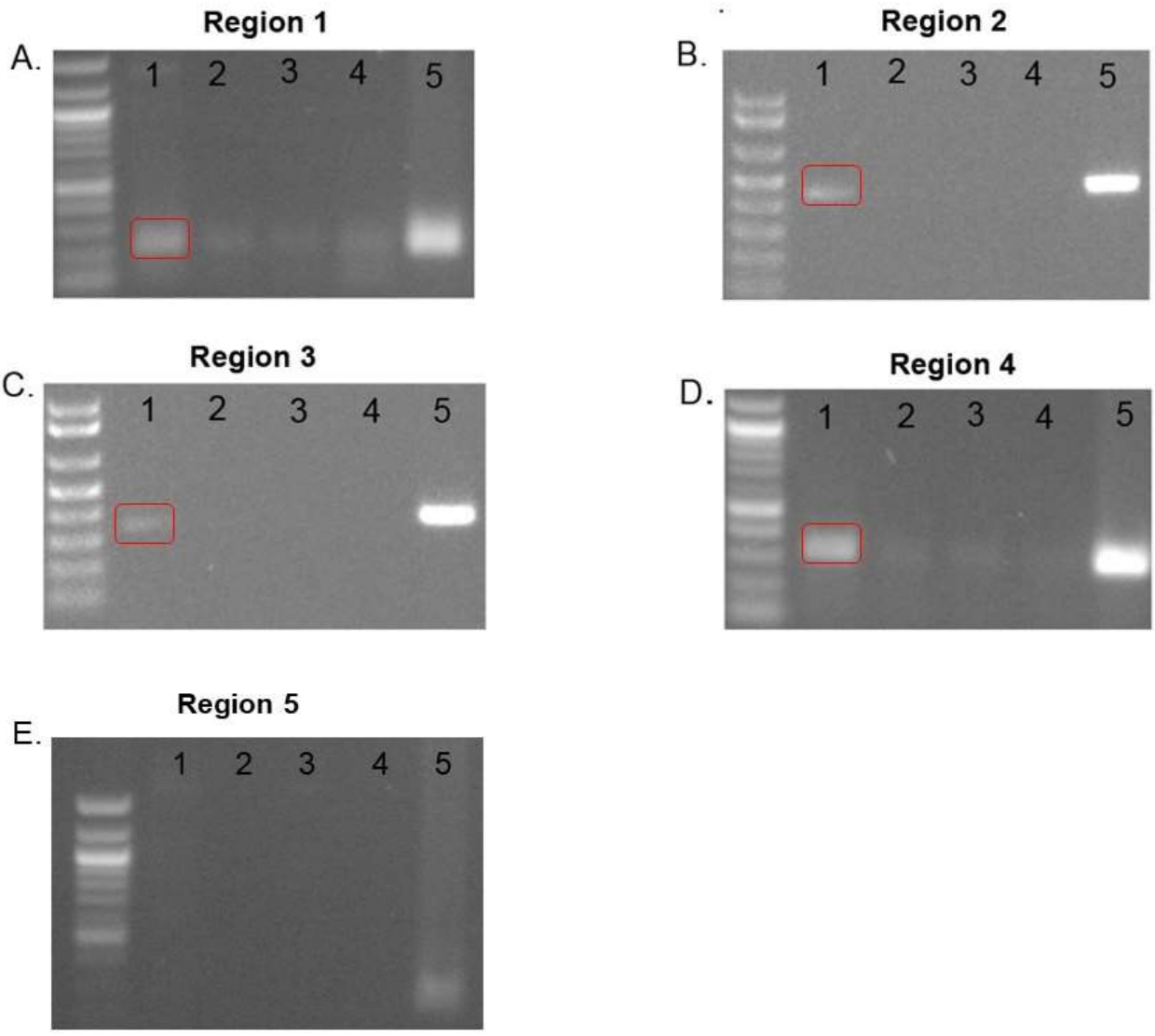

A.
Region 1
1 2 3 4 5
B.
Region 2
1 2 3 4 5
C.
Region 3
1 2 3 4 5
D.
Region 4
1 2 3 4 5
E.
Region 5
1 2 3 4 5

Figure 3.3. DLX2 binds to *Nkx2.2* regulatory elements *in vivo*.

A-D. DLX2 occupies the *Nkx2.2* promoter regions 1, 2, 3 and 4 in the developing forebrain (Lane 1). ChIP was carried out with high affinity purified antibody to DLX2 (Ab). Controls included a negative antibody control where no DLX2 antibody was added, along with negative hindbrain (HB) tissue as DLX2 is not expressed in the developing hindbrain. Enrichment for DLX2 binding was not observed in no-antibody controls (Lane 2 and 4) as well as negative tissue expression controls (Lane 3 and 4). Genomic DNA was also used as a positive PCR control for identification of DLX2 enrichment to chromatin (Lane 5).

E. DLX2 enrichment at R5 was not observed *in vivo.*

Lane 1: FB+Ab, Lane 2: FB-Ab, Lane 3: HB+Ab, Lane 4: HB-Ab, Lane 5: genomic DNA.

ChIP: chromatin immunoprecipitation, FB: forebrain, HB: hindbrain, gDNA: genomic DNA, Ab: antibody.

3.2.4 **DLX2 binds to the *Nkx2.2* regulatory region *in vitro***

ChIP experiments reveal the occupancy of DLX2 on the *Nkx2.2* regulatory region. To investigate specificity of DLX2 binding to *Nkx2.2* regulatory elements, EMSA were performed. *Nkx2.2* regulatory regions identified by ChIP were radiolabelled with [α-^{32}P]-dGTP (Perkin Elmer), incubated with recombinant DLX2 protein and size separated on a polyacrylamide gel to resolve free probe from DLX2 bound probes. Direct binding of DLX2 to labelled R1 and R2 regulatory region was not observed (Figure 3.4, lanes 4 and 6). Direct binding of DLX2 to labelled R3 and R4 regulatory region resulted in shifted bands compared to the free labelled DNA probes *in vitro* due to an increase in the molecular weight of the complex (Figure 3.4, lanes 8 and 11). Furthermore, addition of DLX2-specific antibody to DLX2-*Nkx2.2* probe complexes for R3 and R4 resulted in a supershift due an increase to the molecular weight of the protein-DNA complex (Figure 3.4, lanes 9 and 12). For R4, addition of excess unlabelled *Nkx2.2* probe competitively inhibited binding of labelled *Nkx2.2* to DLX2, demonstrating specificity of DLX2 binding to the *Nkx2.2* regulatory region (Figure 3.4, lane 13).

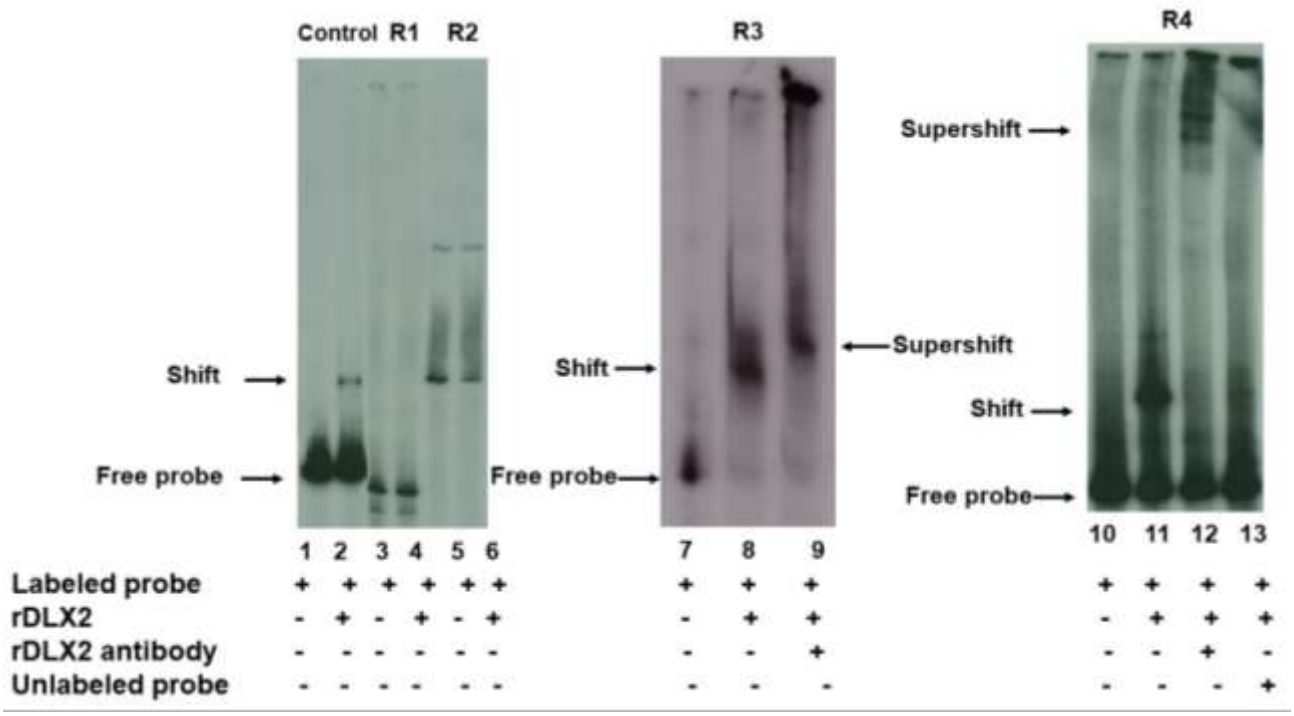


Figure 3.4. EMSA reveals that DLX2 directly binds to *Nkx2.2* regions 3 and 4 *in vitro*.

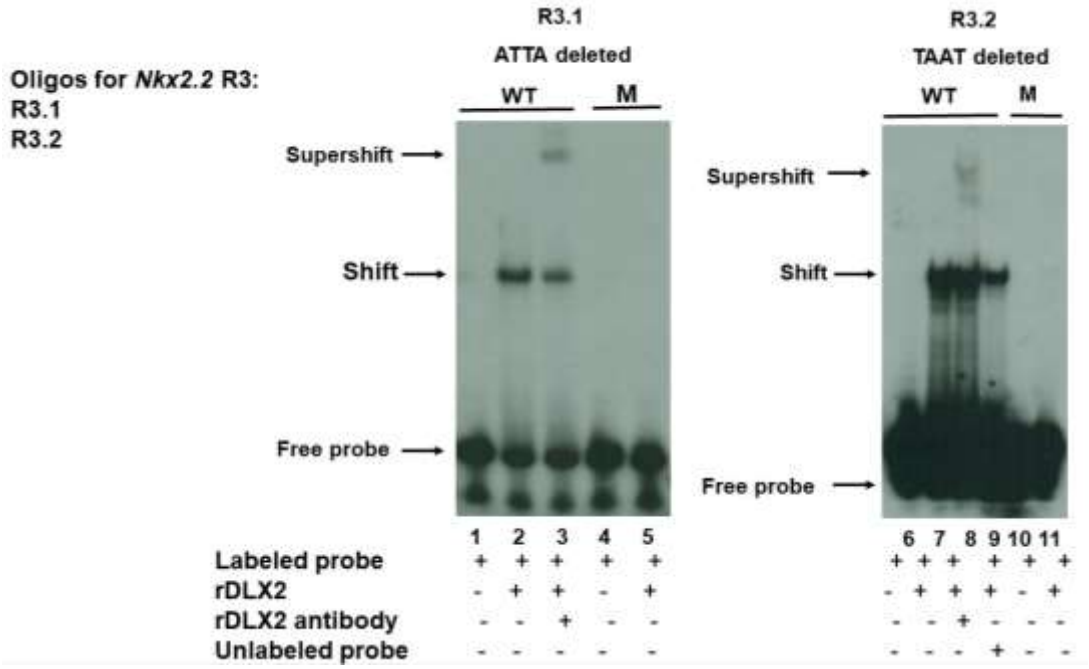
EMSA carried out on radiolabelled ChIP positive *Nkx2.2* promoter regions (R1 to 4) demonstrated no specific binding to regions 1 and 2 (lanes 4 and 6). Specific binding of recombinant DLX2 to regions 3 and 4 of the *Nkx2.2* promoter *in vitro* resulted in a shift of DLX2/*Nkx2.2* complexes in a polyacrylamide gel compared to free labelled DNA probes (lanes 8 and 11). In the presence of DLX2 antibody, a supershift was observed confirming the specificity of binding (lanes 9 and 12). Multiple shifts and supershifts can indicate different patterns of binding or oligomerization of the recombinant protein. Furthermore, addition of excess unlabelled *Nkx2.2* probe to radiolabelled R4 competitively inhibited binding of labelled Nkx2.2 to DLX2 (lane 13).

rDLX2: recombinant DLX2

3.2.5 Critical *Nkx2.2* binding sites specific for DLX2 binding *in vitro*

To further assess the specificity of DLX2 binding to *Nkx2.2*, site-directed deletion of the putative DLX2 binding sites followed by EMSA was also carried out. *Nkx2.2* region 3 contains a TAAT and an ATTA motif, region 4 contains a TAAT and an ATTAAT motif (Figure 3.1). Each of these TAAT/ATTA motifs within the positive EMSA regions R3 and R4 were separately deleted using the overlap extension PCR method (Lee, Shin et al. 2010). Conducting EMSA on each of the deleted binding sites in the *Nkx2.2* R3 and R4 probes showed reduced binding of recombinant DLX2 to the radiolabelled probe in the deleted sites compared to the WT (Figure 3.5). Collectively, these results demonstrate that recombinant DLX2 directly binds to regulatory elements of *Nkx2.2* and supports *Nkx2.2* as a transcriptional target of DLX2.

A.


Oligos for Nkx2.2 R3:
R3.1
R3.2
R3.1
ATTA deleted
WT
M
Supershift
Shift
Free probe
R3.2
TAAT deleted
WT
M
Supershift
Shift
Free probe
1 2 3 4 5
6 7 8 9 10 11
Labeled probe
rDLX2
rDLX2 antibody
Unlabeled probe

B.

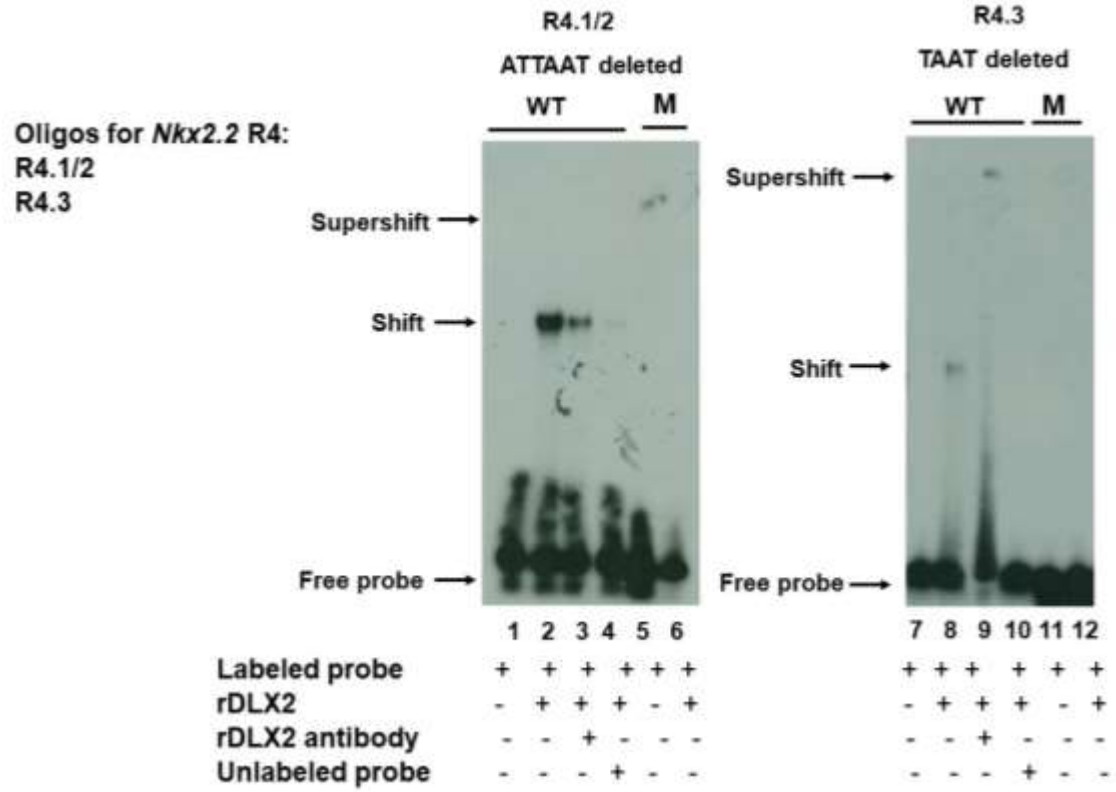

Oligos for Nkx2.2 R4:
R4.1/2
R4.3
R4.1/2
ATTAAT deleted
WT
M
Supershift
Shift
Free probe
R4.3
TAAT deleted
WT
M
Supershift
Shift
Free probe
1 2 3 4 5 6
7 8 9 10 11 12
Labeled probe
rDLX2
rDLX2 antibody
Unlabeled probe

Figure 3.5. Specific binding of rDLX2 at *Nkx2.2* regulatory elements R3 and R4 *in vitro*.

A. EMSA was carried out on radiolabelled *Nkx2.2* promoter region 3. In the WT, addition of rDLX2 to each binding site resulted in a shift revealing specific binding and incubation of probe-rDLX2 protein complexes with a DLX2-specific antibody generated a supershift. Once the TAAT and ATTA motifs in region 3 were separately deleted (M), no binding of labelled probe was observed in the presence of rDLX2.

B. EMSA was carried out on radiolabelled *Nkx2.2* promoter region 4. In the WT addition of rDLX2 resulted in a shift showing specific binding and incubation of probe-rDLX2 protein complexes with a DLX2-specific antibody generated a supershift. Once the TAAT and ATTAAT motifs in region 4 were separately deleted (M), no binding of labelled probe was observed in the presence of rDLX2.

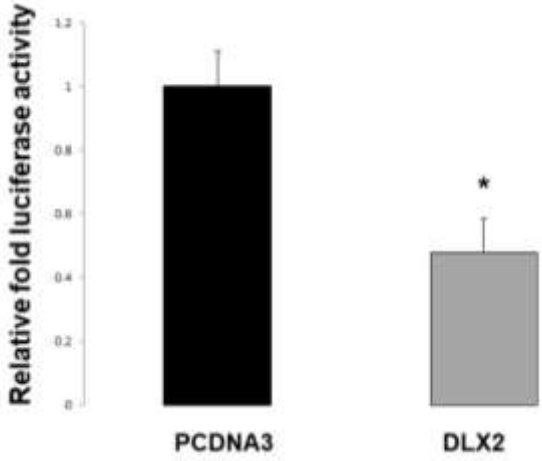
Deletion of the DLX2 binding sites in R3 and R4 further verified the specificity of DLX2 binding to putative motifs identified in the *Nkx2.2* regulatory elements.

rDLX2: recombinant Dlx2; WT: wild-type; M: mutant.

3.2.6 **Dlx2 represses expression of *Nkx2.2 in vitro***

I hypothesized that DLX2 represses *Nkx2.2* expression that may lead to transcriptional repression of oligodendroglial differentiation during forebrain development. To assess the functional consequence of DLX2 binding to *Nkx2.2* regulatory elements *in vitro*, luciferase reporter gene assays were performed. ChIP positive *Nkx2.2* regulatory regions were sub-cloned upstream of a luciferase reporter gene and co-transfected into HEK293 cells with a *Dlx2* expression plasmid. Co-transfection of *Dlx2* expression plasmids with plasmids containing *Nkx2.2* regulatory elements resulted in a significant decrease in luciferase activity *in vitro*, consistent with transcriptional repression *in vivo* (Figure 3.6).

A.



B.

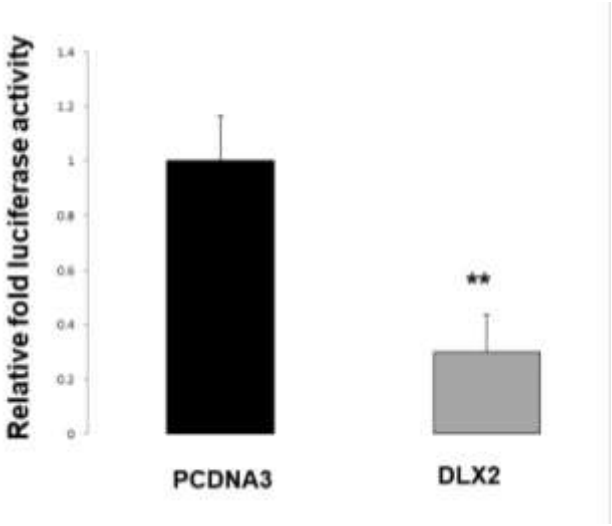


Figure 3.6. *Nkx2.2* is repressed upon *Dlx2* co-expression *in vitro*.

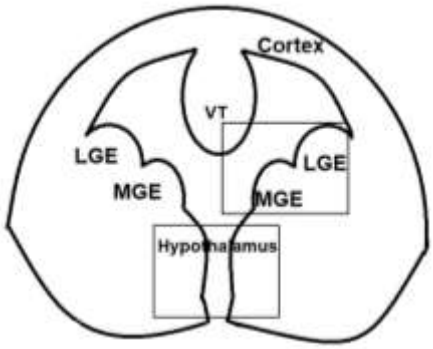
Nkx2.2 R3 and R4 were sub-cloned into pGL3 reporter vectors and co-transfected into HEK293 cells with a *Dlx2* expression plasmid. Co-transfection of *Dlx2* expression plasmids with reporter vectors containing *Nkx2.2* R3 (A) and R4 (B) regulatory elements resulted in significant downregulation of luciferase expression compared to controls. All luciferase results were normalized to β-galactosidase activity.

Error bars represent standard error of the mean. The assay was conducted in three biological and three technical replicates. * = P values <0.05, ** = P values <0.01.

3.2.7 **DLX2 and NKX2.2 spatial expression**

ChIP and EMSA revealed DLX2 binds to *Nkx2.2* regulatory elements. To elucidate the role of DLX2 in the spatial expression of *Nkx2.2* in the forebrain, immunofluorescence staining of WT and *Dlx1/Dlx2* DKO E13.5 tissue sections was performed. *Dlx* genes are expressed in GABAergic interneurons in the subpallial telencephalon by E10.5 (Eisenstat, Liu et al. 1999). DLX1 and DLX2 have an overlapping expression pattern in the VZ and SVZ of the MGE and LGE during development (Figure 1.7) (Panganiban and Rubenstein 2002). IF staining on E13.5 WT sections confirmed expression of DLX2 in basal telencephalon where neuronal progenitor cells in the VZ and SVZ of the LGE and MGE were positive for DLX2 expression (Figure 3.7 A).

Expression of NKX2.2 has been previously assessed in the developing forebrain showing expression in the boundary of alar and basal hypothalamus and also in a limited domain of the ventral thalamus (Puelles, Martinez-de-la-Torre et al. 2012). Here, I assessed the expression of NKX2.2 in the E13.5 WT forebrain. As expected, NKX2.2 was not expressed in the VZ or SVZ in the ganglionic eminences of WT tissue (Figure 3.7 D). Instead, consistent with previous reports, high levels of expression of NKX2.2 were observed in the WT hypothalamus (Figure 3.7 E-F). Co-expression studies of DLX2 and NKX2.2 revealed a minimally overlapping pattern of expression in the ganglionic eminences (Figure 3.7 G). However, both DLX2 and NKX2.2 were expressed in the hypothalamic region (Figure 3.7 H-I). DLX2 was expressed in the periventricular zone, medial zone and lateral zone of the hypothalamus. NKX2.2 expression was mainly observed in the periventricular zone and the lateral zone. The majority of $DLX2^{+}$/$NKX2.2^{+}$ cells were seen in the periventricular zone and the medial zone mainly consisted of only $DLX2^{+}$ cells (Figure 3.7 I).


Cortex
VT
LGE
MGE
LGE
MGE
Hypothalamus

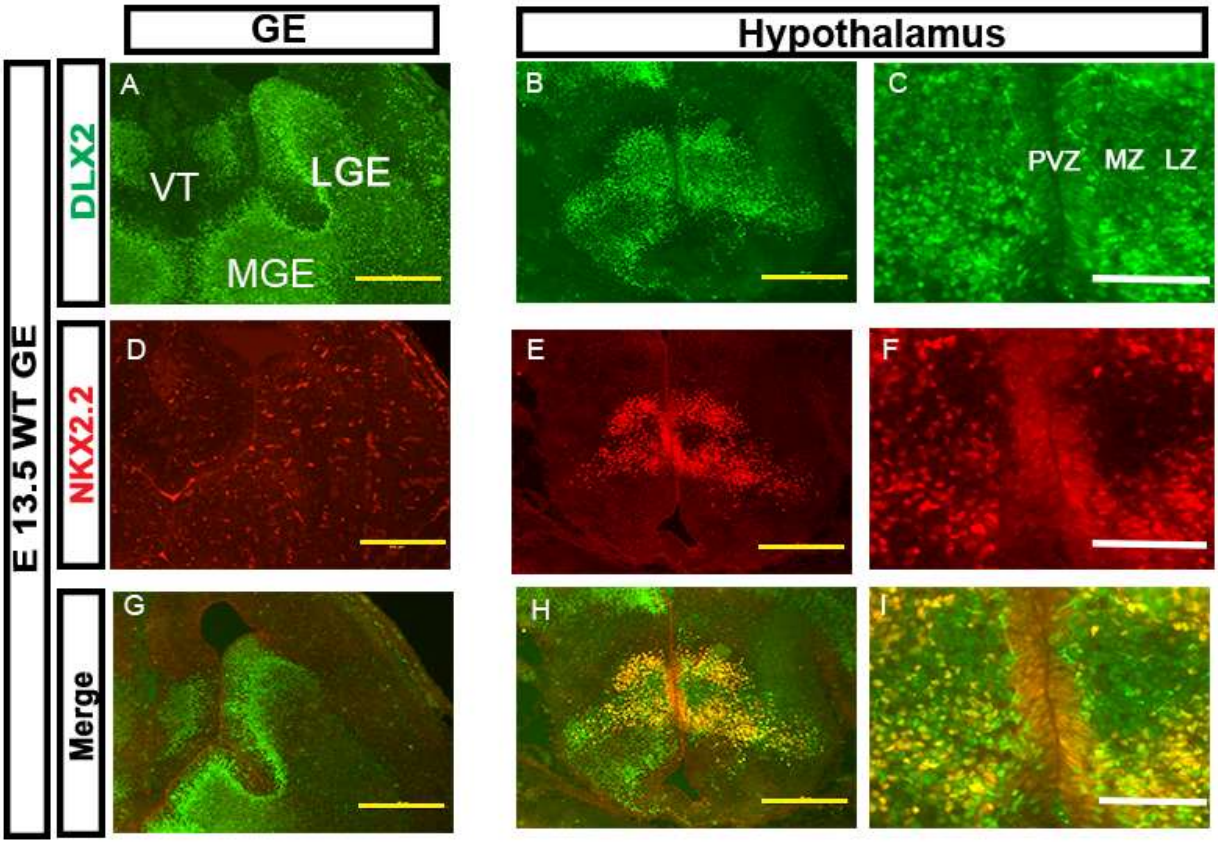

GE
Hypothalamus
E 13.5 WT GE
DLX2
NKX2.2
Merge
A
B
C
D
E
F
G
H
I
VT
LGE
MGE
PVZ
MZ
LZ

Figure 3.7. Spatial expression of DLX2 and NKX2.2 in the E13.5 WT forebrain.

Expression of DLX2 and NKX2.2 was assessed in E13.5 WT forebrain sections using immunofluorescence staining. DLX2 expression was observed in the MGE and LGE (A) as well as the hypothalamus (B and C). NKX2.2 expression was absent in the developing ganglionic eminences (D) but was observed in the hypothalamus (E and F). Co-expression of DLX2 and NKX2.2 is shown in panels G-I. There was no detected co-expression of DLX2 and NKX2.2 in the ganglionic eminences (G). DLX2 and NKX2.2 were co-expressed in the hypothalamic region with overlapping regions of single DLX^+ cells (H and I).

Yellow scale bars represent 100 μm (A, B, D, E, G and H) and white scale bars represent 50 μm (C, F and I).

MGE: Medial ganglionic eminences, LGE: lateral ganglionic eminences, VT: ventral thalamus, PVZ: periventricular zone, MZ: medial zone, LZ: lateral zone, GE: ganglionic eminences.

3.2.8 **NKX2.2 expression is increased in the *Dlx1/Dlx2* DKO forebrain**

To compare the spatial expression pattern of NKX2.2 between the WT and *Dlx1/Dlx2* DKO, IF was performed in *Dlx1/Dlx2* DKO tissue. NKX2.2 expression expanded dorsoventrally and laterally in the hypothalamus of the *Dlx1/Dlx2* DKO compared to WT forebrains (Figure 3.8 G-H). Interestingly, in the *Dlx1/Dlx2* DKO sections, NKX2.2 was expressed in the ventral thalamus (Figure 3.8 E-F). These findings support a role for DLX2 in repression of *Nkx2*.2, where in the absence of *Dlx1/Dlx2*, NKX2.2 expression was increased in the medial zone and lateral zone of the hypothalamus and was markedly increased in the ventral thalamus.

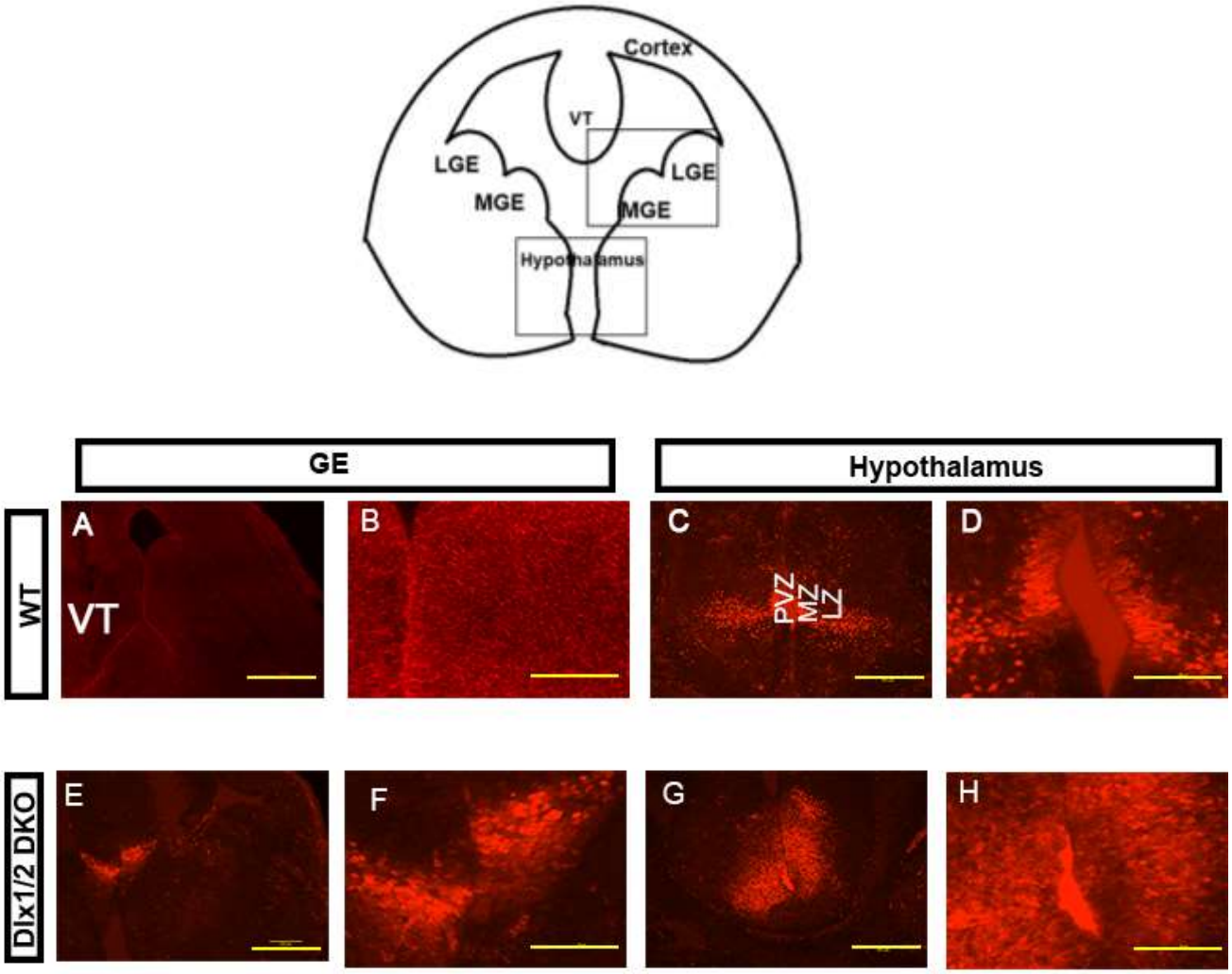


Figure 3.8. In the absence of *Dlx1/Dlx2* expression, NKX2.2 expression is upregulated in the E13.5 forebrain.

In the *Dlx1/Dlx2* DKO, NKX2.2 was expressed in the ventral thalamus where it is not usually expressed at high levels (E-F) and was over-expressed in the hypothalamus with expansion dorsoventrally and laterally into the medial and lateral zones (G-H).

Scale bars represent 100 μm.

MGE: Medial ganglionic eminences, LGE: lateral ganglionic eminences, VT: ventral thalamus, PVZ: periventricular zone, MZ: medial zone, LZ: lateral zone, GE: ganglionic eminences.

3.2.9 *Nkx2.2* transcript levels are increased in the *Dlx1/Dlx2* DKO forebrain

I also assessed the transcript levels of *Nkx2.2* in the WT and *Dlx1/2* DKO ganglionic eminences using qRT-PCR. I found a significant increase (P value= 0.0048) in the expression of *Nkx2.2* in the *Dlx1/Dlx2* DKO ganglionic eminences compared to WT (Figure 3.9). These results are consistent with my hypothesis that *Nkx2.2* is a transcriptional target for DLX2 *in vivo* where *Dlx1/Dlx2* transcription factors play a role in repressing the expression of *Nkx2.2* in the basal telencephalon during forebrain development.

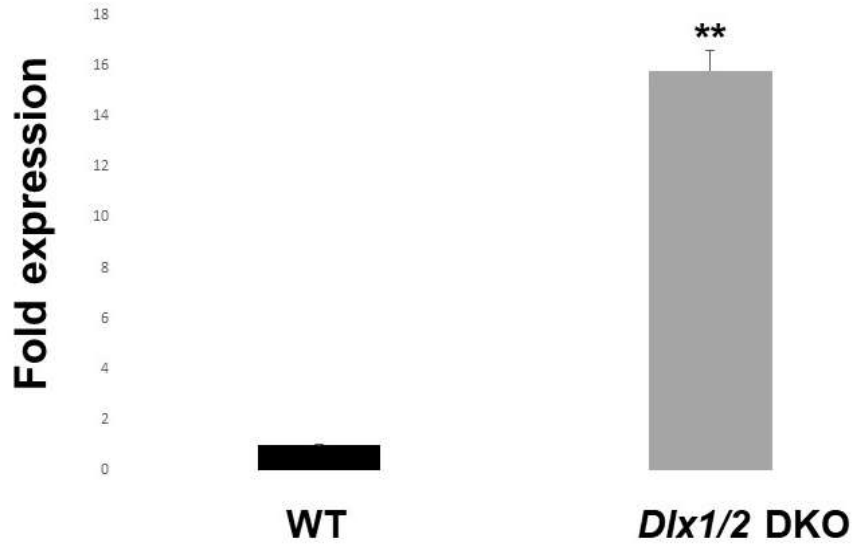


Figure 3.9. In the absence of *Dlx1/Dlx2,* NKX2.2 expression is upregulated in the E13.5 forebrain.

qRT-PCR on RNA extracted from WT and *Dlx1/Dlx2* null ganglionic eminences revealed a significant increase in NKX2.2 expression in DKO forebrains compared to controls.

Error bars represent standard error of the mean.

The assay was conducted in three biological and three technical replicates.

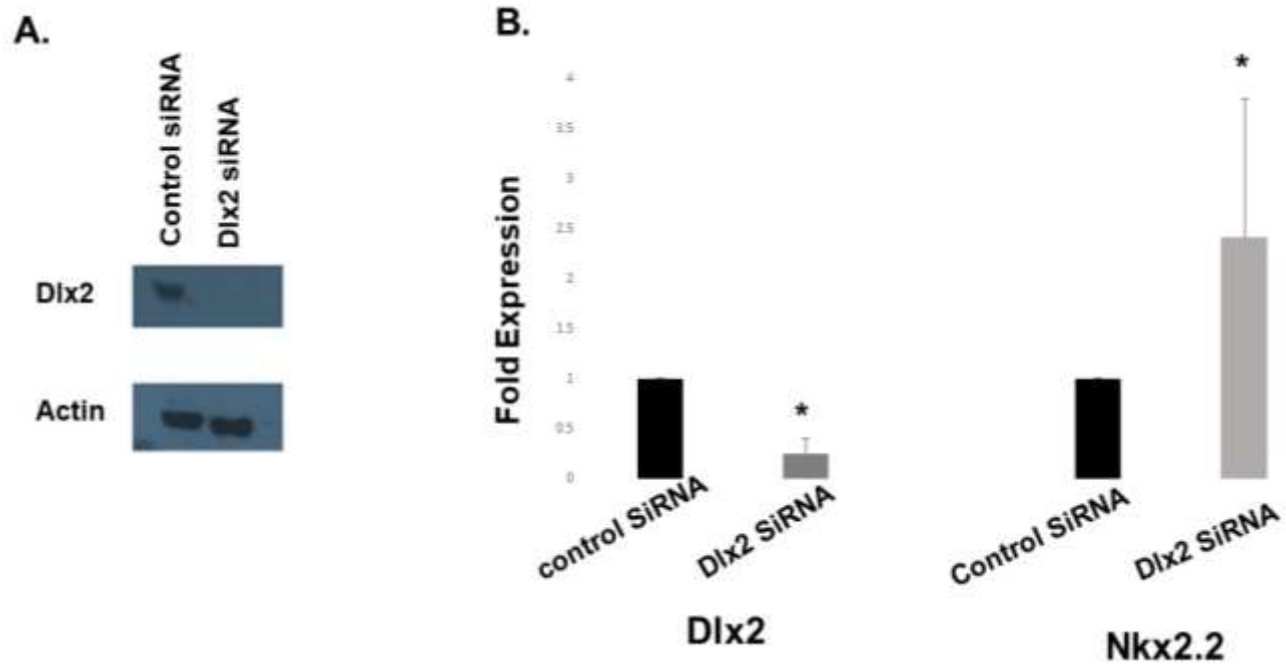
GEs: ganglionic eminences, WT: wild type, DKO: double knockout.

**: P value<0.01.

3.2.10 *Nkx2.2* expression is significantly increased in *Dlx2*-siRNA treated cells and E13.5 GE primary culture

To support the results observed in the *Dlx1/Dlx2* DKO mouse, I also assessed *Dlx2* loss of function in the neuroblastoma cell line SK-N-BE (2) as well as E13.5 primary embryonic forebrain cultures. After successfully knocking down (KD) *Dlx2* expression in SK-N-BE (2) cells using siRNA (Figure 3.10 A), I assessed *Nkx2.2* expression. *Nkx2.2* mRNA expression significantly increased in *Dlx2*-siRNA treated SK-N-BE (2) cells compared to the control-siRNA treated cells ($P \leq 0.05$, Figure 3.10 B).

I also prepared and grew primary cultures from E13.5 ganglionic eminences and determined the effects of *Dlx2* loss of function in E13.5 primary cultures. Transient loss of *Dlx2* expression in primary cultures was conducted using a siRNA pooling technique with two different *Dlx2*-siRNA. Transient DLX2 knockdown experiments using two different *Dlx2*-siRNA in the primary cultures significantly increased *Nkx2.2* expression *in vitro* in *Dlx2*-siRNA treated E13.5 GE primary cultures compared to the control-siRNA treated E13.5 GE primary cultures (P value≤ 0.05, Figure 3.10 C). These results are consistent with *Nkx2.2* expression studies in the E13.5 DKO mouse forebrain and support my hypothesis that DLX2 represses *Nkx2.2* expression *in vivo*.


A.
Control siRNA
Dlx2 siRNA
Dlx2
Actin
B.
Fold Expression
control SiRNA
Dlx2 SiRNA
Dlx2
Control SiRNA
Dlx2 SiRNA
Nkx2.2

C.

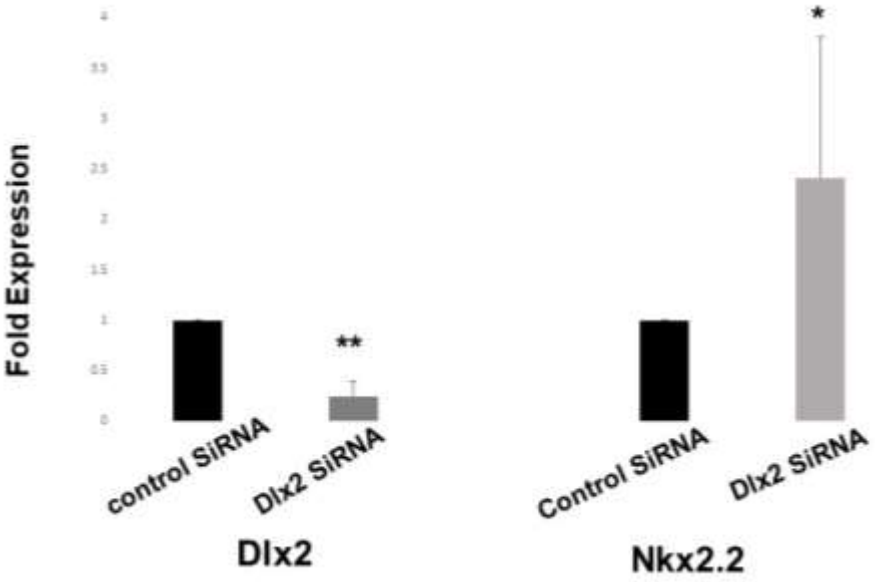

Fold Expression
control SiRNA
Dlx2 SiRNA
Dlx2
Control SiRNA
Dlx2 SiRNA
Nkx2.2

Figure 3.10. *Nkx2.2* expression in *Dlx2*-siRNA treated SK-N-BE (2) cells and *Dlx2*-siRNA treated E13.5 GE primary culture.

Western Blot showing the expression levels of DLX2 in SK-N-BE (2) cells treated with *Dlx2*-siRNA (A). *Nkx2.2* expression was significantly decreased in *Dlx2*-siRNA treated SK-N-BE (2) cells (B). *Nkx2.2* expression was also significantly decreased in E13.5 primary embryonic forebrain cultures treated with 2 different *Dlx2*-siRNA (C).

Error bars represent standard error of the mean.

The assay was conducted in three biological and three technical replicates.

*= P value ≤ 0.05, **= P value ≤ 0.01.

4 Chapter 4: DLX2 promotes GABAergic interneuron migration through activation of *Cxcr4*

4.1 Introduction

Chemotactic factors provide cells with information regarding the direction of migration. In the ganglionic eminences, Semaphorins and Slit1 are responsible for guiding interneurons away from the GEs by interacting with their receptors NRP1/NRP2 and Robo1, respectively (Marin, Yaron et al. 2001, Andrews, Barber et al. 2007). On the other hand, chemoattractants are expressed in the pallium, where they guide the interneurons towards the cortical layers. CXCL12 is an important chemoattractive molecule which interacts with CXCR4 and CXCR7 chemokine receptors, expressed on MGE-derived interneurons (Sanchez-Alcaniz, Haege et al. 2011).

CXCR4/CXCL12 signaling is crucial for the guidance of cortical interneurons from the GE to the neocortex. Disruption of signaling by CXCR4 or CXCL12 results in abnormal migration of interneurons in the forebrain (Zlotnik, Yoshie et al. 2006). Studies on mice deficient for *Cxcr4* or *Cxcl12* showed defects in the localization of GABAergic interneurons in the developing brain, suggesting that mutations in *Cxcl12/Cxcr4* disrupts migration of GABAergic interneurons from the GE to the neocortex (Stumm, Zhou et al. 2003, Tiveron, Rossel et al. 2006, Li, Adesnik et al. 2008, Lopez-Bendito, Sanchez-Alcaniz et al. 2008). Stumm and his colleagues showed that CXCR4 is expressed in tangentially migrating interneurons and striatal neuronal precursors are attracted to CXCL12 *in vitro.* In mice deficient in *Cxcl12* as well as in *Cxcr4*$^{-/-}$ mice, CXCR4-expressing interneurons were reduced in the superficial layers and strongly increased in the deep layers of the developing neocortex while no difference was observed in CXCR4-expressing Cajal-Retzius cells in the mutant animals compared to the WT (Stumm, Zhou et al. 2003). Furthermore, there is a defect in the MZ and SVZ migratory streams in *Cxcr4* mutants, and *Lhx6*-GFP^{+} interneurons generating from the MGE are trapped in the cortical plate (Li, Adesnik et al. 2008). Gene expression profiles in *Dlx1/2* DKO mice showed expression of

Cxcr4 is significantly decreased in MGE and LGE generating GABAergic interneurons (Long, Cobos et al. 2009). Based on these observations, I hypothesised that DLX2 is an upstream regulator of *Cxcr4* and is involved in activating *Cxcr4* in GABAergic interneurons. Activation of *Cxcr4* in GABAergic interneurons can then facilitate their migration to the neocortex through CXCR4/CXCL12 signalling.

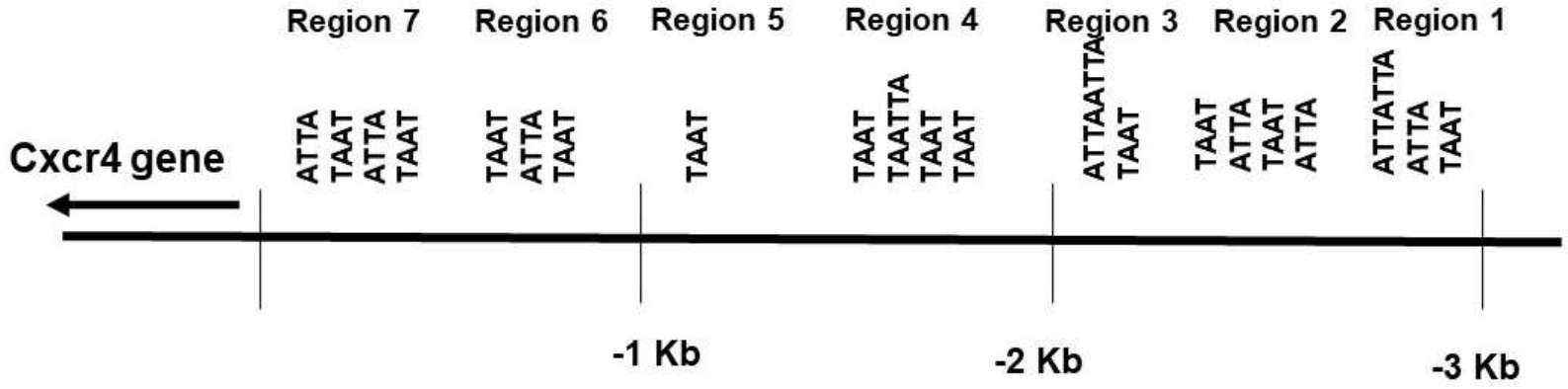
4.2 Results

4.2.1 The *Cxcr4* promoter region contains putative DLX2 binding sites

To identify putative DLX2 binding sites on the *Cxcr4* proximal regulatory region, TAAT/ATTA motifs present approximately 3Kb upstream of the *Cxcr4* gene were identified. There are 25 putative DLX2 binding sites located approximately 3Kb upstream of *Cxcr4* gene. For simplicity, this regulatory region was divided into 7 regions, R1 to R7, for further experiments. Figure 4.1 A shows a schematic of the *Cxcr4* proximal regulatory sequence harbouring putative DLX2 binding sites.

When overlaying the *Cxcr4* regulatory region with the UCSC Genome Browser (NCB137/mm9 assembly) DNase I hypersensitivity, as an indicator of open chromatin, was present (Figure 4.1 B). Furthermore, enrichment of active histone markers such as H3K4me1, H3K4me3 and H3K27ac in the regulatory region of *Cxcr4* was reported in E14.5 brain tissue (Figure 4.1 B). Enrichment of the regulatory region of *Cxcr4* with DNase I hypersensitivity, H3K4me1, H3K4me3 and H3K27ac in E14.5 brain tissue can be an indicator that *Cxcr4* is transcriptionally active during brain development.

A.


Region 7
Region 6
Region 5
Region 4
Region 3
Region 2
Region 1
Cxcr4 gene
ATTA TAAT ATTA TAAT
TAAT ATTA TAAT
TAAT
TAAT TAATTA TAAT TAAT
ATTAATTA TAAT
TAAT ATTA TAAT ATTA
ATTATTA ATTA TAAT
-1 Kb
-2 Kb
-3 Kb

B.

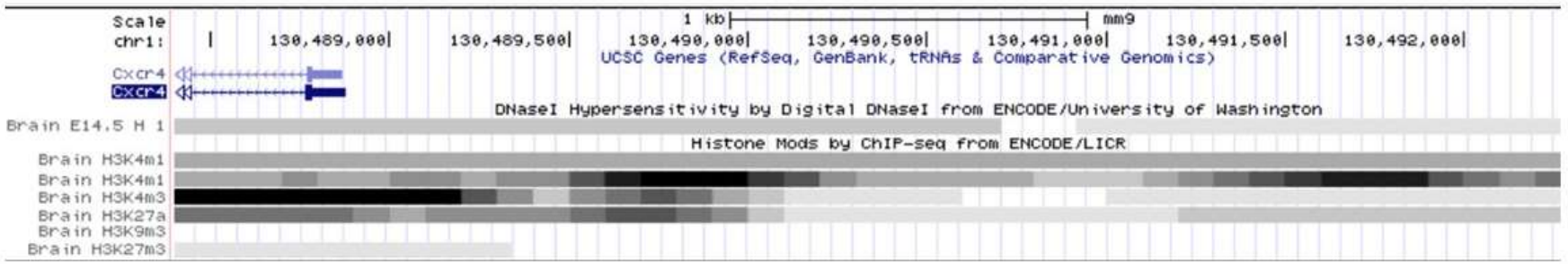

Scale
chr1:
1 kb
mm9
130,489,000
130,489,500
130,490,000
130,490,500
130,491,000
130,491,500
130,492,000
UCSC Genes (RefSeq, GenBank, tRNAs & Comparative Genomics)
Cxcr4
Cxcr4
DNaseI Hypersensitivity by Digital DNaseI from ENCODE/University of Washington
Brain E14.5 H 1
Histone Mods by ChIP-seq from ENCODE/LICR
Brain H3K4m1
Brain H3K4m1
Brain H3K4m3
Brain H3K27a
Brain H3K9m3
Brain H3K27m3

Figure 4.1. Schematic diagram of the *Cxcr4* regulatory region highlighting putative DLX2 binding sites.

A. All the TAAT/ATTA motifs present in the proximal regulatory region approximately 3Kb upstream of *Cxcr4* gene were assigned as putative DLX2 binding sites. There are 25 putative DLX2 binding sites located in the proximal regulatory region of *Cxcr4*.

B. The *Cxcr4* regulatory region was overlaid with the UCSC Genome Browser (NCB137/mm9 assembly). The regulatory region of *Cxcr4* corresponded with DNase I hypersensitivity. Enrichment of active histone modifications H3K4me1, H3K4me3 and H3K27ac in the regulatory region of *Cxcr4* is reported in E14.5 brain tissue.

4.2.2 *Cxcr4* is a transcriptional target of DLX2 *in vivo*

ChIP experiments on E13.5 wildtype GEs were carried out to identify DLX2 transcriptional targets on the *Cxcr4* proximal regulatory region *in vivo*. After crosslinking protein with DNA in E13.5 wildtype GE and precipitating the protein-DNA complexes with DLX2 antibody, primers flanking putative DLX2 binding sites were used to amplify DLX2 bound regulatory regions of *Cxcr4* in a PCR reaction. Immune enrichment obtained by use of the DLX2 antibody was present for all the regions (R1- R7) in the interrogated *Cxcr4* regulatory region *in vivo* (Figure 4.2). Control ChIP experiments performed without antibody or from the hindbrain tissue, where DLX2 is not expressed, were negative. The positive amplicons from ChIP were sub cloned into pGL3 Basic Vector for subsequent experiments.

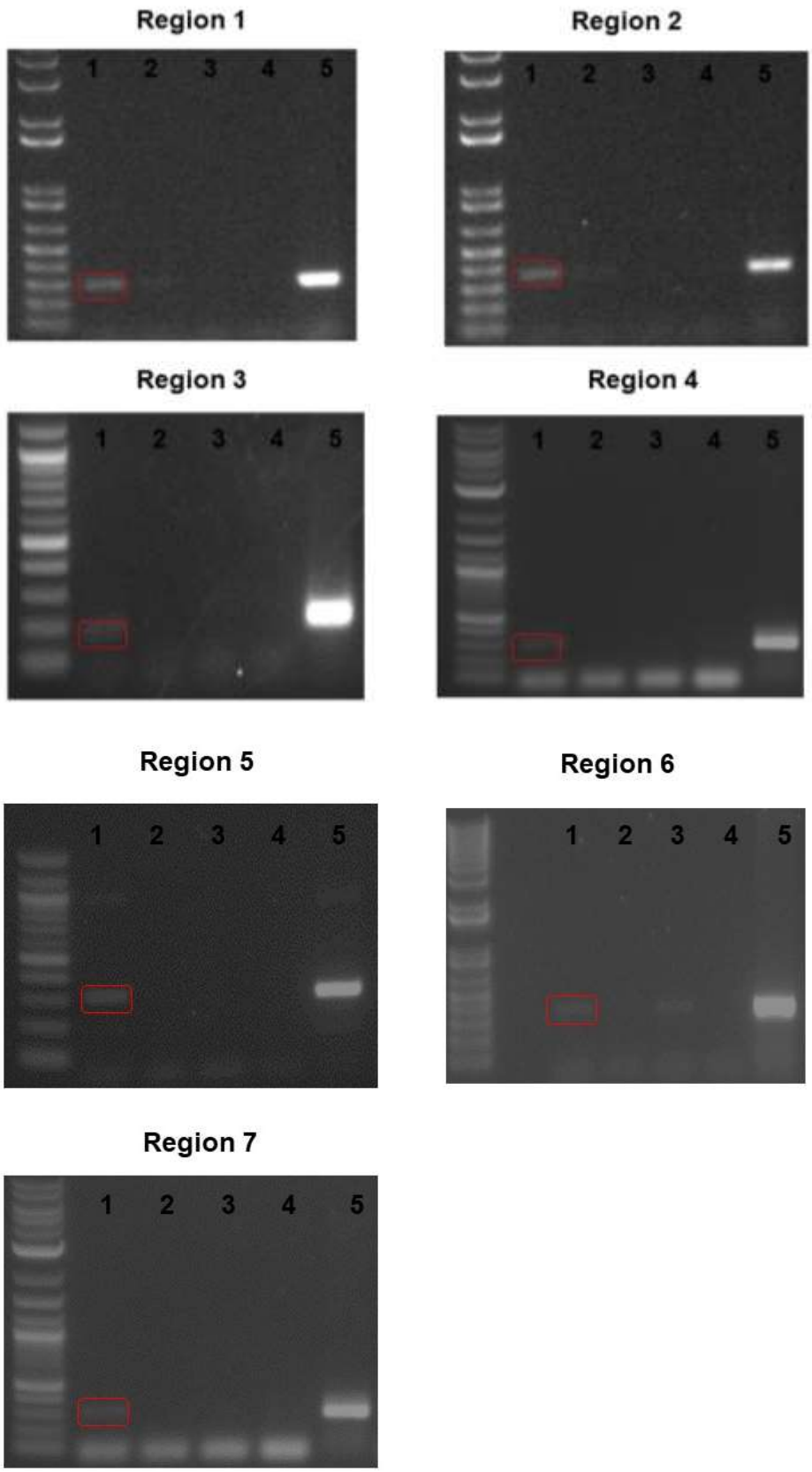

Region 1
1 2 3 4 5
Region 2
1 2 3 4 5
Region 3
1 2 3 4 5
Region 4
1 2 3 4 5
Region 5
1 2 3 4 5
Region 6
1 2 3 4 5
Region 7
1 2 3 4 5

Figure 4.2. DLX2 binds to *Cxcr4* regulatory elements *in vivo.*

DLX2 enrichment at *Cxcr4* region 1 to region7 was observed *in vivo* (Lane 1). ChIP was carried out with high affinity purified antibody to DLX2 (Ab). Controls included a negative antibody control where no DLX2 antibody was added, along with negative hindbrain (HB) tissue as DLX2 is not expressed in the developing hindbrain. Enrichment for DLX2 binding was not observed in no-antibody controls (Lane 2 and 4) as well as negative tissue expression controls (Lane 3 and 4). Genomic DNA was also used as a positive PCR control for identification of DLX2 enrichment to chromatin (Lane 5).

Lane 1: FB+Ab, Lane 2: FB-Ab, Lane 3: HB+Ab, Lane 4: HB-Ab, Lane 5: genomic DNA.

ChIP: chromatin immunoprecipitation, FB: forebrain, HB: hindbrain, gDNA: genomic DNA, Ab: antibody.

4.2.3 **DLX2 binds to the *Cxcr4* regulatory region *in vitro***

To assess the specificity of binding on the regulatory regions of *Cxcr4*, EMSA was performed using [γ-^{32}P]-dGTP radiolabelled oligonucleotides associated with each TAAT/ATTA motif of the *Cxcr4* regulatory region. Using this approach, specific and critical binding sites for DLX2 on the *Cxcr4* proximal promoter region have been characterized. Figures 4.3 to 4.9 reveal specific binding of DLX2 to each TAAT/ATTA motif in the *Cxcr4* regulatory element *in vitro.* Table 4.1 summarizes the results obtained from these EMSA experiments.

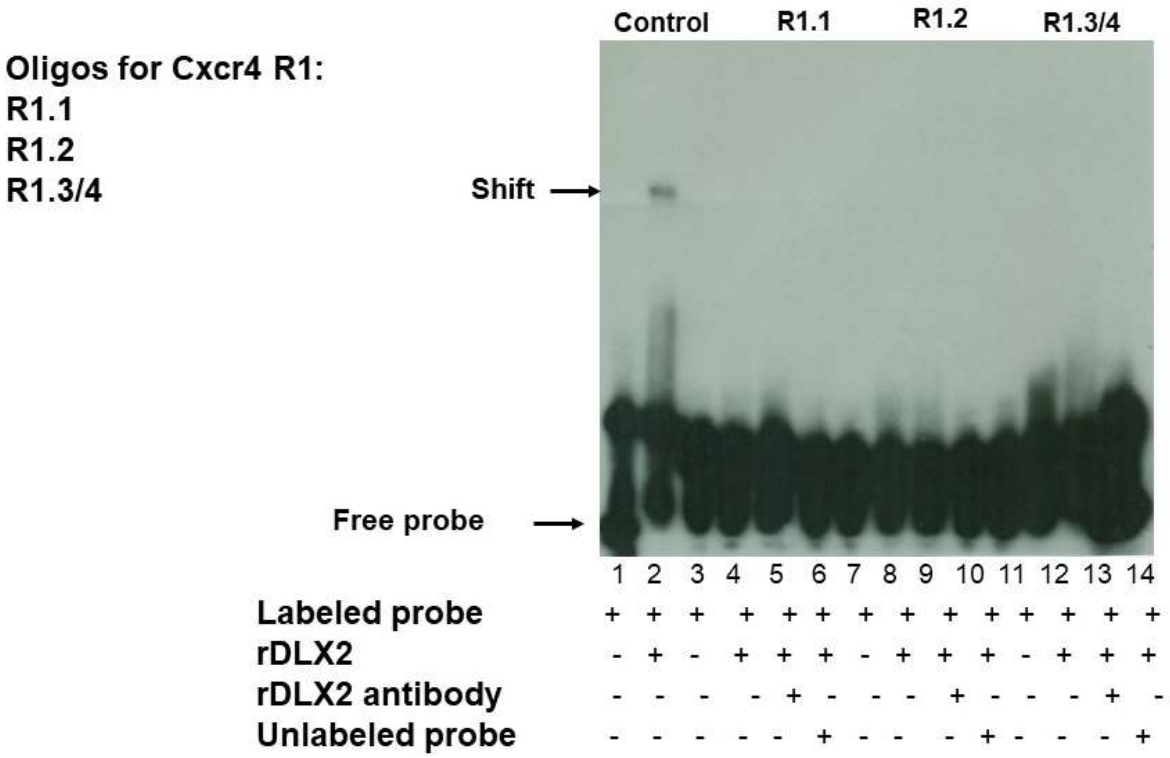


Figure 4.3. EMSA shows DLX2 does not specifically bind to R1 of *Cxcr4 in vitro*.

EMSA was carried out using radiolabelled oligonucleotides for R1.1 (TAAT), R1.2 (ATTA) and R1.3/4 (ATTATTA) of the *Cxcr4* promoter. Specific binding of recombinant DLX2 was not observed for any of the regions (lanes 4, 8, 12). No supershift was observed in the presence of DLX2 antibody (lanes 5, 9, 13). Excess unlabeled probe was added to compete with radiolabelled DNA for binding (cold competition) (lanes 6, 9, 13).

rDLX2: recombinant DLX2

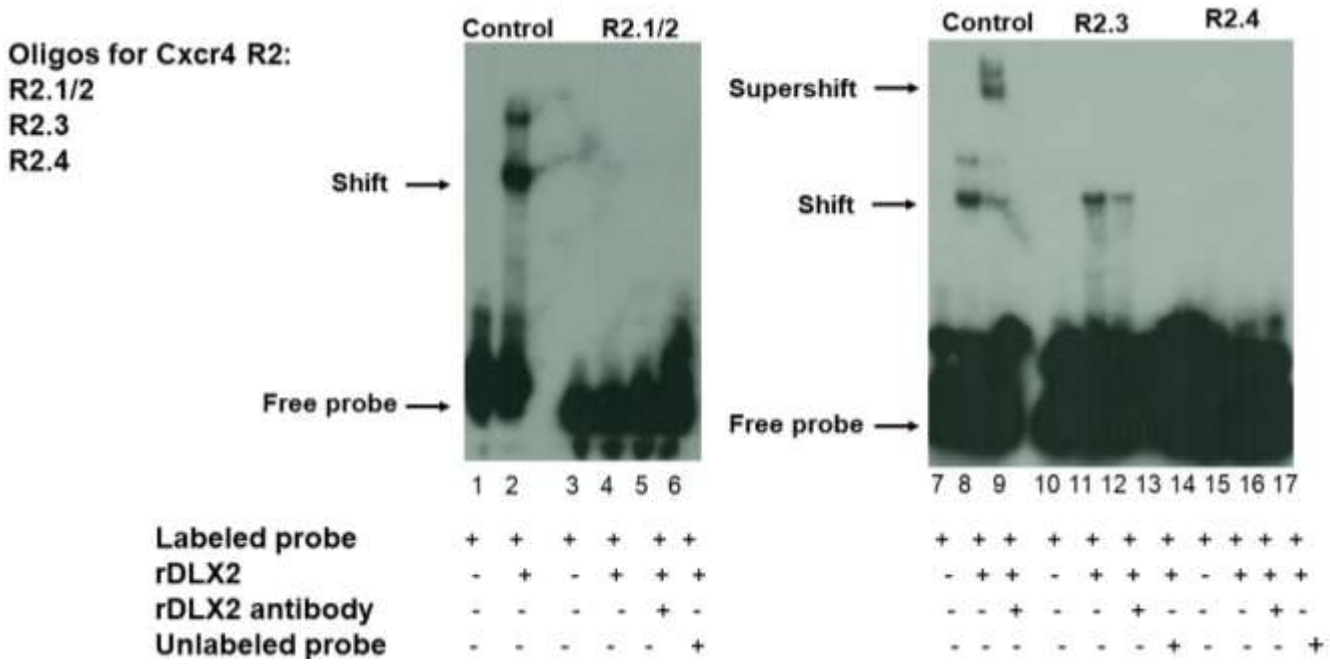


Figure 4.4. EMSA reveals the specific binding of DLX2 to R2 of *Cxcr4 in vitro*.

EMSA was carried out using radiolabelled oligonucleotides for R2.1/2 (ATTA), R2.3 (ATTACTAAT) and R2.4 (TAAT) of the *Cxcr4* promoter. Specific binding of recombinant DLX2 was not observed for R2.1/2 and R2.4 (lanes 4 and 14) but was seen for R2.3 (lane 11). No supershift was observed in the presence of DLX2 antibody for R2.1/2 and R2.4 (lanes 5 and 15). Excess unlabeled probe was added to compete with radiolabelled DNA for binding (cold competition) (lanes 6, 13, 17). Multiple shifts and supershifts in the control samples can indicate different patterns of binding or oligomerization of the recombinant protein.

rDLX2: recombinant DLX2.

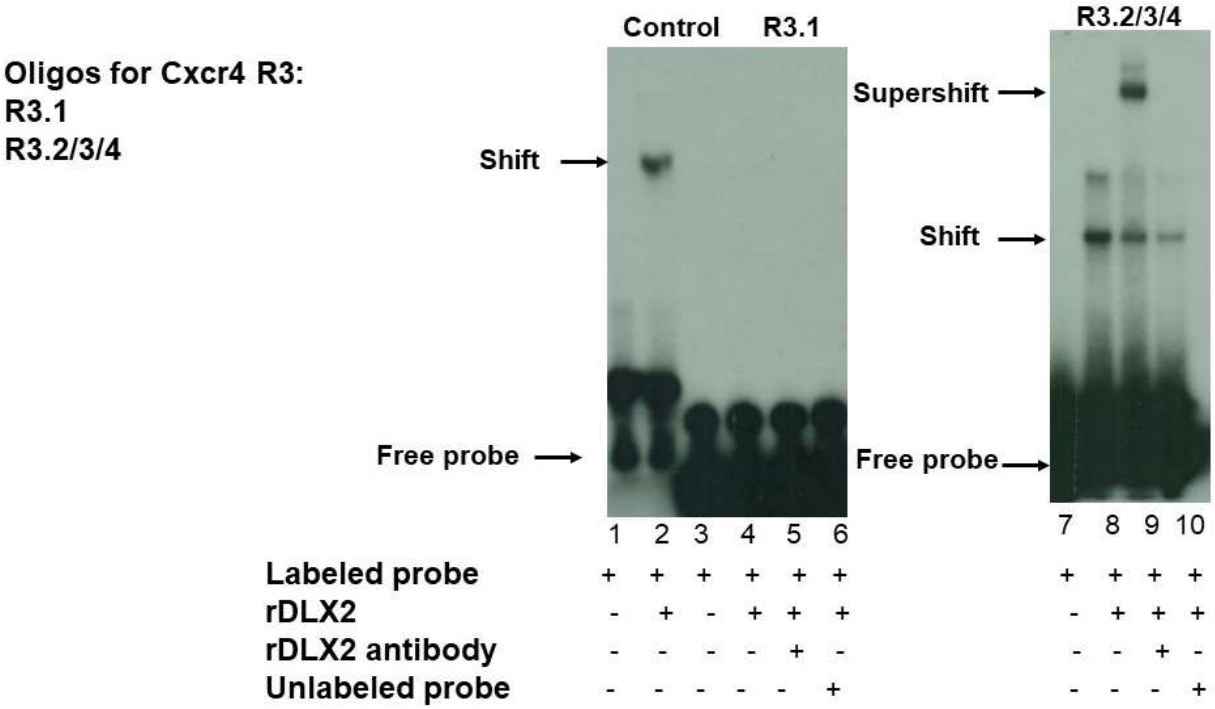


Figure 4.5. EMSA reveals the specific binding of DLX2 to R3 of *Cxcr4 in vitro*.

EMSA was carried out using radiolabelled oligonucleotides for R3.1 (TAAT) and R3.2/3/4 ATTAATTA) of the *Cxcr4* promoter. For R3.1 specific binding of recombinant DLX2 was not observed (lane 4) and no supershift was observed in the presence of DLX2 antibody (lane 5). For R3.2/3/4 specific binding of recombinant DLX2 *in vitro* resulted in a shift (lane 8) and adding the DLX2 antibody revealed a supershift (lane 9). Excess unlabeled probe was added to compete with radiolabelled DNA for binding (cold competition) (lanes 6, 10). Multiple shifts and supershifts in R3.2/3/4 can indicate different patterns of binding or oligomerization of the recombinant protein. Addition of excess unlabeled probe to R3.2/3/4 resulted in a signal decrease in the shift due to competition with the radiolabelled probe (lane 10).

rDLX2: recombinant DLX2

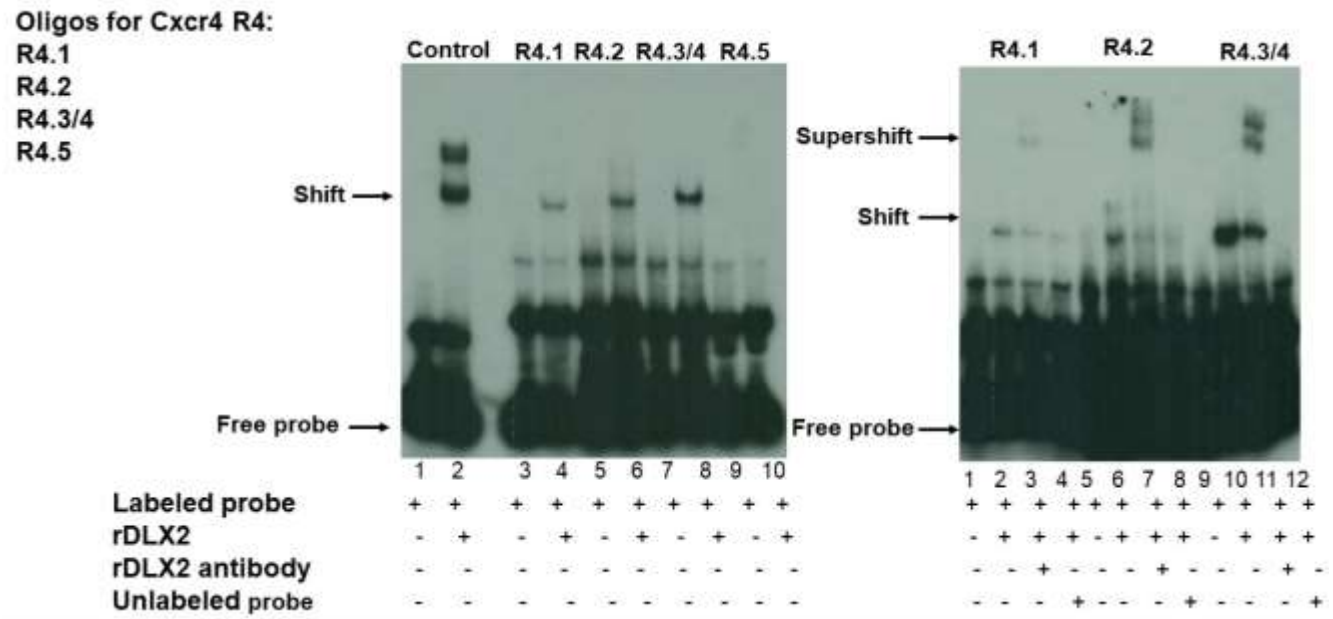


Figure 4.6. EMSA reveals the specific binding of DLX2 to R4 of *Cxcr4 in vitro*.

EMSA was carried out using radiolabelled oligonucleotides for R4.1 (TAAT), R4.2 (TAAT), R4.3/4 (TAATTA), and R4.5 (TAAT) of the *Cxcr4* promoter. Specific binding of recombinant DLX2 for R4.1, R4.2 and R4.3/4 resulted in a shift (left image, lanes 4, 6, 8). No specific binding was observed for R4.5 (left image, lane10). The right image shows addition of DLX2 antibody as well as cold competition for the three positive R4.1, R4.2 and R4.3/4. Specific binding of recombinant DLX2 for R4.1, R4.2 and R4.3/4 resulted in a shift (right image, lanes 2, 6, 10) and adding the DLX2 antibody revealed a supershift (right image, lanes 3, 7, 11). Furthermore, excess unlabeled probe was added to compete with radiolabelled DNA for binding (cold competition) (right image, lanes 4, 8, 12). Multiple shifts and supershifts can indicate different patterns of binding or oligomerization of the recombinant protein.

rDLX2: recombinant DLX2

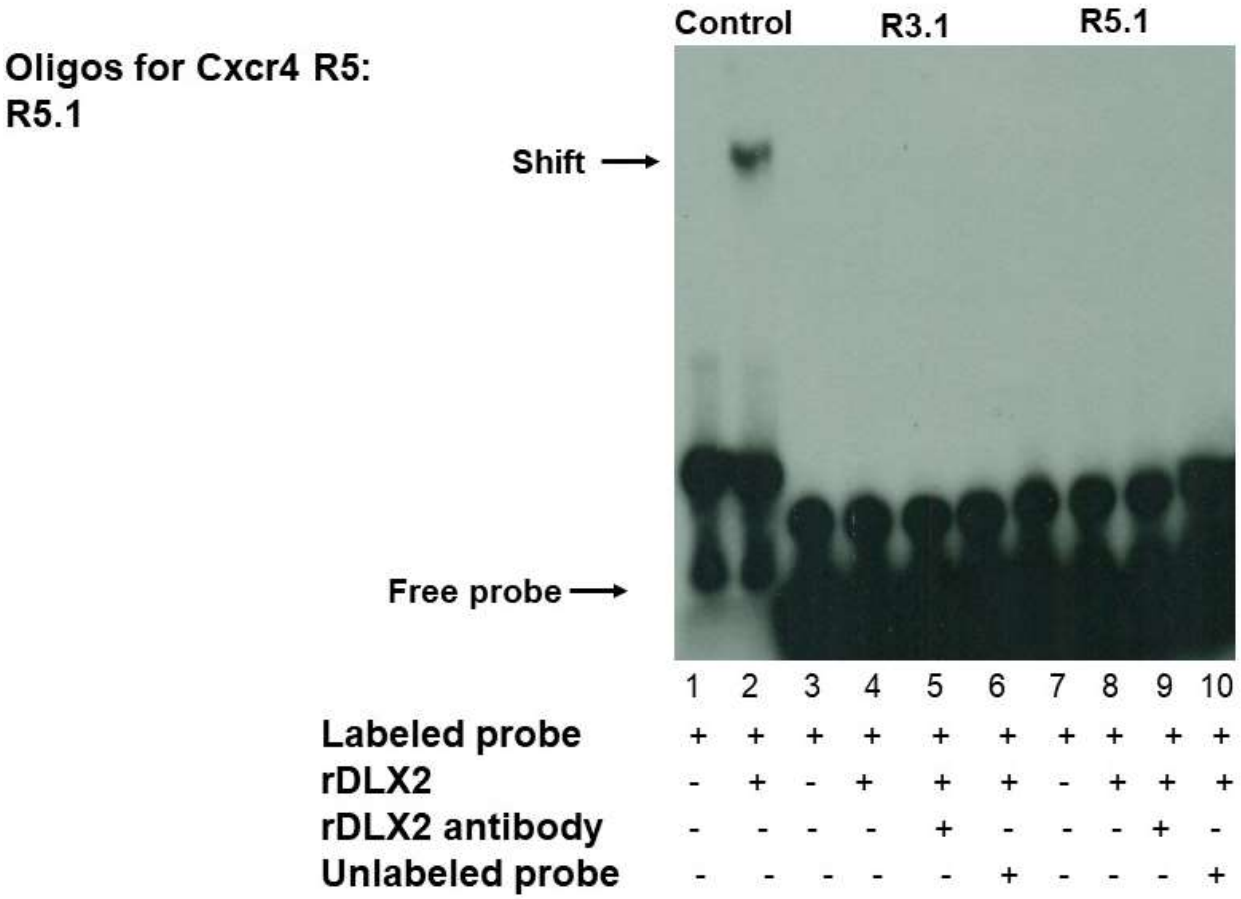


Figure 4.7. EMSA shows DLX2 does not bind to R5 of *Cxcr4 in vitro*.

EMSA was carried out using radiolabelled oligonucleotides for R5.1 (TAAT) of the *Cxcr4* promoter. Specific binding of recombinant DLX2 did not result in a shift (lane 8) and no supershift was observed when DLX2 antibody was added (lane 9). Furthermore, excess unlabeled probe was added to compete with radiolabelled DNA for binding (lane 10). rDLX2: recombinant DLX2

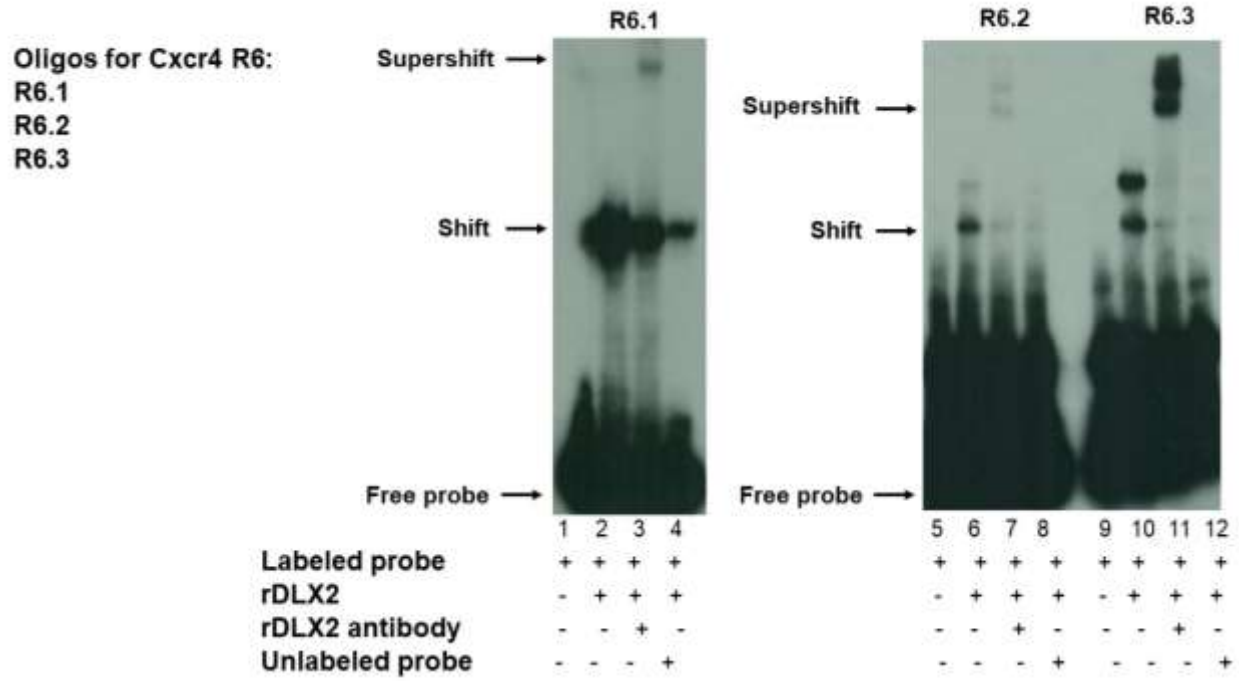


Figure 4.8. EMSA shows the specific binding of DLX2 R 6 of *Cxcr4 in vitro*.

EMSA was carried out using radiolabelled oligonucleotides for each R6.1 (TAAT), R6.2 (ATTA), and R6.3 (TAAT) of the *Cxcr4* promoter. Specific binding of recombinant DLX2 *in vitro* resulted in a shift for all the regions (lanes 2, 6, 10) and adding the DLX2 antibody revealed a supershift (lanes 3, 7, 11). Furthermore, excess unlabeled probe was added to compete with radiolabelled DNA for binding (lanes 4, 8, 12). Addition of excess unlabeled probe to R6.1 resulted in a signal decrease in the shift due to competition with the radiolabelled probe (lane 4). Addition of excess unlabeled probe to R6.2 and R6.3 eliminated the shift (lanes 8 and 12). Multiple shifts and supershifts can indicate different patterns of binding or oligomerization of the recombinant protein.

rDLX2: recombinant DLX2

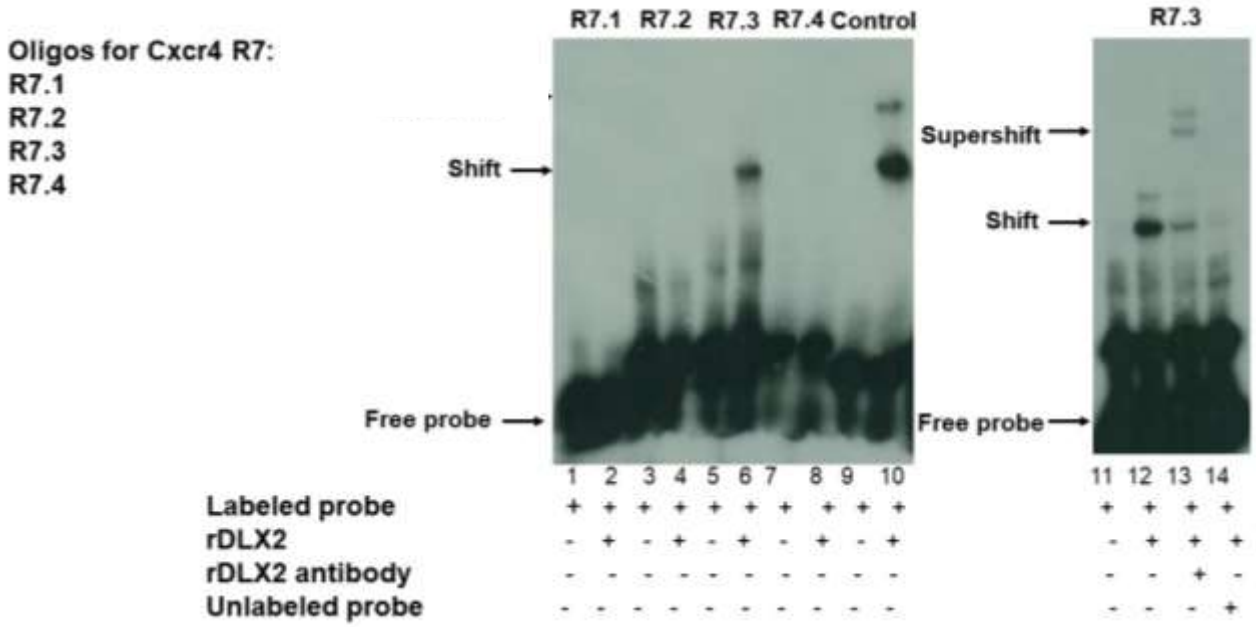


Figure 4.9. EMSA reveals the specific binding of DLX2 to R7 of *Cxcr4 in vitro*.

EMSA was carried out using radiolabelled oligonucleotides for R7.1 (TAAT), R7.2 (ATTA), R7.3 (TAAT), and R7.4 (ATTA) of the *Cxcr4* promoter. No specific binding of recombinant DLX2 was observed for R7.1, R7.2 and R7.4 (lanes 2, 4, 8). For R7.3 specific binding of recombinant DLX2 resulted in a shift (lane 12) and adding the DLX2 antibody revealed a supershift (lane 13). Furthermore, excess unlabeled probe was added to R7.3 to compete with radiolabelled DNA for binding which resulted in a signal decrease (lane 14). Multiple shifts and supershifts in R7.3 can indicate different patterns of binding or oligomerization of the recombinant protein.

rDLX2: recombinant DLX2

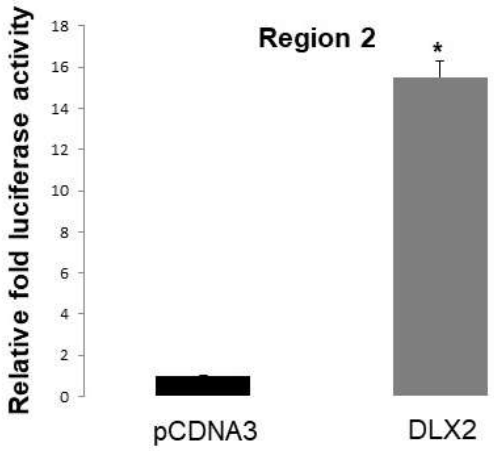
Table 4.1. Summarized results from EMSA for TAAT/ATTA motifs of the *Cxcr4* proximal promoter region.

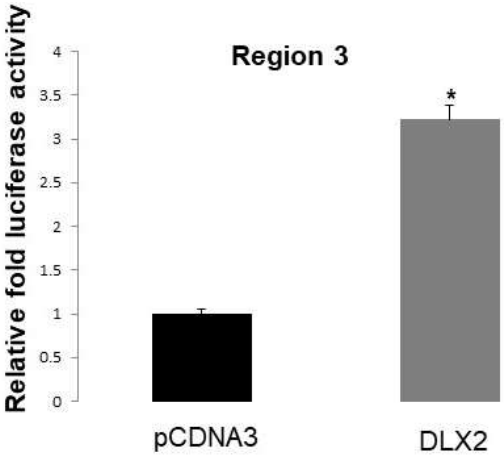
EMSA was carried out using radiolabelled oligonucleotides for each TAAT/ATTA motif of the *Cxcr4* promoter to characterize the specific binding sites for DLX2 on the *Cxcr4* regulatory region. EMSA shows that regions 1 and 5 do not specifically bind to recpmbinant DLX2 *in vitro*.

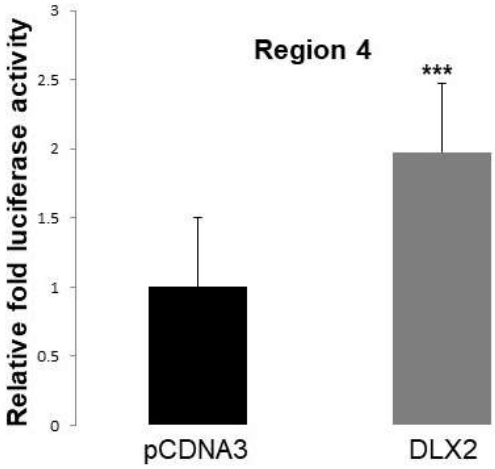
***Cxcr4* regulatory region**	**Oligonucleotides**	**TAAT/ATTA motif**	**EMSA results**
Region 1	R1.1	TAAT	-
	R1.2	ATTA	-
	R1.3/4	ATTATTA	-
Region 2	R2.1/2	ATTA	-
	R2.3	ATTACTAAT	+
	R2.4	TAAT	-
Region 3	R3.1	TAAT	-
	R3.2/3/4	ATTAATTA	+
Region 4	R4.1	TAAT	+
	R4.2	TAAT	+
	R4.3/4	TAATTA	+
	R4.5	TAAT	-
Region 5		TAAT	-
Region 6	R6.1	TAAT	+
	R6.2	ATTA	+
	R6.3	TAAT	+
Region 7	R7.1	TAAT	-
	R7.2	ATTA	-
	R7.3	TAAT	+
	R7.4	ATTA	-

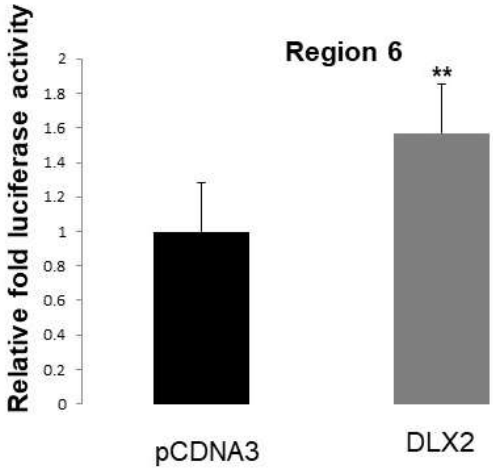
4.2.4 **Dlx2 activates expression of *Cxcr4 in vitro***

To understand the functional consequence of DLX2 binding to the *Cxcr4* regulatory region *in vitro*, luciferase reporter gene assays were conducted. *Cxcr4* regions R2, R3, R4, R6 and R7 showed DLX2 occupancy with ChIP and had specific binding to DLX2 *in vitro* through EMSA. *Cxcr4* R2, R3, R4, R6 and R7 were sub-cloned into pGL3 reporter vectors and co-transfected into HEK293 cells with a *Dlx2* expression plasmid. When the cells were co-transfected with *Cxcr4* reporter vectors and the *Dlx2* expression plasmid, significant activation of luciferase expression was observed *in vitro* for regions 2, 3, 4 and 6 (Figure 4.10). However, there was significant repression of reporter gene expression observed *in vitro* for region 7. All luciferase activities were normalized to β-galactosidase activity. This activation of 4 of 5 regions supports an overall role for DLX2 as a transcriptional activator of *Cxcr4* expression *in vitro*.


Region 2
Relative fold luciferase activity
18
16
14
12
10
8
6
4
2
0
*
pCDNA3
DLX2


Region 3
Relative fold luciferase activity
4
3.5
3
2.5
2
1.5
1
0.5
0
*
pCDNA3
DLX2


Region 4
Relative fold luciferase activity
3
2.5
2
1.5
1
0.5
0
***
pCDNA3
DLX2


Region 6
Relative fold luciferase activity
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
pCDNA3
DLX2

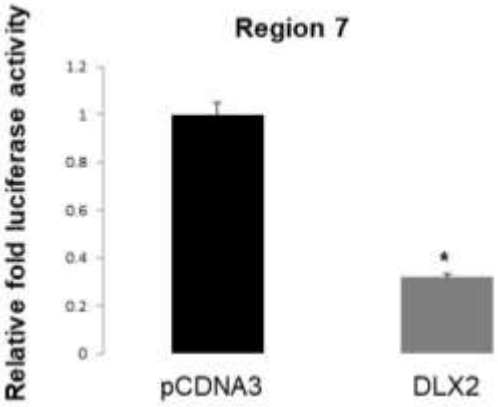

Region 7
Relative fold luciferase activity
1.2
1
0.8
0.6
0.4
0.2
0
*
pCDNA3
DLX2

Figure 4.10. CXCR4 is activated upon DLX2 co-expression *in vitro*.

Cxcr4 sub-regions 2, 3, 4, 6 and 7 were sub-cloned into pGL3 reporter vectors and co-transfected into HEK293 cells with a *Dlx2* expression plasmid, resulting in significant activation of luciferase reporter gene expression *in vitro* for regions 2, 3, 4 and 6 but not for region 7. All luciferase activities were normalized to β-galactosidase activity.

Error bars represent standard error of the mean (SEM).

The assay was conducted in three biological and three technical replicates.

* = P values <0.05, ** = P values <0.05, *** = P values <0.001.

4.2.5 **DLX2 and CXCR4 spatial expression in the developing forebrain**

To assess the role of DLX2 on the temporal and spatial expression of CXCR4 *in vivo*, IF was carried out on E13.5 WT tissue sections. Out of several commercial CXCR4 antibodies tested, only a goat polyclonal antibody corresponding to amino acids 14-40 of the N-terminus of mouse CXCR4 from Abcam (catalog#: Ab1670) showed reproducible and reliable results. IF on E13.5 WT tissue sections using this antibody showed positive staining in the forebrain tissue (Figure 4.11 B, E, H). It is important to note that CXCR4 is a membrane bound G protein coupled receptor and these IF results, especially at higher magnification, demonstrated cellular localization at the plasma membrane (figure 4.11 H). IF staining on E13.5 WT sections confirmed co-expression of DLX2 and CXCR4 in the VZ and SVZ of the LGE and MGE (Figure 4.11 C, F, I). However, in these regions cells positive for only DLX2 and cells positive for only CXCR4 were also detected (Figure 4.11 F, I).

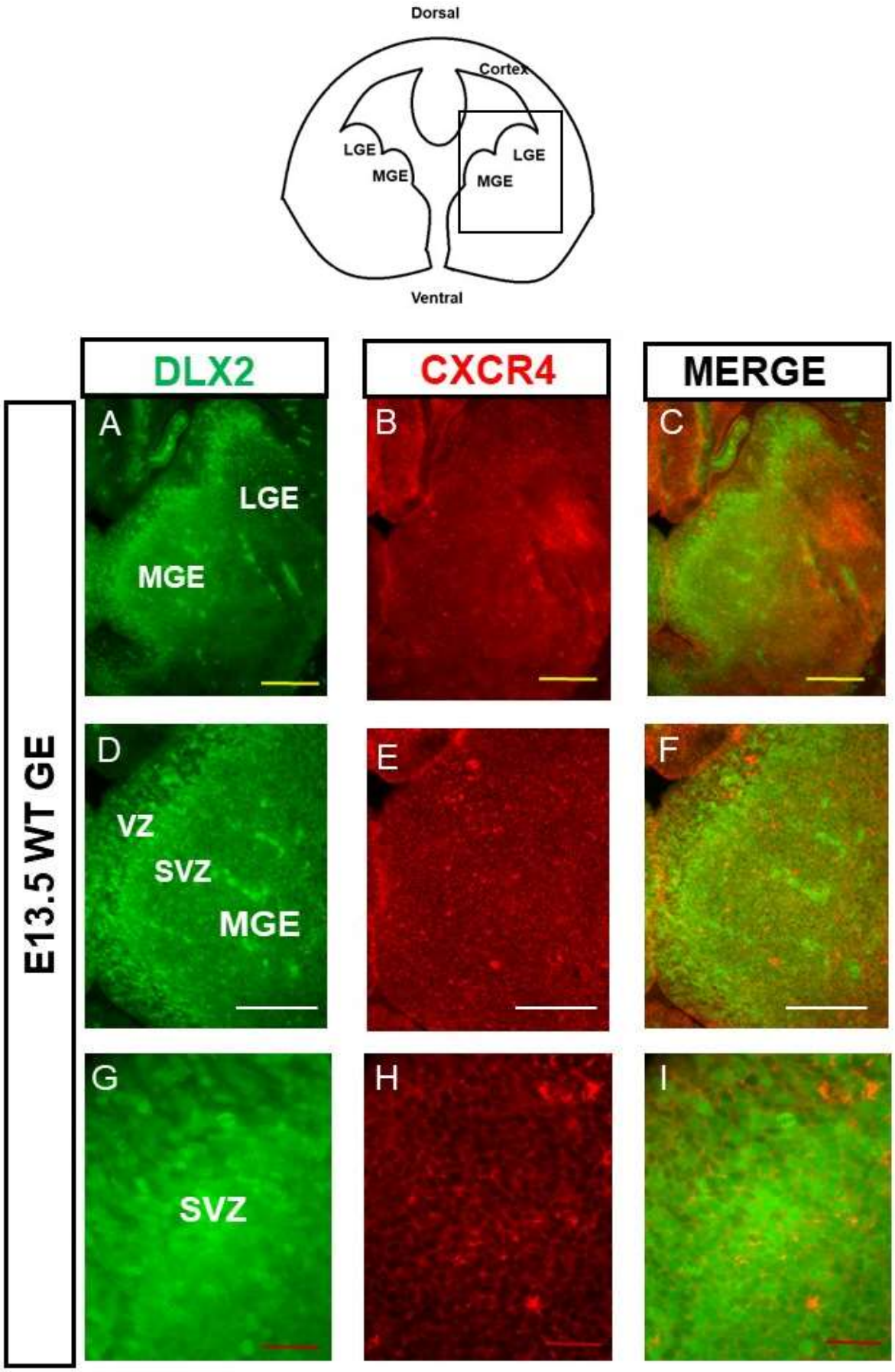

Dorsal
Cortex
LGE
MGE
LGE
MGE
Ventral
DLX2
CXCR4
MERGE
E13.5 WT GE
A
LGE
MGE
B
C
D
VZ
SVZ
MGE
E
F
G
SVZ
H
I

Figure 4.11. Spatial expression of DLX2 and CXCR4 in the E13.5 WT forebrain.

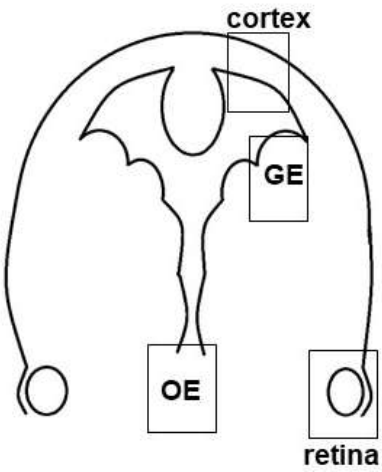
DLX2 expression is shown in the ventricular (VZ) and subventricular zones (SVZ) of the MGE and LGE at 10X (A), 40X (D) and 60X (G) magnification. CXCR4 expression is shown in the ventricular and subventricular zones of the MGE and LGE at 10X (B), 40X (E) and 60X (H) magnification. Co-expression of DLX2 and CXCR4 is also shown (C, F, I).

Yellow scale bars represent 100μm, white scale bars represent 50μm, and red scale bars represent 25μm. Experiments performed with Dr. Q. Jiang, Eisenstat laboratory.

MGE: Medial ganglionic eminences, LGE: lateral ganglionic eminences. VZ: ventral zone, SVZ: subventricular zone.

Furthermore, *in situ* hybridization using previously designed riboprobes (Dr. John Rubenstein, UCSF, USA) for *Cxcr4* was conducted on E13.5 WT tissue. ISH assays showed the presence of *Cxcr4* in the developing cortex, GE, retina and olfactory epithelium (Figure 4.12). To demonstrate co-expression of DLX2 and *Cxcr4*, IHC for DLX2 coupled with ISH for *Cxcr4* was performed. IHC for DLX2 coupled with ISH for *Cxcr4,* showed that *Dlx2* and *Cxcr4* are co-expressed in the GEs of the E13.5 WT forebrain (Figure 4.13). It is important to point out that the DIG molecule is very large so all ISH that use the DIG labelling methods demonstrate a cytoplasmic cellular localization whether studying the expression of a membrane bound protein or nuclear transcription factor. Furthermore, when using chromogenic substrates it is very difficult to show co-localization at the cellular level except at very high magnifications which can explain the weak co-expression shown in Figure 4.13. However, this technique was used to support the conclusion that there is co-localization for DLX2 and *Cxcr4* in the same neuroanatomic region.

To compare the spatial expression pattern of CXCR4 between the WT and *Dlx1/Dlx2* DKO, IHC for DLX2 coupled with ISH for *Cxcr4* was performed in the *Dlx1/Dlx2* DKO tissue. In the absence of *Dlx1/Dlx2*, *Cxcr4* expression is lost in the forebrain (Figure 4.13). These findings support the hypothesis that DLX2 activates *Cxcr4 in vivo*.


cortex
GE
OE
retina

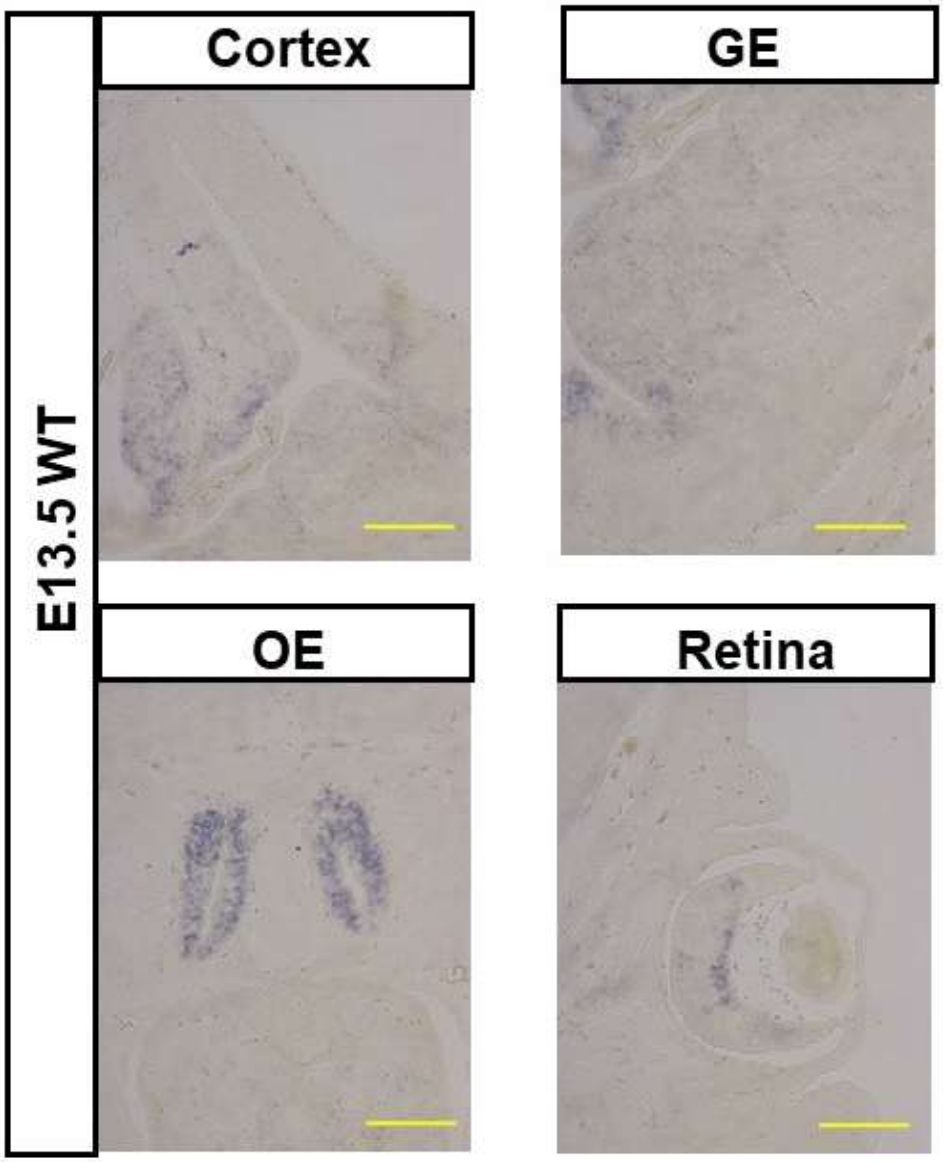

E13.5 WT
Cortex
GE
OE
Retina

Figure 4.12. ISH for *Cxcr4* riboprobe on E13.5 WT tissue.

ISH for *Cxcr4* on E13.5 WT tissue shows that *Cxcr4* is expressed in the ganglionic eminences, olfactory epithelium, cortex and retina.

Scale bars represent 100 μm.

Experiments performed with Dr. Q. Jiang, Eisenstat laboratory.

GE: ganglionic eminences, OE: olfactory epithelium, WT: wildtype.

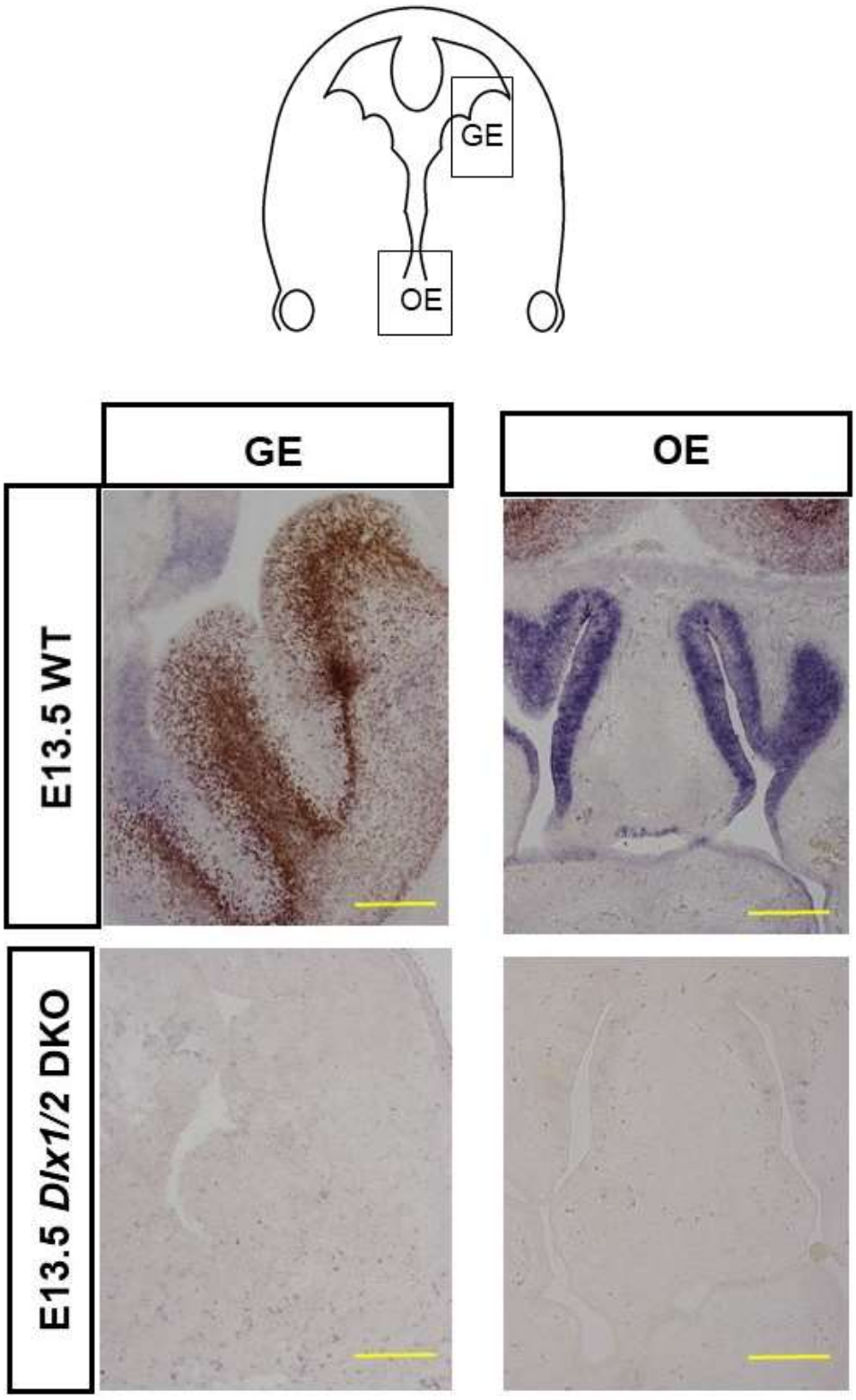

GE
OE
GE
OE
E13.5 WT
E13.5 Dlx1/2 DKO

Figure 4.13. IHC experiment for DLX2 coupled with ISH for *Cxcr4* in E13.5 WT and *Dlx1/2* DKO tissue.

Cxcr4 (blue) is co-expressed with DLX2 (brown) in the ganglionic eminences. In the absence of *Dlx1/Dlx2*, *Cxcr4* expression is downregulated in the E13.5 forebrain. *Cxcr4* expression is also downregulated in the OE in the DKO.

Scale bars represent 100 μm.

Experiments performed with Dr. Q. Jiang, Eisenstat laboratory.

GE: ganglionic eminences, OE: olfactory epithelium, WT: wildtype, DKO: double knockout.

4.2.6 *Cxcr4* transcript levels are decreased in the *Dlx1/Dlx2* DKO forebrain

The transcript levels of *Cxcr4* in the WT and *Dlx1/2* DKO ganglionic eminences were assessed on dissected GE tissue by performing qRT-PCR. There was a significant decrease in the expression of *Cxcr4* in the *Dlx1/Dlx2* DKO ganglionic eminences compared to WT (Figure 4.14). These results are consistent with my hypothesis that *Cxcr4* is a transcriptional target for DLX2 *in vivo* where *Dlx1/Dlx2* transcription factors play a role in activating the expression of *Cxcr4* in the basal telencephalon during forebrain development.

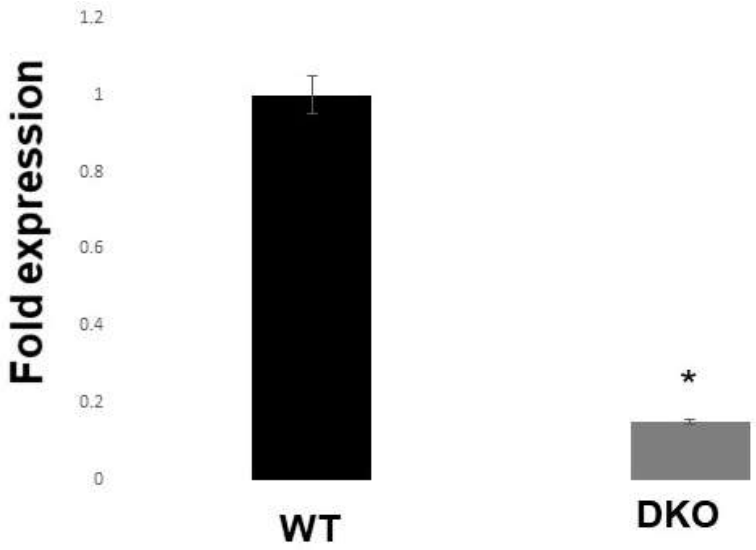


Figure 4.14. In the absence of *Dlx1/Dlx2, Cxcr4* mRNA expression is downregulated in the E13.5 forebrain.

qRT-PCR on RNA extracted from WT and *Dlx1/Dlx2* null ganglionic eminences revealed a significant decrease in *Cxcr4* expression in DKO forebrains compared to controls.

Error bars represent standard error of the mean (SEM).

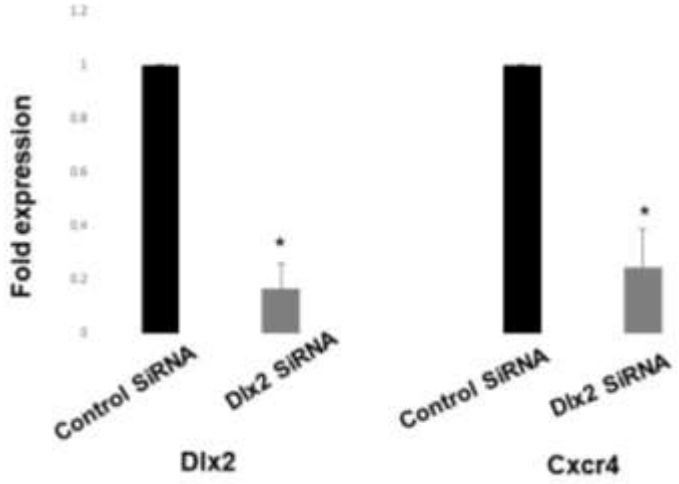
The assay was conducted in three biological and three technical replicates.

WT: wild-type, DKO: double knockout, *: P values <0.05

4.2.7 *Cxcr4* expression significantly decreases in *Dlx2*-siRNA treated SK-N-BE (2) cells and in E13.5 GE primary culture

Additional experiments were conducted to confirm that DLX proteins activate *Cxcr4* gene expression *in vitro*. *Dlx2* was transiently knocked down using *Dlx2*-siRNA in the SK-N-BE (2) neuroblastoma cell line (Figure 3.10 A, Figure 4.15 A) and E13.5 primary forebrain cultures (Figure 4.15 B). Transient knockdown of *Dlx2* expression resulted in a significant reduction of *Cxcr4* expression in SK-N-BE (2) cells (Figure 4.15 A). Expression levels of *Cxcr4* were also assessed in E13.5 forebrain primary cultures. Transient knockdown of *Dlx2* expression using siRNA pooling with two different *Dlx2*-siRNA in E13.5 primary cultures significantly decreased the expression of DLX2 and resulted in a significant loss of *Cxcr4* expression *in vitro* (Figure 4.15 B). These results further support my hypothesis that DLX2 is a transcriptional activator of *Cxcr4* expression in the developing CNS.

A.


Fold expression
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control SiRNA
Dlx2 SiRNA
Control SiRNA
Dlx2 SiRNA
Dlx2
Cxcr4

B.

.

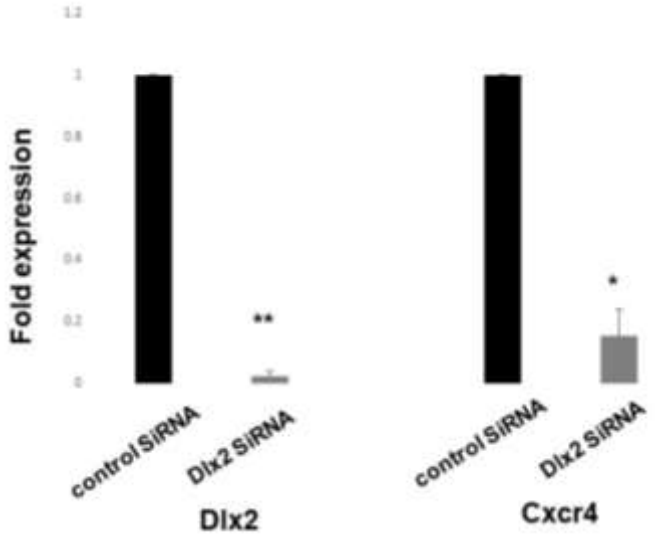

Fold expression
1.2
1
0.8
0.6
0.4
0.2
0
**
*
control SiRNA
Dlx2 SiRNA
control SiRNA
Dlx2 SiRNA
Dlx2
Cxcr4

Figure 4.15. qRT-PCR analysis showing relative expression of *Cxcr4* in *Dlx2*-siRNA treated cells and E13.5 GE primary cultures.

qRT-PCR analysis showing relative expression of *Cxcr4* after siRNA knockdown of *Dlx2* expression in S-KN-BE (2) cells (A). *Cxcr4* expression in E13.5 primary embryonic forebrain cultures treated two different *Dlx2*-siRNA (B).

Error bars represent standard error of the mean (SEM).

The assay was conducted in three biological and three technical replicates.

*= $P \leq 0.05$, **= $P \leq 0.01$

4.2.8 Altering *Dlx2* expression affects GABAergic interneuron migration *in vitro*

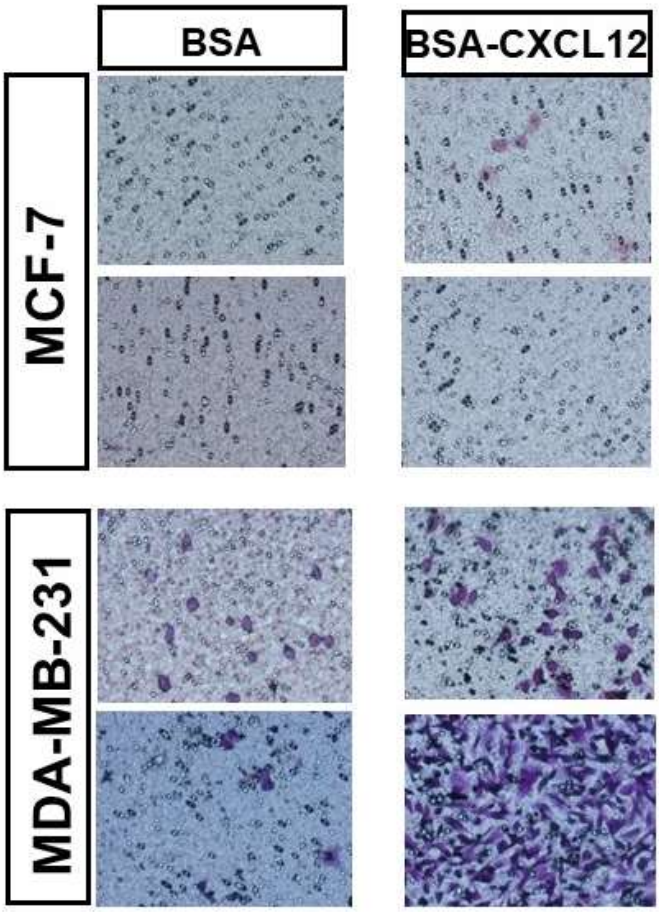
The last aim of the *Cxcr4* project was to investigate the effect of DLX2 on migration of GABAergic interneurons to the neocortex. The experimental approach consisted of preparing organotypic cultures of the E13.5 brain and tracing the migration of interneurons by placing DiI crystals on the organotypic cultures. To prepare the cultures, E13.5 brains were dissected from embryos, embedded in low melting point agarose, sliced into 300µm sections in the vibratome and placed on 8µm polycarbonate membranes. Following these preparations, DiI crystals were carefully placed on the GE using a pin under a dissection microscope. Consequently, the cultures were incubated for different times (24, 30, 48, 72 hours) in the incubator and after fixation, visualized on an Olympus fluorescent compound microscope using rhodamine fluorescence filters. Several attempts were made to perform this assay; however, due to multiple technical issues no useful data was generated to demonstrate the migratory stream of GABAergic interneurons in the embryonic brain (data not shown).

An alternative approach to assess the role of DLX2 in the migration of interneurons was to use the Boyden migration assay to assess the migration of cells *in vitro*. In this assay, Transwell plates containing 8µm polycarbonate membranes were used. MDA-MB-231 cells (Dr. R. Leng, University of Alberta), MCF-7 cells (Dr. R. Leng, University of Alberta), SK-N-BE (2) cells and E13.5 GE primary cultures were placed on the upper layer of the permeable membrane and CXCL12 solution was added to the lower layer beneath the membrane. The cells were allowed to migrate through the pores of the polycarbonate membrane after incubation. Finally, the cells that migrated through to the other side of the inserts were fixed, stained and counted (Boyden 1962, Chen 2005).

4.2.8.1 Cell migration is absent in MCF-7 and present in MDA-MB-231 breast cancer cells

Initially, MDA-MB-231 and MCF-7 breast cancer cell lines were used to establish the assay and test the system. MDA-MB-231 cells have a strong migration capability and were used as positive control, while MCF-7 cells have much weaker migration capability and were used as a negative control. After conducting the Transwell migration assay, cell migration was undetectable using MCF-7 cells and was significantly increased in MDA-MB-231 cells in the presence of CXCL12 (Figure 4.16).

A.


BSA
BSA-CXCL12
MCF-7
MDA-MB-231

B.

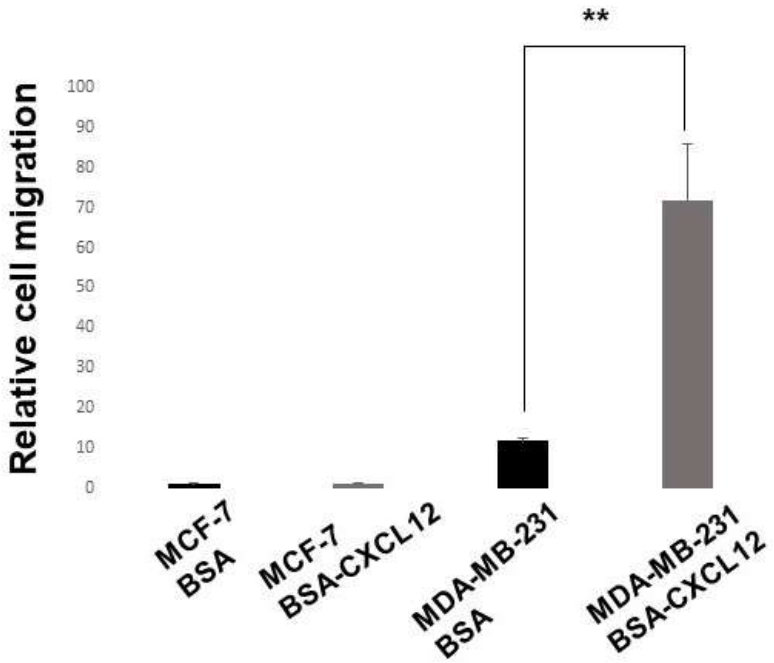

**
Relative cell migration
100
90
80
70
60
50
40
30
20
10
0
MCF-7 BSA
MCF-7 BSA-CXCL12
MDA-MB-231 BSA
MDA-MB-231 BSA-CXCL12

Figure 4.16. Cell migration in MCF-7 and MDA-MB-231 breast cancer cells treated with CXCL12.

A. Cell migration was almost undetectable in MCF-7 and present in MDA-MB-231 cells treated with CXCL12.

B. Relative cell migration was significantly increased in MDA-MB-231 cells in the presence of CXCL12.

Error bars represent standard error of the mean (SEM).

The assay was conducted in three biological and three technical replicates.

**= $P \leq 0.01$.

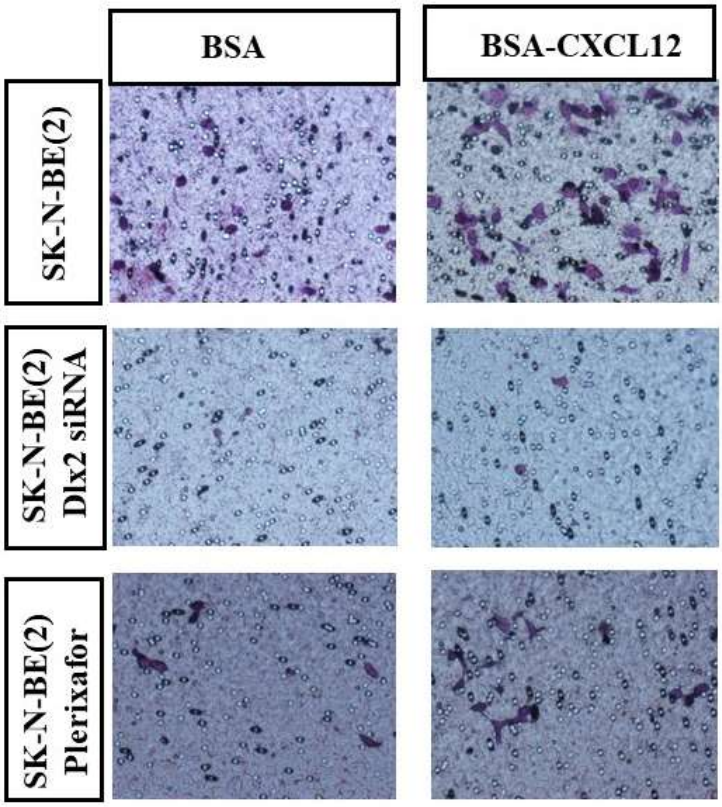
4.2.8.2 Cell migration in SK-N-BE (2) cells

After establishing the assay using the breast cancer cell lines, cell migration was assessed in SK-N-BE (2) cells. Conducting Boyden Transwell assay on SK-N-BE (2) cells revealed that there is a significant increase in cell migration in the presence of CXCL12 (Figure 4.17). Furthermore, *Dlx2* expression was knocked down in SK-N-BE (2) cells using siRNA. Cell migration was significantly decreased in *Dlx2*-siRNA treated cells compared to untreated cells, supporting the contribution of DLX2 to cell migration *in vitro* (Figure 4.17).

I was also interested in exploring the role of Plerixafor (AMD3100, Genzyme Corporation) on cell migration. Plerixafor is a CXCR4 antagonist that inhibits the binding of CXCL12 to its receptor CXCR4. After treating cells with 100mM Plerixafor for an hour, I assessed the migration of cells using the Transwell assay. Similar to *Dlx2*-siRNA treated cells, Plerixafor treated cells have a significant reduction in migration compared to untreated cells *in vitro* (Figure 4.17).

I also conducted the Bonhoeffer stripe assay to confirm the results obtained from the Transwell assay. Indeed, there was a notable increase in cell migration towards the stripes containing CXCL12 in SK-N-BE (2) cells (Figure 4.18 A). Furthermore, cell migration towards CXCL12 containing stripes was significantly decreased in *Dlx2*-siRNA treated cells compared to untreated cells, and there was no difference in cell distribution in the absence and presence of CXCL12 in transiently *Dlx2* knocked down cells compared to untreated cells, further supporting the contribution of DLX2 to cell migration *in vitro* (Figure 4.18 B, C).

A.


BSA
BSA-CXCL12
SK-N-BE(2)
SK-N-BE(2) Dlx2 siRNA
SK-N-BE(2) Plerixafor

B.

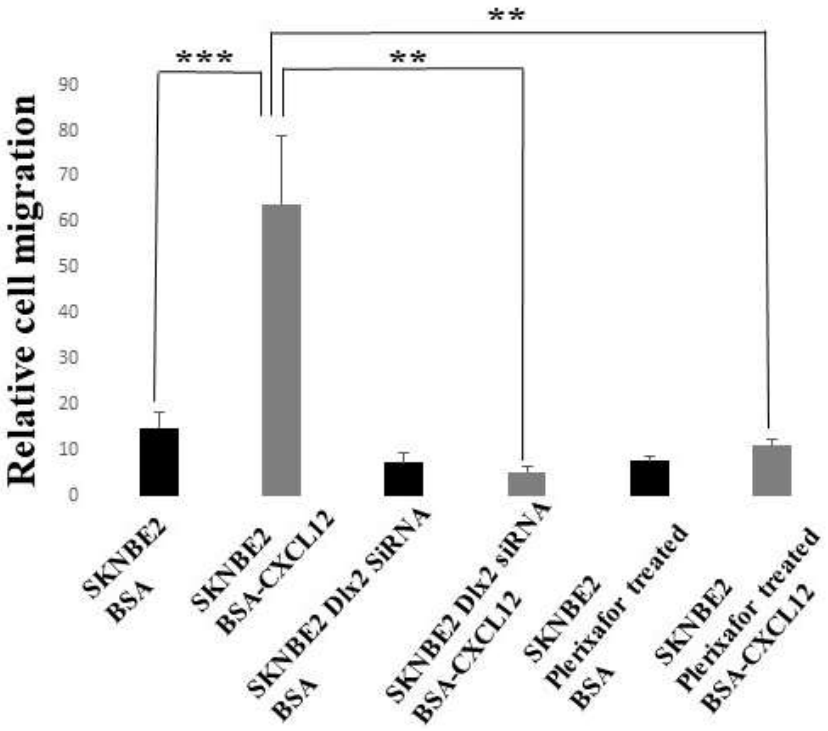

Relative cell migration
***
**
**
0
10
20
30
40
50
60
70
80
90
SKNBE2 BSA
SKNBE2 BSA-CXCL12
SKNBE2 Dlx2 SiRNA BSA
SKNBE2 Dlx2 siRNA BSA-CXCL12
SKNBE2 Plerixafor treated BSA
SKNBE2 Plerixafor treated BSA-CXCL12

Figure 4.17. Cell migration in SK-N-BE (2) cells using the Transwell assay.

A. There was a significant increase in cell migration in SK-N-BE (2) cells in the presence of CXCL12. Cell migration was significantly decreased in *Dlx2*-siRNA treated cells compared to untreated cells in the presence of CXCL12. Plerixafor was used as a CXCR4 antagonist. Plerixafor treated cells also showed a significant reduction in migration compared to untreated cells in the presence of CXCL12.

B. Relative cell migration was quantified in untreated, *Dlx2*-siRNA treated, and Plerixafor treated SK-N-BE (2) cells in the presence or absence of CXCL12.

Error bars represent standard error of the mean (SEM).

The assay was conducted in three biological and three technical replicates.

**= $P \leq 0.01$, ***= $P \leq 0.001$.

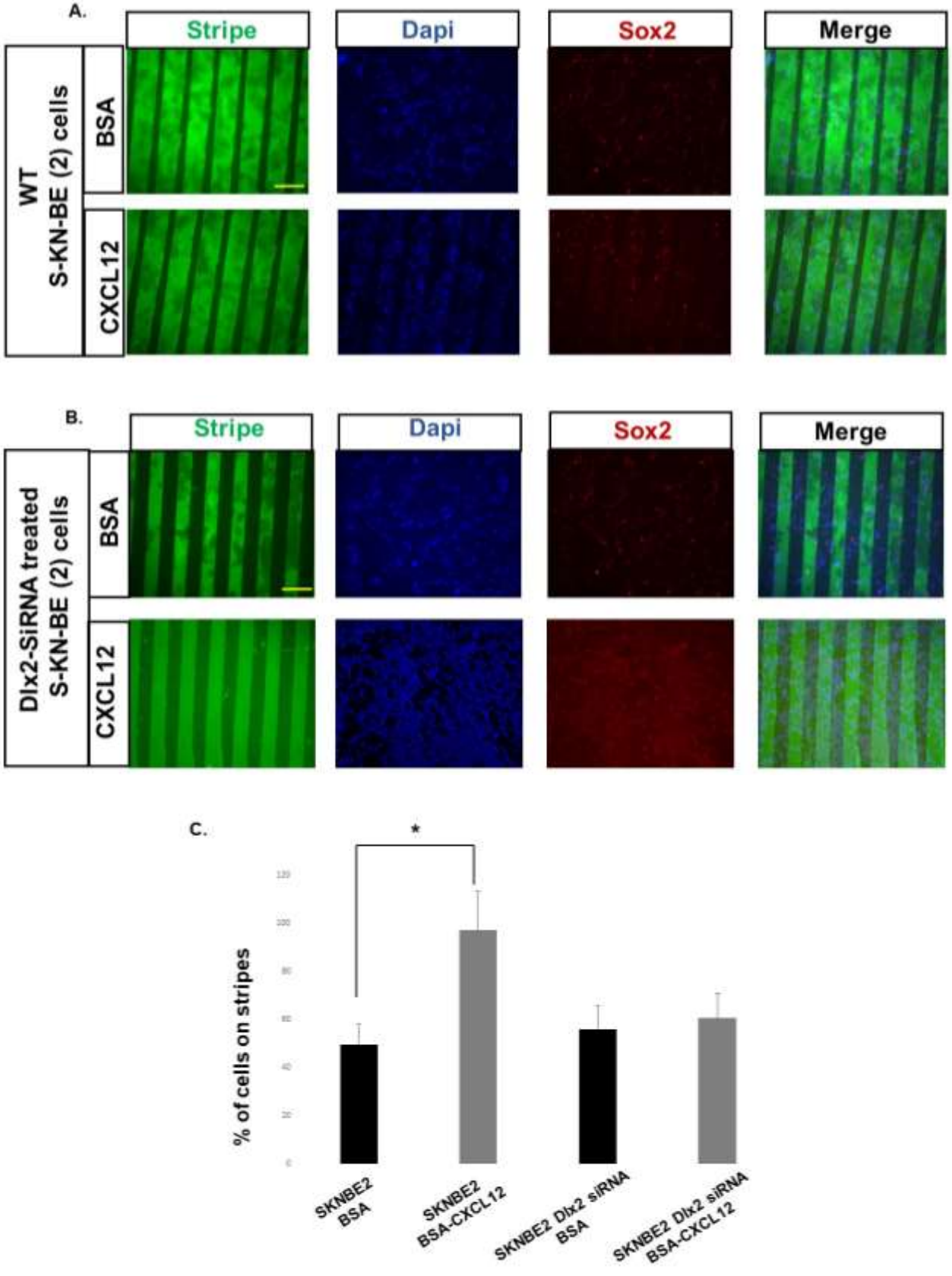

A.
Stripe
Dapi
Sox2
Merge
WT
S-KN-BE (2) cells
BSA
CXCL12
B.
Stripe
Dapi
Sox2
Merge
Dlx2-SiRNA treated
S-KN-BE (2) cells
BSA
CXCL12
C.
*
% of cells on stripes
SKNBE2
BSA
SKNBE2
BSA-CXCL12
SKNBE2 Dlx2 siRNA
BSA
SKNBE2 Dlx2 siRNA
BSA-CXCL12

Figure 4.18. Cell migration in SK-N-BE (2) cells using the stripe assay.

A. There was an increase in cell migration in SK-N-BE (2) cells in the CXCL12 striped area compared to the BSA striped area.

B. Cell migration was decreased in *Dlx2*-siRNA treated cells compared to untreated cells in the presence of CXCL12.

C. The quantification of stripe migration assay is shown. In the untreated SK-N-BE (2) cells the percent of cells on stripes in the presence of CXCL12 was significantly increased compared to the control group (49% in BSA vs 97% in CXCL12). In *Dlx2*-siRNA treated cells there was no significant difference in the presence or absence of CXCL12 (57% in BSA vs 60% in CXCL12). Error bars represent standard error of the mean (SEM).

The assay was conducted in three biological replicates.

*= $P \leq 0.05$.

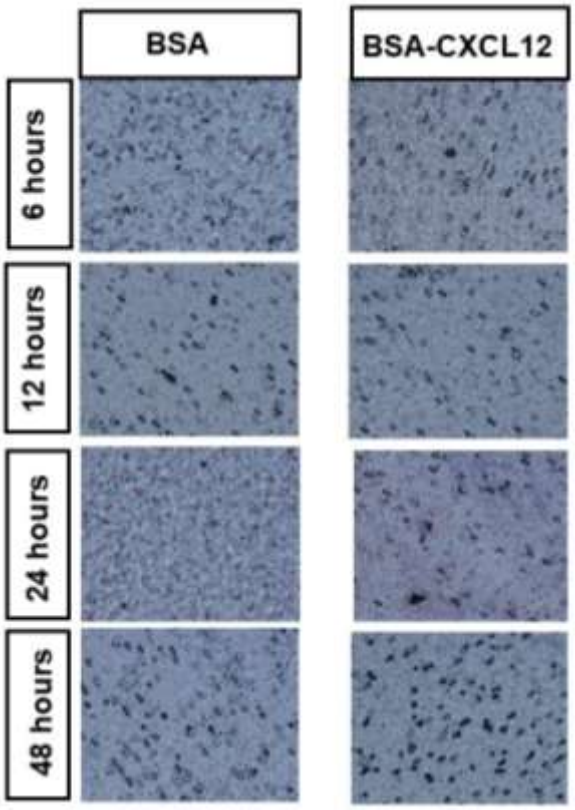
4.2.8.3 Cell migration in E13.5 forebrain primary cultures

For the next step, cell migration in E13.5 primary GE cultures was assessed. Primary cultures were placed on the top layer of the Transwell membranes and CXCL12 was added to the lower layer of the Transwell beneath the membranes. After incubation for different time points (from 6 to 48 hours), the migration of the cells from the top layer of the membranes to the bottom layer of the membranes was assessed. After evaluating the migration of E13.5 primary cells after incubation at different time points, no significant difference in their migration in the presence or absence of CXCL12 was observed (Figure 4.19).

I also performed the Bonhoeffer stripe assay using E13.5 forebrain primary cultures. The primary cultures failed to attach to the plates after stripes were made and the results for this experiment were inconclusive.

Furthermore, migration capabilities at other embryonic time points (E12.5, E14.5, E15.5 and E16.5) during embryonic forebrain development was also assessed. Although migration capability was observed at E13.5 to E15.5, there were insufficient migrating cells to proceed with experiments (Figure 4.20).

A.


BSA
BSA-CXCL12
6 hours
12 hours
24 hours
48 hours

B.

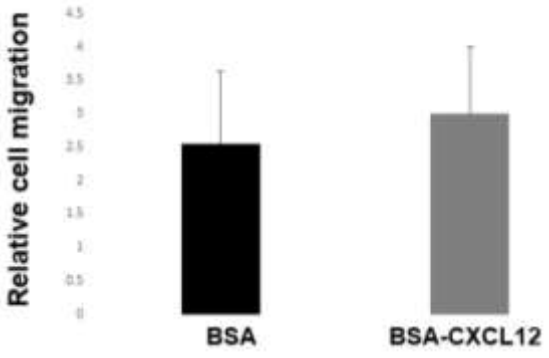

Relative cell migration
BSA
BSA-CXCL12

Figure 4.19. Cell migration assays in E13.5 forebrain primary cultures treated with the chemokine CXCL12.

Examples of individual Transwell experiments are shown in A. Relative cell migration was not statistically significant (B).

Error bars represent standard error of the mean (SEM).

The assay was conducted in N=4 biological and N=3 technical replicates.

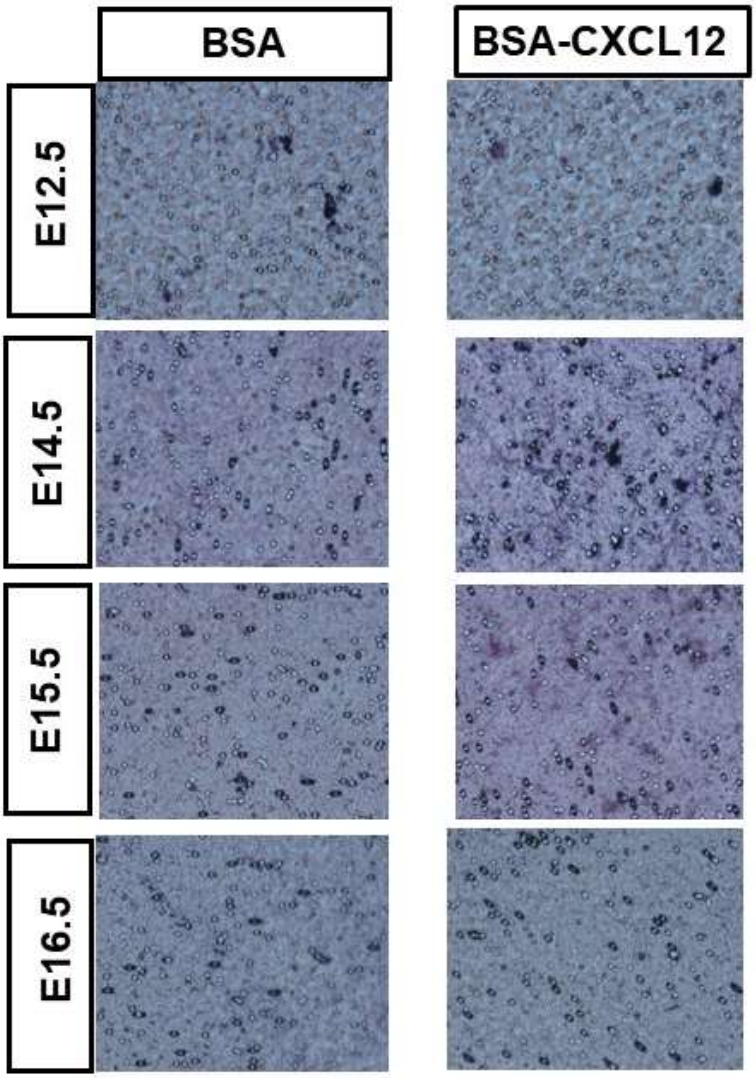


Figure 4.20. Cell migration assays in E12.5, E14.5, E15.5 and E16.5 forebrain primary cultures treated with the chemokine CXCL12.

Examples of individual Transwell experiments are shown. Experiments for E12.5 and E14.5 were performed in N=2 biological replicates and N=3 technical replicates, respectively. Experiments for E15.5 and E16.5 were performed in N=3 biological replicates and N=3 technical replicates.

5 Chapter 5: Phosphorylation of DLX2 during forebrain development

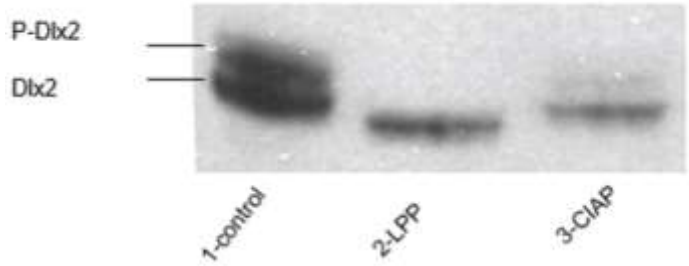
5.1 Introduction

Posttranslational modifications of transcription factors are important for gene expression and regulate transcription factor activity and localization. Current knowledge regarding PTM of DLX2 and whether it affects DLX2 localization and function is very limited. Previous studies in the Eisenstat lab using 1-D gels of E13.5 GE lysates, a time point when DLX2 is highly active during brain development, have shown that DLX2 is dephosphorylated by lambda protein phosphatase (LPP) and to a lesser extent by calf intestine alkaline phosphatase CIAP (Figure 5.1 A, Zhang S and Eisenstat D, unpublished observations). Whilst LPP is capable of dephosphorylating serine, threonine and tyrosine residues in many tissue including the brain, CIAP is mainly active in the intestine, liver and kidney and can explain its lower activity in the ganglionic eminence extracted tissue. Furthermore, two-dimensional gel electrophoresis (2-D) of E13.5 GE lysates confirmed that DLX2 is dephosphorylated by LPP (Figure 5.1 B, Zhang S and Eisenstat D, unpublished observations). In the 2-D gel electrophoresis proteins are separated in one dimension based on their charge and then in another dimension perpendicular to the first based on their molecular mass. Furthermore, while DLX1 is only expressed in the nucleus of the ventral thalamus, DLX2 is expressed both in the nucleus and cytoplasm of the ventral thalamus (Eisenstat, Liu et al. 1999). It is possible that the subcellular localization of DLX2 in the ventral thalamus is determined in part by PTM of DLX2.

More studies need to be carried out to investigate phosphorylation of DLX2 and its role during brain development. The identity of critical serine, threonine or tyrosine residues required for phosphorylation as well as the kinase(s) upstream of DLX proteins have not been characterized. Furthermore, the signaling pathways that regulate DLX function have yet to be

determined. I hypothesized that phosphorylation of critical residues of the DLX2 protein is required for its nuclear localization and transcriptional activity during forebrain development.

A.



B.

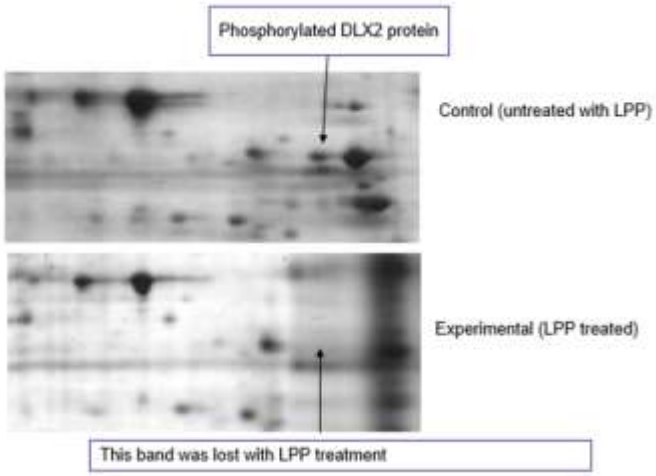


Figure 5.1. DLX2 phosphorylation in mouse E13.5 striatum.

A. 1-D gel immunoblot using the concentrated DLX2 antibody. Lane 1: E13.5 GE tissue lysate shows two bands. Lane 2: E13 GE lysate treated with lambda protein phosphatase (LPP). Lane 3: E13.5 GE lysate treated with calf intestine alkaline phosphatase (CIAP).

B. 2-D gel (silver stain) using E13.5 GE total cell lysate. Upper gel: control. Lower gel: LPP treated sample demonstrates a lost band supporting that DLX2 is a phosphorylated protein. (Zhang S and Eisenstat D, unpublished observations).

5.2 Results

5.2.1 Western Blotting of E13.5 WT GEs shows posttranslational modifications of DLX2

To confirm the presence of posttranslational modifications of DLX2 in the embryonic forebrain, I performed Western Blotting of E13.5 WT ganglionic eminences. Indeed, Western Blotting of tissue extracted from the E13.5 ganglionic eminences using concentrated DLX2 antibody demonstrated the presence of PTM in the detected DLX2. Treating the samples with lambda protein phosphatase eliminated the upper band, suggesting that phosphorylation is a major PTM present in DLX2 (Figure 5.2).

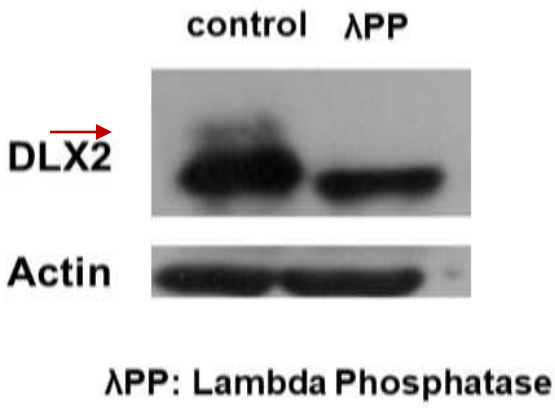


Figure 5.2. Western Blotting on E13.5 WT GEs shows the presence of posttranslational modifications on DLX2.

Immunoblotting indicated the presence of two DLX2 bands. Addition of lambda protein phosphatase removed this upper band (red arrow) consistent with the hypothesis that DLX2 is phosphorylated *in vivo*.

β-actin was used as a loading control.

λPP= lambda protein phosphatase

5.2.2 **DLX2 expression was not detected in several cell lines**

After identifying posttranslational modifications in DLX2 *in vivo*, the next step in this study was to identify sites of phosphorylation on DLX2 by mass spectrometry. To identify PTM using mass spectrometry, at least 100ng of protein was needed. Assessing phosphorylation sites *in vivo* would require sacrificing many mice. To circumvent this issue, I attempted to use cell lines to identify phosphorylation residues on DLX2. At first I sought to identify a cell line that expresses significant levels of DLX2. The expression of DLX2 in Y79, WERI-RB1 (retinoblastoma); COLO 320 (colorectal cancer); SK-N-SH, SH-SY5Y (neuroblastoma); PC-12 (pheochromocytoma), RS4;11 (leukemia); and D341, D283 (medulloblastoma) cells was assessed. The cell lines were chosen based on their biological relevance and their availability in the lab. I then assessed the level of DLX2 expression in the aforementioned cell lines using Western Blotting with concentrated DLX2 specific antibody. Unfortunately, DLX2 expression was not detectable in any of the cell lines (Figure 5.3).

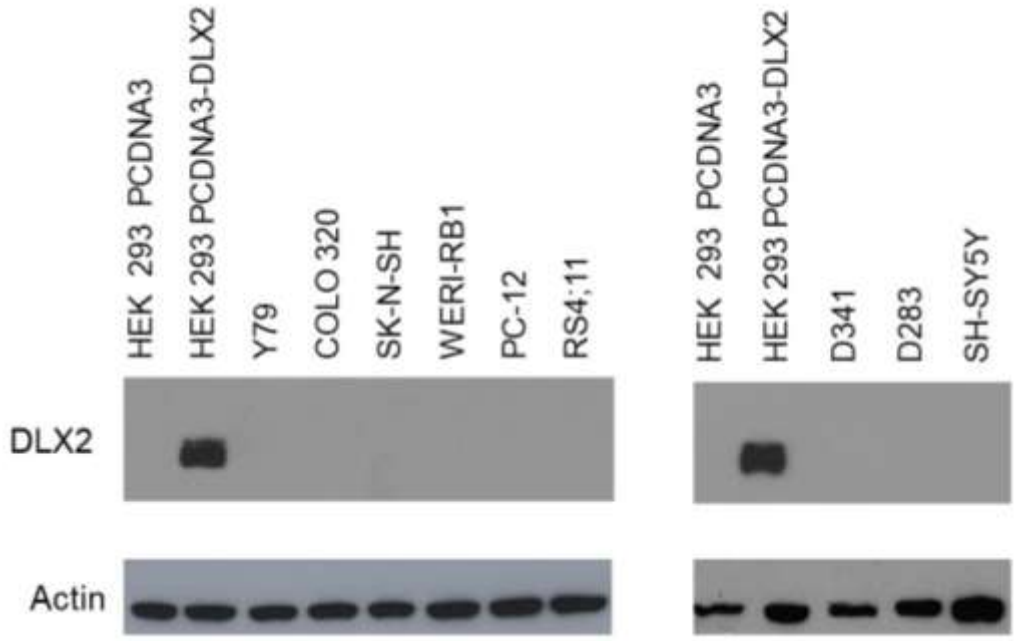


Figure 5.3 DLX2 expression levels in different cell lines using Western Blotting with DLX2 specific antibody.

Cell lines Y79, COLO 320, SK-N-SH, WERI-RB1, PC-12, RS4;11, D341, D283, and SH-SY5Y were chosen based on their biological relevance and their availability in the lab. DLX2 expression was not detected in any of the cell lines.

5.2.3 Immunoprecipitation was not able to isolate DLX2 from E13.5 ganglionic eminences

Since none of the cell lines mentioned above expressed DLX2, I decided to proceed with identifying DLX2 phosphorylation *in vivo*. Consequently, GEs from 20 litters were collected to gather a considerable amount of protein necessary for mass spectrometry (>100ng). Immunoprecipitation using the concentrated DLX2 antibody was then carried out to isolate DLX2 from the tissue. Whilst the concentrated DLX2 antibody is very good for performing ChIP, we have not been very successful in using it for immunoprecipitation experiments on tissue (Eisenstat, personal communication). Immunoprecipitation experiments revealed that DLX2 antibody can successfully isolate DLX2 from HEK 293 cells but is unable to pull down DLX2 from embryonic tissue (Figure 5.4).

A.

B.

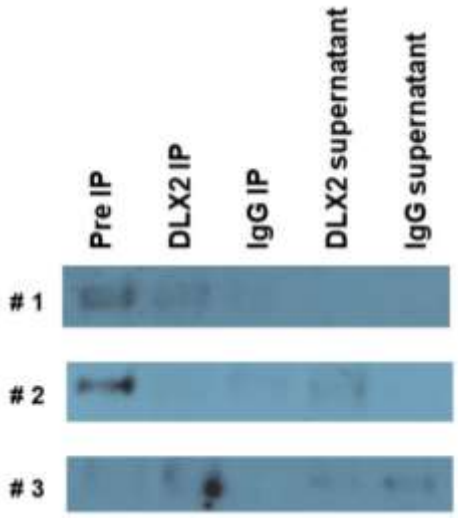


Figure 5.4. Immunoprecipitation using DLX2 antibody to isolate DLX2 from HEK293 cells (A) and E13.5 ganglionic eminences (B).

A. IP experiments successfully isolated DLX2 from *Dlx2* transfected HEK293 cells. The pre-IP consisted of sample before the IP was conducted and was used as a positive control. Concentrated DLX2 antibody was added to isolate DLX2 (DLX2 IP). IgG antibody was used as a negative control (IgG IP).

B. IP experiments were unable to isolate DLX2 from embryonic tissue. Several attempts were made to isolate DLX2 from embryonic tissue.

5.2.4 Initial mass spectrometry experiments did not identify DLX2 potential sites of phosphorylation

Two approaches were taken to identify DLX2 phosphorylation residues *in vivo* by mass spectrometry analysis. In the first approach, concentrated DLX2 antibody was used to isolate DLX2 from E13.5 embryonic tissue and this purified sample was sent for analysis. However as previously mentioned, I was unable to successfully isolate DLX2 from embryonic tissue by immunoprecipitation (Figure 5.4). Nonetheless, samples were sent for mass spectrometry analysis after conducting immunoprecipitation. As expected, results from mass spectrometry analysis showed that the IP was unable to isolate DLX2 protein from the total lysate, less than 1% of the sample included DLX2 protein and detection of phosphorylation was unsuccessful.

To overcome this issue, an alternative approach was used where GEs from 20 litters were dissected and protein lysates were separated with a 10% SDS-PAGE gel. Subsequently, the band corresponding to DLX2 molecular weight (43 KD) was cut out and analysed by mass spectrometry. The results showed that there were 334 proteins in the sample, and detection of phosphorylation on a specific protein was not possible. Therefore, the results for this study are currently inconclusive.

5.2.5 *In silico* analysis for DLX2 phosphorylation

I also performed *in silico* analysis to obtain information on DLX2 phosphorylation. Nine different phosphorylation prediction tools including GPS 3.0, NetPhos 3.1, KinasePhos 2.0, Disphos, PhosphositePlus, Phospho.elm, PhosphoNET, Scansite, and pkaPS were used to predict phosphorylation sites on DLX2.

Results from *in silico* analysis are summarized in Table 5.1.

Table 5.1. *In silico* analysis of DLX2 phosphorylation.

Phosphorylation prediction tools used are GPS 3.0, NetPhos 3.1, KinasePhos 2.0, Disphos, PhosphositePlus, Phospho.elm, PhosphoNET, Scansite, and pkaPS.

Position	NetPhos3.1	GPS 3.0	KinasePhos	Disphos	Phosphosite plus	others
15T	PKC	AGC/GRK		Low score		
20S	PKC					
22T	PKC					
31S	GSK3 (LOW)	AGC/NDR		+	+	
41S	CDC2	AGC/GRK/BARK	IKK	+		
43S	CDC2	AGC/RSK		+		
44S	CDC2/PKC			+		
45S	PKC	AGC/RSK/PKC		+	+	
46S	PKC/CDC2	AGC/PDK1			+	
48S	CDC2		CKI	+		
49S	PKC/CDC2	AGC/PKC		+		
56S	AGC/GRK	AGC/NDR		+		
63T	AGC/GRK/GPRK6	AGC/GRK/GRK		+		
68S	PKC	AGC/RSK				PLK1
69Y	INSR/SYK		MDD	+		
70Y	INSR/SYK			+		
115Y	INSR/SYK			+		
125S	CDC2					
125Y	INSR (LOW)			+	+	
127T	GSK3 (LOW)		PKC	+		
129S	PKC	AGC/PKC		+		
130S	+		CDC2	+	+	
216S	PKA	AGC/AKT/AKT2		+		
235S	CDK5/MAPK	AGC/PDK1	CDC2	+	+	
267S	CDC2	AGC/GRK/BARK	ATM	+		
269S	CDC2		IKK	+		
270S	CDK5/CDC2	AGC/PDK1	CDC2/ATM	+		
305S	PGK/DNAPK	AGC/GRK	ATM			
307T	+	AGC/GRK	MAPK			

There is a lot of discrepancy for phosphorylated residues on DLX2 and many phosphorylated sites have been predicted. Nevertheless, residues 31 serine (S), 41S, 45S, 130S, 235S, 267S and 270S have the highest possibility of being phosphorylated sites *in vivo,* since they were predicted by four or more phosphorylation prediction tools. The locations of high-possibility predicted phosphorylation sites on DLX2 are shown in Figure 5.5. None of the high-possibility sites are located in the homeodomain region. This is expected, since the homeodomain is important for interacting with regulatory elements and may function more effectively if devoid of any posttranslational modification.

Figure 5.5. Location of highly predicted phosphorylation sites on DLX2 protein.

Highly predicted phosphorylated sites shown in red are residues predicted to be phosphorylated *in vivo* using four or more phosphorylation prediction tools. The homeodomain region is underlined and italicized. Predictions indicate that the homeodomain is devoid of high potential posttranslational modifications by serine (S), threonine (T) and tyrosine (Y) phosphorylation.

6 Chapter 6: Discussion

6.1 Interaction of DLX2 with DNA binding sites on *Nkx2.2* and *Cxcr4* regulatory regions

DLX transcription factors bind to specific sequences on *cis* regulatory regions known as DNA binding sites. Since homeodomain transcription factors generally recognize TAAT/ATTA motifs (Catron, Iler et al. 1993, Damante, Fabbro et al. 1994), all the TAAT/ATTA sites within 3kb of the proximal promoter of *Nkx2.2* and *Cxcr4* were interrogated as candidate sites for DLX2 binding. ChIP based PCR assays revealed that DLX2 occupied several of these canonical homeodomain DNA binding motifs within the interrogated regulatory regions. ChIP positive regions were then further characterized using EMSA to assess binding specificity, and luciferase reporter assays to determine the functional consequences of co-expression with a *Dlx2* expression construct, respectively, *in vitro*.

Transcription factors can recognize hundreds of DNA binding sites with different binding affinities. Therefore, a single DNA binding sequence may not be able to demonstrate the specificity of binding for a particular transcription factor. To account for variability within DNA binding sites a position weight matrix (PWM) is used. PWM is a more comprehensive presentation of the DNA binding motif that considers variability within consensus binding sites and gives a higher weight to more conserved positions (Stormo 2000). Affolter and his colleagues analyzed protein binding microarrays (PBMs) to obtain 8 nucleotide binding sequences for over 190 homeodomain transcription factors. Based on their PBM analysis, the PWM for DLX2 was NT/CAATTA/GN with N corresponding to any nucleotide and T and A having a slightly higher weight at positions two and seven, respectively (Affolter, Slattery et al. 2008). Another study used high throughput SELEX and ChIP sequencing (ChIP-seq) to analyze

specific DNA binding sequences of human transcription factors and specified NYAATTAN as the PWM for DLX2 (N, any nucleotide; and Y, Pyrimidine (C or T))(Jolma, Yan et al. 2013). Based on these aforementioned studies, the 6 nucleotide sequence C/TAATTA is a more comprehensive binding sequence for DLX2 and should be considered when interrogating any regulatory region of interest for potential DLX2 binding in the future.

To regulate gene expression, transcription factors interact with *cis* regulatory regions located proximal or distal from the gene. ChIP-seq using a DLX1 antibody on E16 mouse tissue extracted from the hypothalamus showed that only ~11% of the peaks from sequencing were located in the promoter region and over 80% of peaks were from intergenic or intronic regions (Lee, Kim et al. 2018). ChIP-seq is an unbiased approach to identify all DNA sequences in the genome that are associated with a transcription factor and do not rely on the specific binding sequence to identify interactions between a specific transcription factor and DNA. Therefore, future directions for this project will include identifying DNA binding sites throughout the genome using ChIP-seq experiments with DLX2 antibody on E13.5 forebrain to get unbiased results on all DNA sequences associated with DLX2. Results from ChIP-seq are expected to show enrichment of DLX2 binding of proximal and distal regulatory elements which can then be further characterized and validated. To confirm candidate DLX2 transcriptional targets identified by ChIP-seq, one would repeat the ChIP experiments combined with PCR using primers. EMSA assays would determine the specificity of interactions and confirm whether the DLX2 interaction with the candidate target sequences is direct or indirect. Moreover, site directed mutagenesis followed by EMSA and luciferase reporter assays can further validate the results gained from ChIP-seq. RNA-seq on WT and DKO E13.5 forebrain can also be conducted to interrogate the expression levels of different genes in the presence and absence of *Dlx1* and *Dlx2*.

Transcription factors recognize DNA through a cooperation between base and shape readout. Base readout is the direct interaction of the transcription factor with DNA. DNA binding sites and PWM only show the shape readout. Transcription factors can also recognize DNA structure such as DNA bending, flexibility in conformation and electrostatic capability (Slattery, Zhou et al. 2014). Furthermore, flanking sequences and presence of other cofactors can influence specificity of binding. Consequently, the most comprehensive approach to identify transcription factor binding specificity is to take into account DNA shape as well as sequence (Inukai, Kock et al. 2017). Utilizing models that predict transcription factor binding based on DNA sequence as well as shape improved upon models that were only using sequence (Zhou, Shen et al. 2015).

6.2 Role of DLX2 in cell fate decision

Dlx1 and *Dlx2* have a critical function in regulating cell fate decisions. *Dlx* genes control cell fate by regulating target genes with different functions. I hypothesized that DLX2 directly mediates transcriptional repression of oligodendroglial differentiation in determination of neuronal *versus* glial cell fates through inhibition of *Nkx2.2* and simultaneously mediates transcriptional activation of GABAergic interneuron migration through promotion of *Cxcr4* expression during forebrain development.

Transcription factors regulate cell fate specification and differentiation through three modes of action. The first mechanism is transcriptional activation where, in the presence of a specific transcription factor, a certain cell fate is determined. On the other hand, in the absence of the same transcription factor, cells will acquire an alternate cell fate. This default pathway is present in pancreatic development. *Pdx1*, a homeodomain containing transcription factor, is involved in different stages of pancreatic development. During mid-pancreatic development,

Pdx1 is required for the formation of the exocrine pancreas. Without *Pdx1*, acinar tissue does not form and the ductal tissue is truncated (Hale, Kagami et al. 2005).

The second mode of action is a feed-forward mechanism. In a feed-forward loop, specification is achieved by activation of transcription factor B by transcription factor A, followed by the activation of transcription factor C by A/B, and so on and so forth. In a subtype of nerve cord neurons in *Drosophila*, known as Tv1 and dAp neurons, a feed-forward cascade is utilized to generate the Neuropeptide like precursor protein 1, Nplp1, which is important for specification of this set of neurons. In the Tv1 and dAp neurons, the COE family member collier (*Col*), encoding a COE/EBF transcription factor, is expressed and activates a feed-forward mechanism. *Col* activates the Apterous (*Ap*) LIM-homeodomain factor and the Eyes absent (*Eya*) transcriptional co-factor. Subsequently, *Ap*, *Eya*, and *Col* act together to activate the bHLH transcription factor Dimmed (*Dimm*) which will activate *Nplp1* (Baumgardt, Miguel-Aliaga et al. 2007).

The third mechanism is active repression where in the presence of a specific transcription factor a certain cell fate is inhibited. An example of transcriptional repression in cell fate decision is evident in development of two cell types during *Drosophila* myogenesis: founder cells (FCs) and fusion-competent myoblasts (FCMs). The zinc-finger repressor Tramtrack plays a role in the specification of FCM cell fate by inhibiting FC cell differentiation. In the absence of *tramtrack69*, FCMs cells take on a FC cell fate (Ciglar, Girardot et al. 2014). Another example for transcriptional repression is seen in cell fate decisions in mesenchymal precursor cells. Differentiation of mesenchymal precursor cells into osteoblasts or adipocytes is regulated by the transcription factor Zfp521. During mesenchymal precursor differentiation, Zfp521 acts as a cell

fate switch important for osteoblast commitment by directly repressing adipocyte determinant factor *Zfp423* (Addison, Fu et al. 2014).

It is evident that these three mechanisms need to work together during cell fate decisions in development of both invertebrates and vertebrates. Specifically, transcriptional activation and active repression of the alternative transcriptional program play hand in hand during cell fate specification to promote one cell fate and antagonize another cell fate. The Eisenstat lab has established that *Dlx1* and *Dlx2* genes are key transcription factors in determination of neuronal *versus* glial cell fates that act as transcriptional activators to promote interneuron cell identity and at the same time act as repressors to inhibit oligodendrogenesis. DLX1 and DLX2 positively regulates the *Gad2/Gad1* gene products, GAD65 and GAD67, which are involved in GABA synthesis in GABAergic interneurons (Le, Zhou et al. 2017). DLX1 and DLX2 also restrict the expression of *Olig2*, favouring GABAergic interneuron cell fate over oligodendrocytes (Petryniak, Potter et al. 2007).

6.3 DLX2 represses *Nkx2.2* expression during forebrain development

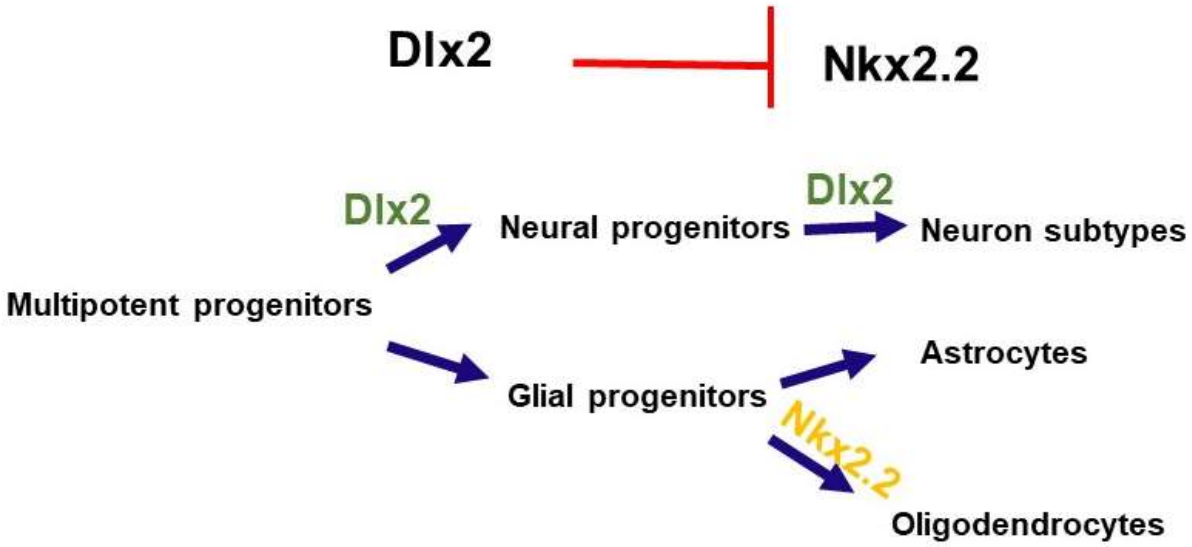
Dlx1 and *Dlx2* genes are important cell fate determinants where they positively promote GABAergic interneuron differentiation at the expense of oligodendrocyte progenitor cell differentiation. Since the expression of *Nkx2.2*, an important factor involved in oligodendrocyte differentiation, increases in *Dlx1/2* DKO mice (Petryniak, Potter et al. 2007), I proposed that DLX2 also represses the cell fate of oligodendrocyte differentiation by negatively regulating *Nkx2.2.* ChIP-based PCR of embryonic mouse forebrain demonstrated that DLX2 occupies regions containing putative DLX2 binding sites located in the proximal promoter region of *Nkx2.2*. Conducting EMSA to validate ChIP positive regions showed that only two regions (R3 and 4) were EMSA positive while four regions were positive for ChIP (R1 to R4). These results

support that direct interaction between DLX2 and promoter region of *Nkx2.2* is only present in R3 and R4. Regulation of R1 and R2 by DLX2 is most likely indirect where DLX2 is part of a complex with other transcription factors and cofactors. Co-expression of DLX2 and NKX2.2 was not observed in the ganglionic eminences of WT mice, and quantitative RT-PCR showed an increase in transcript levels of *Nkx2.2* in embryonic *Dlx1/2* DKO forebrain tissues as well as in neuroblastoma cells and forebrain primary cultures with transient knockdown of *Dlx2* as compared to the WT. In the developing brain, NKX2.2 is expressed in a limited domain of the ventral thalamus and also in the boundary of alar and basal hypothalamus (Puelles, Martinez-de-la-Torre et al. 2012). In this study I detected NKX2.2 expression at E13.5 in the hypothalamus by immunofluorescent staining. In the *Dlx1/Dlx2* DKO forebrain, NKX2.2 expression was significantly increased in the alar and basal domains of hypothalamus. Furthermore, NKX2.2 expression was ectopically detected in the ventral thalamus in the *Dlx1/Dlx2* DKO forebrain. Similar phenotypes have been seen in *Pax6*$^{-/-}$ mutants, where NKX2.2 expression is not restricted to the ventral thalamus and is expressed throughout the thalamus. Moreover, in mice lacking *Pax6* the axons of the thalamic neurons move to the hypothalamus instead of the telencephalon (Clegg, Li et al. 2015). Overexpression of NKX2.2 in *Pax6* null mice, similar to the phenotype observed in *Dlx1/Dlx2* DKO mice, may be the result of DLX and PAX6 interactions at the transcriptional level. Performing ChIP re ChIP experiments can identify protein-protein interactions between DLX2 and PAX6. Furthermore, looking at expression levels of NKX2.2 downstream targets such as *Pdgfra*, *Mbp*, and *Sirtuin 2* (*Sirt2*) in *Dlx1/2* DKO mice as well as *Pax6*$^{-/-}$ mice can provide further information on the interaction between DLX2, PAX6 and *Nkx2.2*. *Pdgfra* is a negative regulator for oligodendrogenesis and by directly repressing *Pdgfra,* NKX2.2 is involved in promoting oligodendrocyte differentiation (Zhu, Zhao et al. 2014). *Mbp*

is another target for NKX2.2. During early stages of development a high concentration of NKX2.2 inhibits expression of *Mbp* by recruiting HDAC1 and competing with the transcriptional activator Pura to prevent premature synthesis of myelin (Wei, Miskimins et al. 2005). Furthermore, in CG4, an OPC cell line, NKX2.2 directly binds to *Sirt2* and with cofactor HDAC-1 represses *Sirt2*. *Sirt2* promotes *Mbp* expression and negative regulation of *Sirt2* by NKX2.2 prevents premature oligodendrocyte differentiation (Ji, Doucette et al. 2011).

My data supports the hypothesis that *Nkx2.2* is a downstream target of DLX2 and is negatively regulated by DLX2. As NKX2.2 is required for oligodendrocyte differentiation and myelination, repression of *Nkx2.2* by DLX2 in the ventral forebrain contributes to the role of *Dlx* genes in interneuron differentiation and reinforces a critical role for *Dlx* genes in neuronal versus glial cell fate specification during forebrain development (Figure 6.1). This work is further substantiated by ongoing work in the laboratory characterizing *Olig2* and *Myt1* as transcriptional targets directly repressed by DLX2 during forebrain development.

A.


Dlx2
Nkx2.2
Dlx2
Dlx2
Neural progenitors
Neuron subtypes
Multipotent progenitors
Astrocytes
Glial progenitors
Nkx2.2
Oligodendrocytes

B.

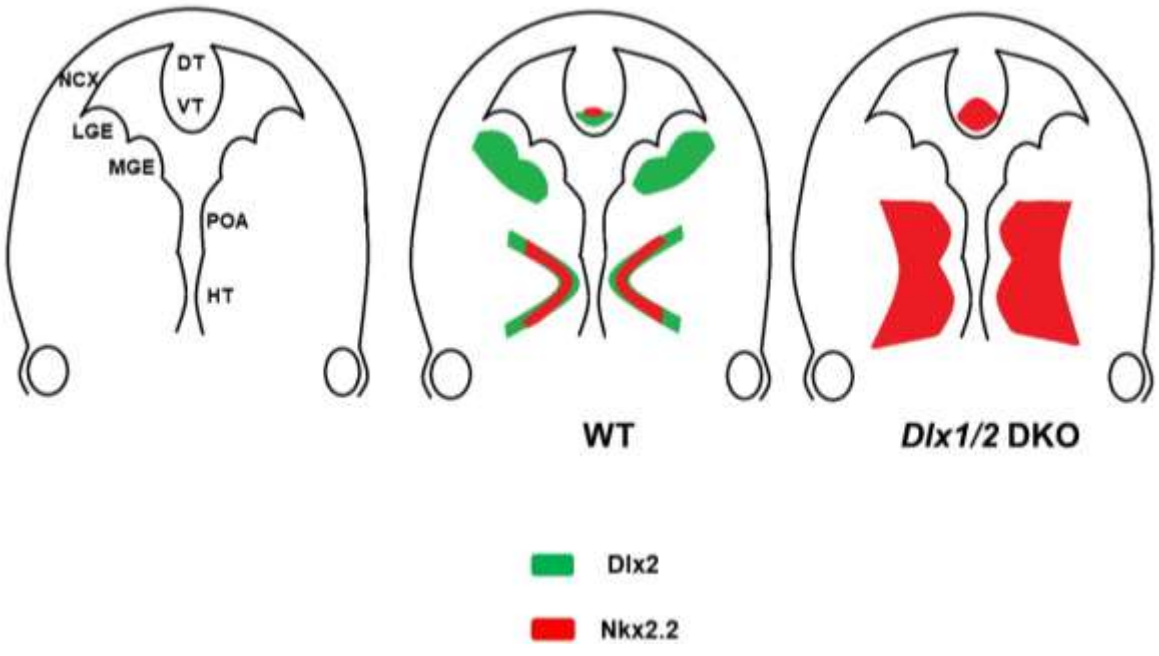

NCX
DT
VT
LGE
MGE
POA
HT
WT
Dlx1/2 DKO
Dlx2
Nkx2.2

Figure 6.1. A diagram depicting the role for DLX2 in neural vs glial cell fate specification during forebrain development.

A. DLX2 is important for the specification and differentiation of GABAergic interneurons. DLX2 negatively regulates *Nkx2.2* to promote neuronal identity and repress an oligodendroglial cell fate in GABAergic interneurons.

B. Pattern of expression of DLX2 and NKX2.2 in the WT and *Dlx1/2* DKO developing murine brain.

In the WT (middle panel), there is no overlapping pattern of expression for DLX2 and NKX2.2 in the ganglionic eminences. However, both DLX2 and NKX2.2 are expressed in the hypothalamic region. In the *Dlx1/2* DKO (right panel), NKX2.2 expression specifically increases in the hypothalamus and ventral thalamus of the *Dlx1/2* DKO compared to WT forebrains.

NCX: neocortex, LGE: lateral ganglionic eminences, MGE: medial ganglionic eminences, DT: dorsal thalamus, VT: ventral thalamus, POA: pre-optic area, HT: hypothalamus, WT: wild-type, DKO: double knockout.

6.4 DLX2 is involved in migration of interneurons by activating *Cxcr4*

The majority of cortical interneurons originate from the subcortical ganglionic eminences and migrate tangentially to reside in the neocortex (Marin and Rubenstein 2001). DLX2 is expressed in GABAergic interneurons and is a crucial transcription factor for differentiation and migration of these inhibitory interneurons (Anderson, Eisenstat et al. 1997, Anderson, Qiu et al. 1997, Stuhmer, Anderson et al. 2002, Le, Zhou et al. 2017). *Dlx1/Dlx2* DKO mice have a complete loss of tangential migration of GABAergic interneurons from the subpallium into the cortex resulting in a reduced number of inhibitory interneurons in the cortex (Anderson, Eisenstat et al. 1997, Anderson, Qiu et al. 1997, Pleasure, Anderson et al. 2000, Marin and Rubenstein 2001).

Several factors including integrins, neurotrophic factors, chemoattractive and repulsive cues have been identified in neuronal migration (Marin 2013). For example, NRP2 was found to be important for the migration of GABAergic interneurons, since in the absence of *Dlx1/Dlx2*, *Nrp2* expression increases. Neuropilins are co-receptors for Semaphorins and send repressive cues to direct the migration of interneurons away from the striatum towards the cortex. The loss of tangential migration in the *Dlx1/2* DKO may be in part related to the loss of DLX mediated repression of *Nrp2* resulting in responsiveness of interneurons to Semaphorin signaling (Marin, Yaron et al. 2001, Le, Du et al. 2007). CXCL12/CXCR4 signalling also has an important role in cell migration in the developing CNS. Mice deficient in *Cxcl12* or *Cxcr4* have defects in interneuron migration and show altered lamination of GABAergic interneurons in cortical layers (Stumm, Zhou et al. 2003, Tiveron, Rossel et al. 2006, Li, Adesnik et al. 2008, Lopez-Bendito, Sanchez-Alcaniz et al. 2008). I hypothesized that *Cxcr4* is a downstream target of DLX2 and that

by activating *Cxcr4* expression, DLX2 promotes the tangential migration of GABAergic interneurons in part through the CXCR4/CXCL12 signalling pathway.

I validated *Cxcr4* as a transcriptional target of DLX2. I showed that DLX2 occupies all the *Cxcr4* regulatory regions *in vivo* by performing ChIP on E13.5 ganglionic eminences and also confirmed specificity of binding *in vitro* in five regions (R2, R3, R4, R6, R7) by EMSA. After conducting luciferase reporter assays R2, R3, R4 and R6 were activated *in vitro*. Interestingly, two motifs containing TAATTA sequence, which is the currently known specific binding site for DLX2, are located in R3 and R4 and were positive for ChIP and EMSA and activated by luciferase reporter assays. Further assessment of these motifs using site directed mutagenesis followed by EMSA and luciferase needs to be conducted to validate them as direct binding sites for DLX2 *in vitro*. I also demonstrated that in the absence of *Dlx1/Dlx2* expression in the developing forebrain there is a significant decrease in the expression of *Cxcr4 in vivo*. Transient knockdown of DLX2 in S-KN-BE (2) cells and E13.5 primary cultures also resulted in a significant decrease in *Cxcr4* expression. The reduction of *Cxcr4* expression in *Dlx1/Dlx2* knockout tissue as well as in transiently knocked down cells supports my hypothesis that DLX2 activates *Cxcr4* expression *in vivo*. Immunofluorescence staining on E13.5 WT tissue sections confirmed DLX2 and CXCR4 are co-expressed in the VZ and SVZ of the LGE and MGE. Furthermore, *in situ* hybridization using riboprobes for *Cxcr4* coupled with IHC for DLX2 showed similar results on the WT forebrain. ISH on E13.5 *Dlx1/2* DKO tissue revealed the loss of expression of *Cxcr4* in the absence of DLX2 *in vivo* revealing the importance of DLX2 for *Cxcr4* expression during development.

To elucidate the effect of DLX2 on CXCL12/CXCR4 mediated migration, the Boyden Transwell assay was performed on the neuroblastoma cell line SK-N-BE (2). SK-N-BE (2) cells

have a significant increase in cell migration in the presence of CXCL12. Transient knockdown of *Dlx2* expression in SK-N-BE (2) cells using siRNA significantly decreased cell migration in *Dlx2*-siRNA treated cells compared to untreated cells, supporting the contribution of DLX2 to cell migration. Furthermore, treating cells with the CXCR4 antagonist, Plerixafor, had similar results to *Dlx2* knockdown experiments, validating the importance of CXCR4 in cell migration. Long and his colleagues showed expression of *Cxcr4* mRNA decreases in *Dlx1/Dlx2* DKO tissue extracted from E15.5 mouse basal ganglia (Long, Cobos et al. 2009). Moreover, CXCR4/CXCL12 signalling is important for positioning of interneurons in cortical layers (Stumm, Zhou et al. 2003, Tiveron, Rossel et al. 2006, Li, Adesnik et al. 2008, Lopez-Bendito, Sanchez-Alcaniz et al. 2008). However, the mechanism by which CXCR4/CXCL12 signalling is involved in tangential migration of GABAergic interneurons from the subpalium to the neocortex and how it is activated is not well known. This study is the first to show DLX2 as a functional activator of CXCR4/CXC12 mediated migration.

6.5 *Cxcr7* as a potential target for DLX2

Whilst CXCR4 was considered to be the only receptor for CXCL12 for quite some time, more recent studies have identified an alternative receptor for CXCL12 known as CXCR7 (Balabanian, Lagane et al. 2005, Sierro, Biben et al. 2007). Similar to CXCR4, CXCR7 is also a seven transmembrane receptor. However, unlike CXCR4, Gαi signalling pathway does not seem to be the pathway by which CXCR7 mediates signalling. Studies have shown that CXCR7 interacts with β-arrestin and activates the MAP kinase pathway (Rajagopal, Kim et al. 2010). CXCR7 has also been known to act as a decoy and regulate CXCR4/CXCL12 signalling through ligand sequestering (Boldajipour, Mahabaleshwar et al. 2008). Another mode of action for

CXCR7 is forming heterodimers with CXCR4 and modifying CXCR4 signalling (Levoye, Balabanian et al. 2009, Decaillot, Kazmi et al. 2011, Singh, Arya et al. 2013).

Studies on CXCR7 function in brain development are limited. In a study by Wang *et al.*, CXCR7 was reported to be important for migration of cortical interneurons (Wang, Li et al. 2011). In this study it was shown that CXCR7 was co-expressed with CXCR4 on progenitor cells of the LGE, MGE and CGE. Furthermore, *Cxcr7* and *Cxcr4* knockout mice have similar histological phenotypes with defects in lamina distribution of *Lhx6* expressing cells where the number of *Lhx6* expressing cells in the cortical plate is increased whilst a reduction in *Lhx6* positive cells in the SVZ and MZ is seen. Interestingly, real-time imaging of *Lhx6*-GFP^{+} cortical slices showed *Cxcr4*$^{-/-}$ and *Cxcr7*$^{-/-}$ null mice have different defects in interneuron movement and leading process length resulting in distinctive migratory properties for cortical migration. Furthermore, simultaneous disruption of CXCR4 and CXCR7 increased phenotypic defects. As expected, treatment of *Cxcr7*$^{-/-}$ null mice with the CXCR4 inhibitor AMD3100 had similar phenotypes to *Cxcr7*$^{-/-}$ and *Cxcr4*$^{-/-}$ null mice. These data show that CXCR4 and CXCR7 do not have redundancy in regulating the migration of GABAergic interneuron and cannot compensate for each other's function (Wang, Li et al. 2011).

While I did not study the function of DLX2 knockdown on CXCR7 expression and function, my knockdown experiments along with CXCR4 inhibition in SK-N-BE (2) cells suggests a distinctive role for CXCR7 in interneuron migration. Indeed, loss of migration of neuroblastoma cells in the presence of CXCL12 is more severe in DLX2 knockdowns compared to Plerixafor treated cells. There is 88% loss of migration in *Dlx2*-siRNA treated SK-N-BE (2) cells vs. 79% loss of migration in Plerixafor-treated SK-N-BE (2) cells. These results suggest that DLX2 can regulate migration through factors other than CXCR4 and that CXCR7 could also be a

downstream target of DLX2 that signals distinctively from CXCR4. Further experiments need to be conducted to assess the interplay between DLX2 and CXCR7 and to understand the role of CXCR7 in cortical migration.

6.6 DLX2 as a therapeutic target for diseases involving CXCR4/CXCL12 signalling

CXCR4/CXCL12 signalling is involved in many biological processes including regulation of homeostasis, adaptive immune responses, tissue formation during morphogenesis, and cell migration during development. Studies have shown that dysregulated expression of CXCR4 and CXCL12 is implicated in autoimmune diseases, viral infections, and cancer. CXCR4/CXCL12 signalling has an important role in hematopoietic stem cell homeostasis, and these chemokines are key players in bone marrow colonization during ontogenesis (Sugiyama, Kohara et al. 2006). Furthermore, CXCR4 has a principal function in organizing responses between the innate and adaptive immune systems by participating in lymph node organization and regulating leukocyte trafficking (Stein and Nombela-Arrieta 2005). During bacterial infection, CXCR4 enhances adaptive immune responses by translocating neutrophils to the lymph nodes (Hampton, Bailey et al. 2015). During development, CXCR4 is highly expressed in B cells (Payne, Drinkwater et al. 2009), and B cell production is absent in mice deficient in CXCR4 (Ma, Jones et al. 1998).

In cancer, CXCL12/CXCR4 signaling is involved in tumour cell survival, proliferation and metastasis (Domanska, Kruizinga et al. 2013), and the expression of CXCR4 is a prognostic marker for cancers such as breast, ovarian, and pancreatic adenocarcinoma. During metastasis, CXCR4 is involved in transendothelial migration of metastatic cells to secondary target sites by activating G protein (Yagi, Tan et al. 2011). In HIV infections, CXCR4 is the co-receptor

involved in the entry of the virus into T cells using the envelope glycoprotein gp120 (Feng, Broder et al. 1996). The inhibition of CXCR4 by its antagonist, AMD3100, blocks HIV-1 and HIV-2 infection by preventing cell entry (Donzella, Schols et al. 1998).

The **w**arts, **h**ypogammaglobulinemia, **i**nfections and **m**yelokathexis (WHIM) syndrome is an uncommon congenital immunodeficiency disorder resulting from heterozygous mutations of CXCR4 and characterized by chronic non-cyclic neutropenia. WHIM is inherited as an autosomal dominant disease which presents with recurrent bacterial infections. Patients with WHIM have leucopenia (low concentration of blood leucocytes) and show warts on their hands and feet as well as the genitalia which can lead to cancer (Gorlin, Gelb et al. 2000).

During brain development, CXCL12/CXCR4 signalling is involved in guidance of cortical interneurons from the GE to the neocortex. Mice deficient in CXCL12 or CXCR4 have defects in interneuron migration and show altered expression of GABAergic interneuron precursors in cortical layers (Stumm, Zhou et al. 2003, Tiveron, Rossel et al. 2006, Li, Adesnik et al. 2008, Lopez-Bendito, Sanchez-Alcaniz et al. 2008).

The involvement of CXCR4/CXCL12 signalling in various biological processes and diseases signifies the importance of understanding the mechanisms by which CXCR4/CXCL12 is regulated and is a crucial step for designing novel drugs and therapies. I hypothesized that DLX2 directly mediates transcriptional activation of GABAergic interneuron migration through activation of *Cxcr4* expression. My results gained from ChIP, EMSA, luciferase, qRT-PCR, IF and ISH experiments suggest DLX2 activates *Cxcr4* expression during murine brain development. Furthermore*, in vitro* transient knockdown studies revealed the importance of *Dlx2* expression in responsiveness of neuroblastoma cells to the CXCL12 chemokine. Indeed,

identifying upstream regulators involved in CXCR4-mediated migration can present novel opportunities for pharmacologic intervention for neuronal migration disorders.

Furthermore, CXCR4 inhibitors have side effects that limit their application. In cancer for instance, therapeutic targeting of CXCR4 promotes the mobilization of bone marrow stem cells from their niche at the same time as decreasing metastasis (Devine, Flomenberg et al. 2004, Broxmeyer, Orschell et al. 2005). In fact, CXCR4 inhibitors are currently administered for patients with non-Hodgkin lymphoma and multiple myeloma in transplantation to mobilize hematopoietic stem cells for harvesting (Devine, Vij et al. 2008, Pusic and DiPersio 2010). Consequently, studying the CXCR4 upstream signaling pathway may help identify targets that could potentially be investigated as therapeutic options for CXCR4 dependent cancers.

6.7 Posttranslational modifications in DLX2

Transcription factors are key elements in signaling cascades; therefore, they need to be regulated in response to signaling events. Transcription factor regulation can occur at different stages from DNA synthesis to posttranslational modifications. Phosphorylation of serine, threonine and tyrosine residues on transcription factors is a common PTM that can affect the localization, DNA binding capability, stability and protein interactions and consequently regulate transcription factor activity. A good example of the role of transcription factor phosphorylation on its activity and localization is seen in retinal development. VAX2 is an *Emx*-related homeodomain transcription factor that is required during two distinct time points in retinal development. At E9.5- E11.5, VAX2 promotes differentiation of the optic nerve and inhibits retinal differentiation in the optic vesicle by repressing *Pax6* (Mui et al. 2005). Postnatally at P10, VAX2 is involved in dorsoventral polarity in the retina (Mui et al. 2002). Between these two developmental windows the retina is differentiated by *Pax6* and *Vax2* is downregulated.

Although regulation of VAX2 could be achieved by turning the *Vax2* gene on and off, a more efficient and precise regulation is explained through the shuffling of this transcription factor between the nucleus and the cytoplasm. During early retinal development, VAX2 is localized to the nucleus where it is involved in optic nerve differentiation. On the other hand, during retinal development this gene is excluded from the nucleus to allow the activation of *Pax6.* The cytoplasmic localization of VAX2 is achieved by phosphorylation at S170, most likely by protein kinase A. VAX2 phosphorylation is antagonized by SHH which promotes the localization of VAX2 into the nucleus (Kim and Lemke 2006).

Indeed, intracellular shuttling of transcription factors between the cytoplasm and the nucleus by PTM can explain a mechanism for a precise spatial and temporal regulation of transcription factor activity. Since previous experiments in the Eisenstat lab on E13.5 GE tissue had identified likely phosphorylation of DLX2, I hypothesized that phosphorylation of critical residues of the DLX2 protein is required for its nuclear localization and transcriptional activity during forebrain development. Whilst experiments revealed the likely presence of posttranslational modifications in DLX2, specifically phosphorylation, the lack of a suitable DLX2 antibody to isolate DLX2 from brain tissue by standard immunoprecipitation prevented me from further identifying residues on DLX2 that are phosphorylated by mass spectrometry. Using *in silico* analysis, I was able to identify highly predicted phosphorylation residues. The availability of *in silico* analysis programs is an important tool to predict phosphorylated residues, protein kinases and the pathway by which *Dlx* genes function in development. Results obtained from *in silico* analysis can help future investigations to more precisely define the roles of phosphorylation in the function of *Dlx* genes. Moreover, linking DLX function to developmental signalling pathways may facilitate the development of small molecule inhibitors and other

therapeutic strategies towards treatment of disorders that result from an imbalance between excitation and inhibition in the forebrain.

7 Chapter 7: Conclusion and future directions

My data supports the hypothesis that *Nkx2.2* is negatively regulated by DLX2 which contributes to the role of *Dlx* genes in neuronal versus glial cell fate specification during forebrain development. Future directions for this project include ChIP-seq for DLX2 to identify all the sites that DLX2 is interacting within the genome. Data obtained from ChIP-seq can then be validated with *in vitro* assays. Furthermore, RNA-seq on *Dlx1/Dlx2* WT and DKO forebrain can also compare the expression of many different genes in the presence and absence of *Dlx1/Dlx2*. ChIP re ChIP experiments can also be conducted to identify protein-protein interactions between DLX2 and other transcription factors. In addition, expression levels of NKX2.2 downstream targets such as *Pdgfra*, *Mbp*, and *Sirt2* in *Dlx1/2* DKO mice can be assessed.

DLX2 can also act as a transcriptional activator to promote the expression of *Cxcr4*. *In vivo* and *in vitro* experiments validated *Cxcr4* as a downstream target of DLX2. Furthermore, cell migration was significantly reduced when *Dlx2* was transiently knocked down in SK-N-BE (2) cells, supporting the contribution of DLX2 to cell migration *in vitro*. These results support a key role for DLX2 as an upstream activator of *Cxcr4* with important functions in GABAergic interneuron migration. Future directions for this project can include assessing expression levels of CXCR7 in *Dlx1/2* DKO tissue as well as *Dlx2*-siRNA treated SK-N-BE (2) cultures. Furthermore, migration of SK-N-BE (2) cells can be examined in the presence of CXCR7 inhibitor, CCX771 (ChemoCentryx), and compared to the migration of SK-N-BE (2) cells when treated with Plerixafor. In addition, migration of SK-N-BE (2) cells when treated with both CXCR4 and CXCR7 inhibitors can be assessed and compared to the migration of *Dlx2*-siRNA treated SK-N-BE (2) cells to determine whether there are similar results when comparing loss of

migration. In addition, further assessment of the role of DLX2 in CXCR4/CXCL12 mediated migration can be determined *in situ*.

Western Blot experiments showed the presence of posttranslational modifications in DLX2 and *in silico* analysis predicted several phosphorylation residues. Future directions will include determining the localization of the phosphorylated DLX2 protein by assessing expression of DLX2 in nuclear and cytoplasmic fractions of GE tissue using a phospho-specific DLX2 antibody (to be developed) in a Western Blot assay and detailed analysis of subcellular expression of phospho-DLX2 during embryonic forebrain development. Furthermore, kinases involved in DLX2 phosphorylation could be identified using *in vitro* kinase assays. For the *in vitro* kinase assay recombinant DLX2 protein is radiolabeled using [γ-^{32}P]-ATP and incubated with different kinases. After incubation, the protein-kinase complex is resolved on a SDS-PAGE gel and visualized by autoradiography. Cyclin-dependent kinase 5 (Cdk5), Protein kinase C (PKC), Tyrosine-protein kinase CSK , Class III phosphoinositide 3-kinase (PIK3C3), and AMP-activated protein kinase (AMPK) are some of the kinases that can be investigated in the *in vitro* kinase assay since they are active during brain development. Once kinases are identified, they can be challenged by kinase-specific inhibitors. Furthermore, the role of candidate kinases in mediating DLX2 phosphorylation can be validated by knockdown experiments. The functional activity of DLX2 phosphorylation mutants could also be assessed *in vitro* by co-transfecting these mutants with luciferase reporter genes for previously validated DLX2 targets activated (*Gad1, Gad2*) or repressed (*Nrp2, Olig2*) during forebrain development.

My work reinforces a critical role for *Dlx* genes and their downstream targets in maintaining the balance of excitation to inhibition during brain development. Understanding the regulation of GABAergic interneuron diversity will enable new therapeutic approaches to disorders linked to

an imbalance of excitatory/inhibitory interneuron populations such as autism spectrum disorders, schizophrenia and epilepsy as well as neuronal migration defects. My phosphorylation study on DLX2 represents an exciting direction in the overall research program to identify the importance of posttranslational modifications on DLX2 function and subcellular localization and could place DLX2 downstream of one or more signaling pathways essential for forebrain development.

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