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and I12a/I12b

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ABSTRACT

Dlx genes are involved in the formation of the forebrain, branchial arches, sensory organs, and limbs. In the forebrain, *Dlx* genes are expressed in restricted domains in the telencephalon and diencephalon. The telencephalon is one of the most complex structures in the vertebrate central nervous system, but despite variable morphologies of the adult telencephalon, the patterning and basic organization is conserved amongst vertebrates.

Cis-acting regulatory sequences (CREs) that contribute to *Dlx* expression in the telencephalon were previously identified in the intergenic region between *Dlx5* and *Dlx6* (I56i and I56ii), between *Dlx1* and *Dlx2* (I12b), and upstream of *Dlx1* (URE2) in zebrafish, mouse, and human. I hypothesize that these CREs contribute to defining distinct subtypes of interneurons during forebrain development. In this study, we have investigated the differential activity of intergenic CREs in the developing zebrafish brain using the established transgenic lines *Tg(6kb-dlx1a/dlx2a:EGFP)* and *Tg(1.4kb-dlx5a/dlx6a:EGFP)* in which the green fluorescent protein gene (GFP) was used as a reporter whose expression was controlled by *dlx* CREs. The two intergenic fragments target reporter transgenes with overlapping patterns of expression in the telencephalon but also show differential activity in the dorsal region of the brain at the level of the pallium. These results suggest that the *dlx1a/2a* CREs are involved in *dlx* regulation in a set of cells occupying the subpallium, whereas the *dlx5a/6a* CREs are involved in *dlx* regulation in a set of cells that migrate out of the subpallium and travel in a ventral to dorsal manner to occupy the pallium. Co-localization of GFP with GAD65/67 and with molecular markers of the interneuron subtype, calretinin, indicates that the *dlx* CREs target the reporter constructs to GABAergic interneurons, with at least one subtype

expressing calretinin. Results from this study reflect a dynamic regulation of *dlx* gene expression in the developing zebrafish forebrain through several regulatory elements with distinct and overlapping functions.

RESUME

Les gènes *Dlx* sont impliqués dans la formation du prosencéphale, des arcs branchiaux, des organes sensoriels, et des membres du corps. Dans le prosencéphale, les gènes *Dlx* sont exprimés dans des zones spécifiques du télencéphale et du diencéphale. Le télencéphale est l'une des structures les plus complexes du système nerveux central des vertébrés, mais malgré une morphologie variables du télencéphale adulte chez diverses espèces, sa modélisation et son organisation basale sont conservées parmi les vertébrés.

Des séquences régulatrices agissant en cis contribuant à l'expression des gènes *Dlx* dans le télencéphale ont déjà été identifiées dans les régions intergéniques de *Dlx5* et *Dlx6* (éléments I56i et I56ii); *Dlx1* et *Dlx2* (élément I12b); ainsi qu'en amont de *Dlx1* (élément URE2). Ces éléments ont été identifiés dans le génome du poisson zèbre, la souris et l'humain. Mon hypothèse est que ces éléments régulateurs agissant en cis contribuent à définir les sous-types distincts d'interneurones au cours du développement du prosencéphale. Dans cette étude, nous avons étudié l'activité différentielle des éléments régulateurs agissant en cis au cours du développement du cerveau du poisson zèbre. Ceci a été accompli avec les lignées de poissons transgéniques *Tg(6kb-dlxa/dlx2a:EGFP)* et *Tg(1.4kb-dlx5a/dlx6a:EGFP)* dans lesquelles la protéine GFP (Green Fluorescent Protein) a été utilisée comme rapporteur, son expression étant sous le contrôle des éléments régulateurs retrouvés dans les régions intergéniques des gènes *Dlx1* et *Dlx2*, ainsi que *Dlx5* et *Dlx6*. Ces deux fragments intergéniques ciblent les transgènes rapporteurs avec des patrons d'expression se chevauchant dans le télencéphale mais montrent aussi une activité différentielle dans la région dorsale du cerveau au niveau du

pallium. Ces résultats suggèrent que les éléments régulateurs agissant en cis dans la région intergénique *dlx1a/2a* sont impliqués dans la régulation des gènes *Dlx* dans un ensemble de cellules migrant en dehors du subpallium d'une façon ventrale à dorsale pour occuper le pallium. La colocalisation de GFP avec GAD65/67 et avec des marqueurs moléculaires, tel la calrétinine, pour les sous-types d'interneurones, indiquent que les éléments agissant en cis des gènes *Dlx* ciblent les rapporteurs aux interneurones GABAergiques, avec au moins un sous-type exprimant la calrétinine. Les résultats de cette étude reflètent la régulation dynamique de l'expression des gènes *Dlx* dans le développement du prosencéphale du poisson zèbre par moyen d'éléments régulateurs agissant en cis avec des patrons d'expression distincts et des fonctions chevauchantes.

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ABBREVIATIONS

OE: olfactory epithelium

ac: anterior commissure

PTd; dorsal part of the posterior tuberculum

DVe: diencephalic ventricle

DT: dorsal thalamus

TeO: optic tectum

GT: griseum tectale

PO: preoptic region

PTv: ventral part of the posterior tuberculum

T: midbrain tegmentum

poc: postoptic commissure

Sv: ventral division of S

M4: telencephalic migrated area

EmT: eminentia thalami

M3: migrated area of the EmT

lfb: lateral forebrain bundle

M1: migrated pretectal area

Sd: dorsal division of the subpallium

OB: olfactory bulbs

Pv: ventral division of the pallium

Pd: dorsal division of pallium

Pr: pretectum

N: region of the nucleus of the medial longitudinal fascicle

VT: central thalamus (prethalamus)

E: epiphysis

b: basal optic tectum

Hr: rostral hypothalamus

Hi: intermediate hypothalamus

1. INTRODUCTION

1.1 Distal-less (*Dll*) genes

Distal-less is one of the earliest known genes expressed in the developing insect limb. Originally identified in *Drosophila*, *Dll* is required for the proximodistal patterning of insect appendages, including the legs and antennae (Panganiban 2000). Along with the appendages, *Dll* is required for the formation of parts of the peripheral nervous system and has been shown to be expressed in the optic lobe of the brain and glial cells of the ventral nerve cord (Panganiban and Rubenstein 2002). *Drosophila* mutants lacking *Dll* genes lack rudimentary larval limbs and as a result die (Cohen and Jurgens 1989).

1.2 Vertebrate Homologs of *Dll* (*Dlx*)

Although only one *Dll* gene has been found in invertebrates thus far, there are numerous *Dll*-related genes identified in vertebrates named, *Dlx*. There may be upwards of 8 *Dlx* genes in vertebrates including the chick, zebrafish, rat, newt, mouse, axolotl, and frog (Zerucha and Ekker 2000). Vertebrate distal-less genes (*Dlx*) are thought to have arisen via a tandem gene duplication event followed by a series of duplication events, producing at least 3 pairs of *Dlx* genes, each linked to a *Hox* cluster (Simeone, Acampora et al. 1994). A separate genome duplication event in teleosts resulted in at least 2 different *Dlx* genes not seen in any other vertebrates. *Dlx* genes are involved in the formation of the forebrain, branchial arches, sensory organs, and limbs. Mammals, including mouse and humans, have 6 *Dlx* genes (Porteus, Bulfone et al. 1991), whereas, zebrafish have 8 (Ekker, Akimenko et al. 1992; Akimenko, Ekker et al. 1994). Like *Dll* genes in insects, *Dlx* genes are required for the proper development of vertebrates. During

early development, *Dlx* paralogs have been shown to exhibit overlapping patterns in their temporal and spatial expression (Panganiban and Rubenstein 2002).

1.3 Genomic organization of *Dlx* genes in vertebrates

The *Dlx* genes are organized in three bigene clusters separated by short intergenic regions (<12kb) and convergently transcribed (Figure 1.1). The three bigene clusters include: *Dlx1/2*, *Dlx5/6*, and *Dlx3/4* (Simeone, Acampora et al. 1994; Nakamura, Stock et al. 1996; Stock, Ellies et al. 1996) and two genes found in zebrafish not linked to other *Dlx* genes, *dlx2b* and *dlx4a* (Ekker, Akimenko et al. 1992; Akimenko, Ekker et al. 1994). The *Dlx* genes are grouped in two general classes based on similar sequence motifs in the proteins they encode, including the homeodomain: *Dlx1/4/6* and *Dlx2/3/5*, yet it is not known whether there are any significant functional differences between the two groups (Stock, Ellies et al. 1996; Ellies, Stock et al. 1997).

1.4 *Dlx* Expression in the Telencephalon

Four *Dlx* genes, *Dlx1*, *Dlx2*, *Dlx5*, and *Dlx6*, are expressed in the forebrain in two highly restricted domains: one is the telencephalon and the other the diencephalon (Eisenstat, Liu et al. 1999; Puelles, Kuwana et al. 2000; Zerucha, Stuhmer et al. 2000). *Dlx* genes display a spatio-temporal expression, whereby, *Dlx2* is expressed the earliest in the progenitors, followed by *Dlx1*, *Dlx5*, and finally *Dlx6* (Liu, Ghattas et al. 1997; Eisenstat, Liu et al. 1999; Zerucha, Stuhmer et al. 2000) (Figure.1.2). Studies show that *Dlx* genes mark the majority of GABAergic cortical interneurons from their birth in the

Figure 1.1. Organization of the vertebrate Dlx genes. (A) A schematic representation of the vertebrate bi-gene clusters. One gene family (*Dlx2*, *Dlx3*, *Dlx5*) is shown on the left and the other gene family (*Dlx1*, *Dlx4*, *Dlx6*) is shown on the right. (B) A schematic representation of the zebrafish *dlx1a/2a* locus. There are three exons associated with each gene. The intergenic region (7.3kb) contains the enhancers I12a (550bp) and I12b (400bp), and located upstream of *dlx1a* is the upstream regulatory element, URE2 (900bp). (C) A schematic representation of the zebrafish *dlx5a/6a* locus. There are three exons associated with each gene. The intergenic region (3.5kb) contains the enhancers I56i (440bp) and I56ii (310bp). Genes are shown in blue, enhancers in red, the direction of gene transcription is represented by arrows, and the intergenic region is indicated by double arrow heads. Image not drawn to scale.

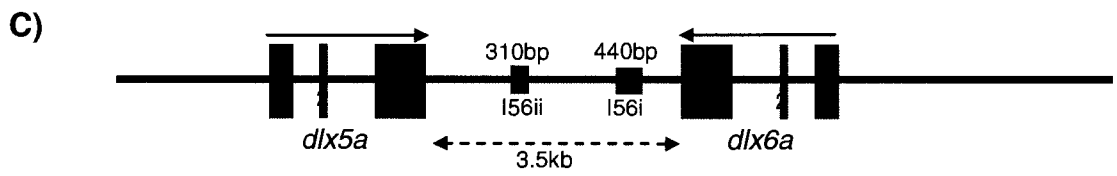
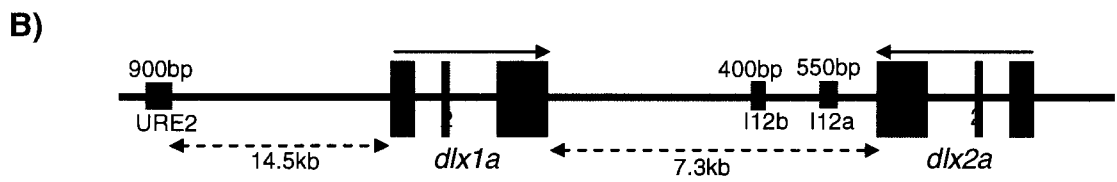
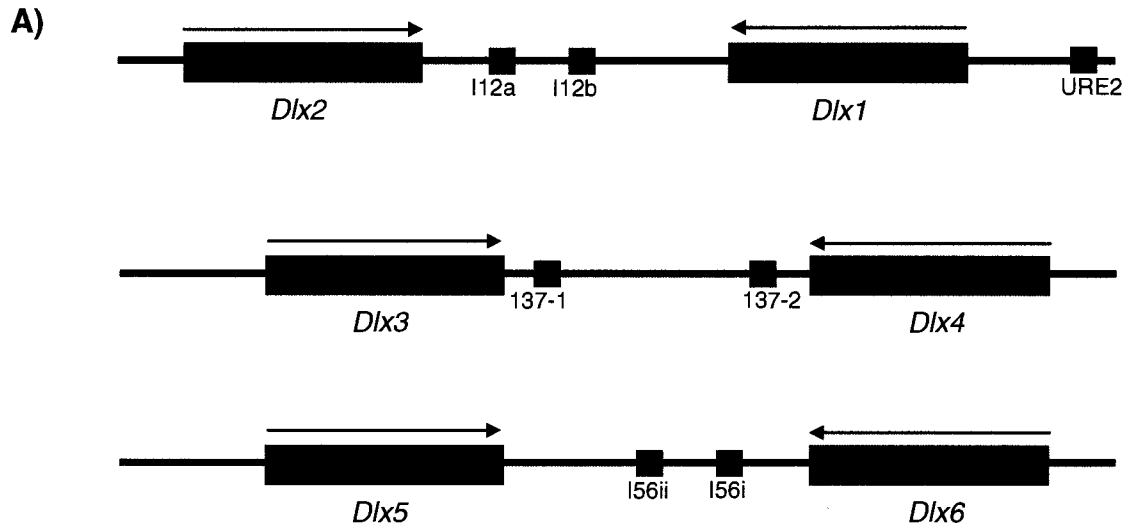
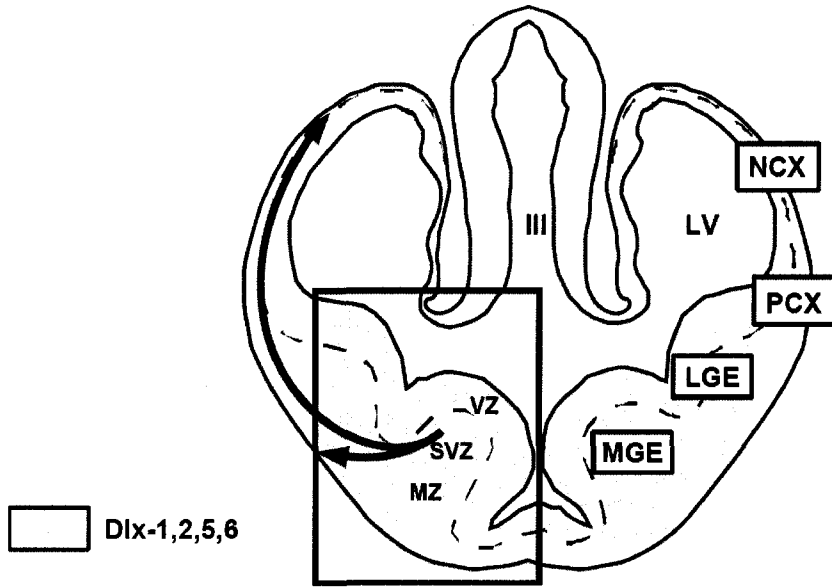
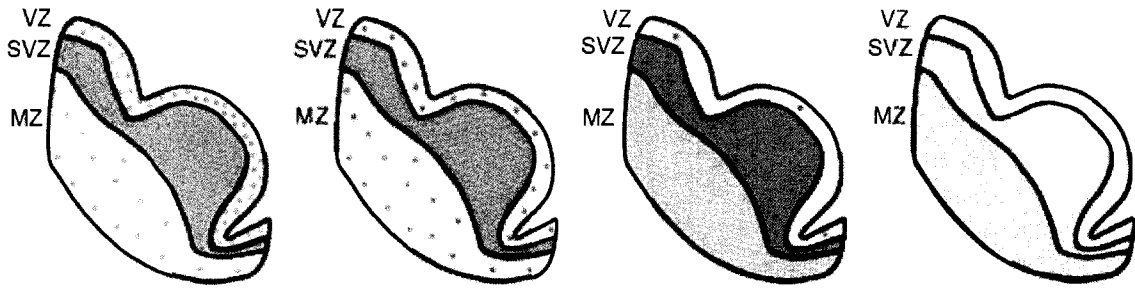


Figure 1.2. Expression domains of the *Dlx* genes in the mouse telencephalon. (A) Schematic representation of an E12.5 coronal section of the mouse telencephalon showing the combined expression of *Dlx* transcripts (yellow). The arrow indicates tangential migration from the subpallium to the pallium. (B) The expression patterns of *Dlx1*, *Dlx2*, *Dlx5*, and *Dlx6* in the basal ganglia. *Dlx 1* and *Dlx2* are mainly expressed in the SVZ and scattered cells are found in the VZ and MZ. *Dlx5* is mainly expressed in the SVZ with moderate expression in the MZ and scattered cells in the VZ. *Dlx6* is mainly expressed in the MZ and moderately expressed in the SVZ. (C) Proposed model for the spatio-temporal expression of *Dlx* genes during brain development. Abbreviations: LGE, lateral ganglionic eminence; LV, lateral ventricle; MGE, medial ganglionic eminence; LV, lateral ventricle; PCX, palliocortex; SVZ, subventricular zone; VZ, ventricular zone; III, third ventricle. Adapted from Panganiban and Rubenstein, 2002.

A)



B)



C)

Dlx2 → Dlx1 → Dlx5 → Dlx6

basal telencephalon through their migration to the cortex in adult mice, suggesting that the *Dlx* genes have a role in their development (Anderson, Eisenstat et al. 1997; Stuhmer, Anderson et al. 2002; Stuhmer, Puellas et al. 2002).

Previous studies show that *Dlx1/2*^{-/-} mutant mice die within the first few hours after birth and show a distinct neural phenotype. There is also a major block in neurogenesis in the lateral ganglionic eminence (LGE) and subsequent lack of differentiation and migration of projection neurons (Anderson, Qiu et al. 1997; Marin, Anderson et al. 2000). These cells do not migrate out of the proliferative region and accumulate while exhibiting partial differentiation (Anderson, Qiu et al. 1997). There is, therefore, a >75% decrease in neocortical GABAergic neurons and >95% decrease in hippocampal and olfactory bulb interneurons in *Dlx1/2*^{-/-} mutant mice (Anderson, Eisenstat et al. 1997; Anderson, Qiu et al. 1997; Marin, Anderson et al. 2000; Marin, Yaron et al. 2001). This marked reduction is due to the lack of tangential migration of immature interneurons born in the ventricular and subventricular zone of the ventral telencephalon. Furthermore, there is a lack of *Dlx5*, and *Dlx6* expression in cells located in the subventricular zone (SVZ) of these mutants (Anderson, Qiu et al. 1997; Zerucha, Stuhmer et al. 2000). With the knowledge that the Dlx2 protein binds to the I56i enhancer sequence, it has been suggested that Dlx1 and Dlx2 may act as positive regulators of the *Dlx5/6* expression through this intergenic enhancer. These cumulative defects suggest that Dlx1 and Dlx2 are upstream regulators of *Dlx5* and *Dlx6* and that Dlx1 and Dlx2 play a role in the development and migration of GABAergic neurons (Zerucha, Stuhmer et al. 2000; Stuhmer, Anderson et al. 2002).

Dlx single mutants tend to have more subtle defects in forebrain development, as

opposed to double mutants such as the *Dlx1/2*^{-/-} mutants. *Dlx2* mutants, however, die shortly after birth and *Dlx1* mutants within the first month due to craniofacial and/or enteric nervous system defects (Qiu, Bulfone et al. 1995; Acampora, Merlo et al. 1999; Cobos, Calcagnotto et al. 2005). Mice lacking *Dlx1* show a marked reduction in calretinin and somatostatin interneuron subtypes and show behavioural and histological signs of epilepsy (Cobos, Calcagnotto et al. 2005). Taken together, these results suggest that *Dlx* genes show considerable redundant function in forebrain development. However, they may be required in a time dependent manner or required for specific functions.

1.5 Telencephalon

The telencephalon is one of the most complex structures in the vertebrate central nervous system. Throughout evolution, the cerebral cortex has undergone significant growth relative to other parts of the brain thereby, predominately affecting its surface area as well as the rise of elaborate functional subdivisions. Further to this, the neocortex has acquired a laminar pattern not readily apparent in non-mammalian vertebrates. Despite variable morphologies of the adult telencephalon, the basic patterning and organization is conserved amongst vertebrates.

The telencephalic vesicles are paired evaginations of the anterior forebrain derived from cells located in the anterior most portion of the neural plate. The main components of the mammalian telencephalon include the pallium (roof), which is primarily the cerebral cortex (considered the 'thinking' part of the mammalian brain) and hippocampus, and the subpallium (base), constituting the striatal, pallidal, and telencephalic stalk (Puelles, Kuwana et al. 2000). The pallium is divided into four main subdivisions: the medial, dorsal, lateral, and ventral pallium (Puelles, Kuwana et al.

2000). Along with other neural components, the telencephalon is essential for integrating and processing sensory information while formulating and effecting a behavioural response. In humans, the telencephalon is involved with consciousness, language, memory and emotions (Puelles Et al. 1999). Previous studies have subdivided the human cerebral cortex into more than 50 areas with remarkable conservation of areal divisions within a given species. This concept suggests a highly conserved and regulated regional specification program throughout development (Wullimann and Rink 2002).

1.6 Comparative Studies

Comparative studies of telencephalic development have led to promising information of evolution, patterning, and brain function. This knowledge will contribute to understanding common telencephalic properties conserved amongst vertebrates. These studies will also help to elucidate the mechanisms by which cytoarchitecture, connectivity, and function of the telencephalon have diverged throughout evolution. While the evolution of brainstem structures (cerebellum) and the subpallial forebrain has become well understood, the evolution of the pallium still needs clarification (Wullimann and Mueller 2004). Since the *Dlx* genes are highly conserved between fish and mammals, the migration of various subtypes of GABAergic interneurons into the pallial forebrain of zebrafish should help give us insights into the evolutionary relationship behind the conservation and divergence of function seen in the telencephalon.

Previous studies, until recently, have been limited to include neuroanatomical and morphological studies due to the overt differences with the mammalian system. Some of these differences include the eversion process that shapes the fish telencephalon, in contrast to the evagination process that generates the telencephalic hemispheres in all

other vertebrates (Nieuwenhuys, Veening et al. 1988; Wullimann MF 2006). There is also an absence of primary motor and sensory telencephalic areas along with varying morphogenesis and organizations of areas such as the cerebellum (Mione, Baldessari et al. 2008). Recent advances, however, in gene technology, specifically in mice and zebrafish, are identifying genes crucial to forebrain development. Zebrafish are an excellent model for biological research based on embryology and ease of genetic manipulation. Several characteristics in particular make this model ideal for studying development. During the first 24 hours period, they are an optically clear organism, allowing for easy observation of deep and superficial organs and structures. Their embryonic development is rapid and most vertebrate structures, including a compartmentalized brain, can be observed within a 48h period, thereby facilitating developmental analysis (Westerfield 2000; Wullimann MF 2006).

1.7 Telencephalic eversion versus evagination

The zebrafish has recently been introduced as a neurobiological and neurogenetic model system. Despite the presence of basic developmental units of brain development, the teleostean forebrain exhibits a different mode of telencephalic hemisphere development in comparison to tetrapod forebrain development. A considerable portion of the pallial telencephalon is laterally everted in the telencephalon resulting in the medial pallium (hippocampus homolog) dislocated to the adult lateral telencephalic periphery (Wullimann and Rink 2002). Typical for the lateral pallium in tetrapods, the most lateral pallial division seen in teleost receives the densest zone of secondary olfactory input, therefore, suggesting that a complete topological eversion of the entire teleostean pallium is unlikely (Wullimann and Rink 2002; Wullimann and Mueller 2004).

1.8 Mouse Telencephalon

The early development of the mammalian brain consists of the anteriorly located forebrain (prosencephalon), the more medial midbrain (mesencephalon), and caudally located hindbrain (rhombencephalon). During development, the forebrain becomes subdivided into the anterior telencephalon and the more caudal diencephalon. The telencephalon eventually forms the cerebrum, the hippocampus, and the olfactory lobes. Like the zebrafish, the telencephalon consists of the ventrally located subpallium and the dorsally located pallium. The diencephalon forms the thalamus and the hypothalamus. The olfactory bulbs are bilateral evaginations situated between the septal and cortical anlagen of the prosencephalic neural plate (Gilbert 2000; Wullimann and Mueller 2004).

1.9 Zebrafish Telencephalon

Despite the recent introduction of zebrafish as a neurogenic model, substantial progress regarding adult regionalization has been made based on the specific localized presence of transmitters and related neurochemicals, and neuronal connections (Mione, Baldessari et al. 2008). In teleosts, the embryonic subpallium is divided into a ventral portion which is presumed to form the adult ventral nuclei and a dorsal portion presumed to form the adult dorsal nuclei (Wullimann and Mueller 2004). During larval stages, the dorsal subpallium continues over the anterior commissure whereas, caudal to the anterior commissure, the ventral subpallium is replaced by the preoptic region. The ventral subpallium, therefore, is restricted to a precommissural domain and is considered to be of septal formation. The more rostral portion to the anterior commissure of the dorsal subpallium is thought to be homologous to the basal ganglia components of tetrapods (Wullimann and Rink 2002). The distribution of dopamine receptors (Vacher, Pellegrini

et al. 2003), substance P (Sharma, Berthoud et al. 1989), and LANT6 (Reiner and Northcutt 1992) are considered to be lines of evidence supporting this general interpretation of the telencephalic divisions. The neurochemistry and connectivity of the dorsal telencephalic area of the teleost are consistent as pallium and the ventral area as subpallium.

Recent studies of early neuronal development of the zebrafish telencephalon show there are many more postmitotic, early differentiated neurons present in the pallium in comparison to the subpallium (Wullimann and Mueller 2004). There are also restricted expression domains of regulatory genes (i.e., *Dlx1/2*) typically expressed early in the amniote subpallium that show similar expression domains restricted to the presumptive subpallial ventral telencephalic areas in the zebrafish (Zerucha, Stuhmer et al. 2000; Wullimann and Rink 2002). Consistent with this notion is the concept that other zebrafish genes (i.e., *Emx1/2*) are exclusively expressed in the dorsal pallial domain conforming to the amniote pattern (Wullimann and Mueller 2004).

1.10 Interneurons

Two classes of neurons make up the neuronal network in the telencephalon: excitatory projection neurons and inhibitory interneurons. Interneurons are crucial for telencephalic function since they regulate principal neuron activity via inhibition and along with projection neurons, maintain the spatio-temporal balance of excitation and inhibition of brain function (Sillito and Kemp 1983; Soltesz 2006). Projection neurons use glutamate as their main neurotransmitter, whereas, interneurons use GABA (γ -amino butyric acid) + several neuromodulators (Soltesz 2006).

Interneurons are anatomically, molecularly, and physiologically heterogenous.

Aside from GABA, interneurons can express various neurotransmitters including: somatostatin (SOM), neuropeptide-Y (NPY), vasoactive-intestinal peptide (VIP), neuronal-nitric oxide synthase (nNOS) and the calcium-binding proteins: parvalbumin (PV), calbindin (CB), and calretinin (CR). There may be one or more combinations of the previous molecular markers that define each subtype of interneuron and therefore, it is difficult to distinguish between different subtypes based on one specific parameter. Recent studies suggest that there is a minimum of 16 different interneuronal species that inhabit the CA1 region of the hippocampus, and with further study this number will increase (Soltesz 2006).

Several studies have shown that parvalbumin-, calretinin-, and somatostatin-positive interneurons constitute distinct and non-overlapping groups of GABAergic neurons in the rat frontal cortex. These neurons have different electrophysical properties and account for more than 80% of all GABAergic neurons in the sensory cortex (Anderson, Marin et al. 2001; Marin, Yaron et al. 2001). It is not known precisely where and when each subtype of interneuron originates during embryogenesis, however, *in utero* fate-mapping experiments in mice have studied spatio-temporal origin of these major subtypes (Butt, Fuccillo et al. 2005). The medial ganglionic eminence (MGE) appears to give rise to the parvalbumin- and somatostatin-expressing interneuron subgroups, whereas the calretinin- and vaso-intestinal peptide (VIP)- and neuropeptide Y (NPY)-expressing interneurons derive from the caudal ganglionic eminence (CGE) (Ghanem, Yu et al. 2007).

1.11 Glutamic Acid Decarboxylase (GAD)

Glutamic acid decarboxylase is an enzyme that catalyzes the formation of the inhibitory neurotransmitter GABA. In mammals, GAD exists in two isoforms encoded by two different genes, *Gad1* and *Gad2* with the isoforms being Gad65 and Gad67. *Gad1* and *Gad2* are expressed in the brain where GABA is used as a neurotransmitter, therefore, where there is GAD, there is GABA (Atkinson, Kaufman et al. 1993).

Previous studies examined the immunohistochemical detection of GAD revealing a sharp contrast between the ventral (subpallial) and dorsal (pallial) region of the adult zebrafish telencephalon. The ventral area showed a rich distribution of GAD-positive cells, whereas, the dorsal area characteristically, showed scattered moderate to strong GAD-positive cells in most areas or low, moderate, or rich areas of GAD-positive cells in other areas. This allows for a clear differentiation between zones and subzones in the dorsal telencephalon. In the ventral telencephalon, it can be seen that the GAD expression pattern is rather rich in comparison to the dorsal GAD expression pattern in adult zebrafish (Mueller, Vernier et al. 2006).

1.12 Calretinin

Calretinin (CR) is a 29-kD calcium binding protein belonging to the EF-hand protein family. The EF-hand calcium binding proteins have been implicated as selective neuronal markers due to their presence mainly in specific neurons in both the central nervous system and the peripheral nervous system (Celio 1990). Calcium binding proteins are thought to maintain calcium homeostasis by buffering the intracellular free calcium concentration which results in the buffering of high calcium concentrations seen in activity including neurotransmitter-induced depolarization (Miller 1991).

Previous studies using immunohistochemical detection of calretinin in adult zebrafish revealed the presence of calretinin in both pallial and subpallial regions in a characteristic pattern. Calretinin immunohistochemistry revealed differences in immunoreactivity of the subdivisions of the adult zebrafish telencephalic lobes. The dorsomedial portion of the telencephalon shows a rich expression pattern of calretinin in the pre and supracommissural medial zone, but not in the more rostral region. There is also a heterogeneous expression pattern dorsoventrally, as demonstrated by different combinations of markers distinguishing dorsal and ventral parts (Castro, Becerra et al. 2006).

1.13 Somatostatin

Somatostatin is synthesized throughout the peripheral and central nervous system as preprosomatostatin before it is cleaved in a tissue-specific manner into two bioactive proteins, somatostatin-14 and somatostatin-28 (Patel 1999). Somatostatin has been shown to affect both exocrine and endocrine cell proliferation and secretion, as well as affecting cognitive, locomotor, and sensory functions. Somatostatin is involved in development as evidenced by its' capacity to affect neuronal migration in the developing rodent nervous system (Yacubova and Komuro 2002).

Previous studies have reported the cloning of zebrafish cDNAs encoding somatostatin precursors termed PPSSII, PPSSI, and PPSSIII (Argenton, Zecchin et al. 1999). PPSS2 was shown to be expressed exclusively in the pancreas, whereas, PPSSI was expressed in both the pancreas and the central nervous system. At 24hpf, PPSSI is expressed in two bilateral cell clusters in the telencephalon, whereas at 28hpf the

expression is reduced and at 30hpf, the expression is no longer observed (Devos, Deflorian et al. 2002).

In goldfish, three somatostatin genes were studied in the brain by in-situ hybridization, PPSI, PPSII, and PPSIII. PPSI was detected in several pallial and subpallial areas of the telencephalon. Cells were scattered close to the surface of the brain in both the lateral and dorsal areas, with the strongest signal being detected in the lateral part of the ventral telencephalon packed against the ventral border. PPSI and PPSIII, showed a similar expression pattern, whereas, PPSII was not detected in the telencephalon (Canosa, Cerda-Reverter et al. 2004).

1.14 Parvalbumin

Parvalbumin consists of 109 amino acids and exists in two isoforms, alpha and beta, in vertebrates (Hsiao, Tsai et al. 2002). Parvalbumin is a member of the calmodulin family (CaM) and contains 2 functional calcium binding domains. The tissue distribution of the β -type in tetrapods tends to be more restrictive than the α -type. The α -type, for example, is detected in skeletal myofibrils, endocrine glands, the brain, bones, and kidney (Gosselin-rey and Gerday 1977). The β -type, however, is expressed specifically in the thymus and hair cells of the Corti organ (Hapak, Zhao et al. 1994).

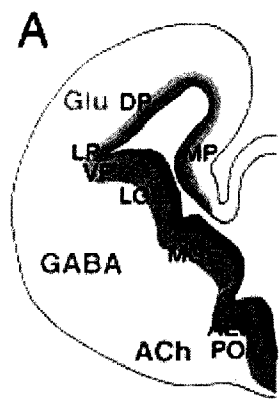
Two β -type zebrafish homologues of PVALB3a and PVALB 3b, related to chicken CPV3 and mammalian oncomodulin were studied by in-situ hybridization (Hsiao, Tsai et al. 2002). In very early development of the zebrafish (i.e. 18hpf), parvalbumin can be detected in the mucous cells, olfactory epithelium of the nasal epithelium, yolk syncytial layer, and extraembryonic tissue. Unlike chicken CPV3, which is expressed exclusively in the thymus, PVALB3a and PVALB3b were undetectable in

the thymus. Results suggest that PVAL3b and PVALB3a expression is distinct and developmentally regulated during early embryogenesis (Hsiao, Tsai et al. 2002).

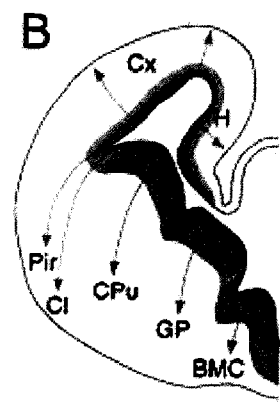
1.15 Interneuron Migration in the Telencephalon

During development, cell migration plays an essential role in the formation and patterning of the telencephalon. Once the patterning and regionalization of progenitors has occurred, cell migration will commence. Interneurons originate from various places in the brain, however in mammals, most GABAergic interneurons derive from the medial ganglionic eminence, thereby traveling from the subpallium to the cerebral cortex and hippocampus (Anderson, Eisenstat et al. 1997). There are two main types of cell migration: radial and tangential. Radial migration occurs when cells migrate from their progenitor zone along the radial glial fibres towards the cerebral cortex where they differentiate into glutamatergic neurons (Marin and Rubenstein 2003) (Figure 1.3). Tangential migration includes cells that migrate orthogonally to the direction of radial

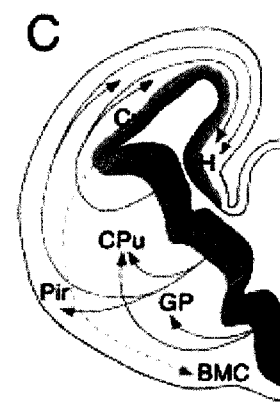
Figure 1.3. Different modes of migration in the developing telencephalon give rise to distinct neurons expressing various neurotransmitters. (A) The main types of neurotransmitters are derived from distinct progenitor populations in the telencephalon. The cholinergic (red) and GABAergic (blue) neurons originate from the ventral AEP/POA and LGE, MGE, respectively. The glutamatergic (green) neurons derive from the VP, LP, DP, and MP. (B, C) These distinct neurons follow radial (B) and tangential (C) migration to reach their final destinations in the telencephalon. The arrows indicate the directions of migration. Abbreviations: Ach, acetylcholine; AEP, anterior entopeduncular area; BMC, basal magnocellular complex; Cl, claustrum; Cpu, caudate-putamen nucleus; Cx, cortex; DP, dorsal pallium; GABA, γ -aminobutyric acid; GP, globus pallidus; Glu, glutamate; H, hippocampus; LGE, lateral ganglionic eminence; LP, lateral pallium; MGE, medial ganglionic eminence; MP, medial pallium; Pir, piriform cortex; POa, anterior preoptic area; VP, ventral pallium. Adapted from Rossant and Tam, 2002.



Progenitor Zones



Radial Migrations



Tangential Migrations

migration into the cerebral cortex where they primarily differentiate into GABAergic interneurons (Marin and Rubenstein 2003; Soltesz 2006). Interneurons will follow highly specified routes to reach the cortex. In order to avoid the developing striatum in the earlier stages of development, interneurons will migrate via a superficial or deeper pathway. Superficial traveling interneurons tend to migrate solo in the marginal zone or the subplate. Deeper traveling interneurons tend to migrate in compact clusters through the lower intermediate zone, eventually occupying a deeper position in the cortex. In addition to the routes the interneurons take in averting the striatum, they will travel solo or in groups (Soltesz 2006).

1.16 Radial Migration

Most of the current knowledge of the mechanisms behind radial migration has been studied in the developing cerebral cortex. Radial migration has been recognized as the primary mechanism whereby developing glutamatergic projection neurons arrive at their destinations in the telencephalon (Marin and Rubenstein 2003; Soltesz 2006). It has been shown that during the development of the cerebral cortex, different waves of migration position the neurons within different layers in the cortical plate. The first set of neurons migrate out of the cortical ventricular zone and constitute the preplate. The second set of neurons form the cortical plate, further dividing the preplate into the marginal zone and the subplate (Angevine and Sidman 1961). The cerebral cortex progresses in development upon successive waves of radial migration, positioning neurons in different layers of the cortical plate. As a result, the marginal zone and subplate contain the earliest-generated neurons of the cortex and the cortical plate, the progressively older neurons (Marin and Rubenstein 2003).

1.17 Tangential Migration

Despite the fact that radial migration is the primary mechanism whereby developing neurons arrive at their final destination in the telencephalon, analysis of Golgi-stained sections revealed the existence of tangentially migrating neurons as well. Studies involving the distribution of transcription factors such as *Dlx2* in the developing embryonic mouse telencephalon suggested that cells originating in the subpallial (subcortical) telencephalon may migrate tangentially into the pallium (Wullimann and Rink 2002). It has now been shown that GABA-producing neurons are born in the subpallium and migrate via several tangential streams into the pallium (Cobos, Puelles et al. 2001).

In rodents, most of the interneurons in the hippocampus, neocortex, and olfactory bulb are derived from the embryonic basal ganglia (Lois and Alvarez-Buylla 1994). The immature interneurons will migrate in distinct spatiotemporal routes to reach their final destinations where they will differentiate. Distinct subtypes of interneurons have characteristic spatiotemporal origins. Parvalbumin and somatostatin expressing interneurons derive primarily from progenitors located in the medial ganglionic eminence, whereas, calretinin expressing interneurons derive from the caudal ganglionic eminences (Ghanem, Yu et al. 2007).

Transplantation experiments using DiI labeling in slice cultures have provided evidence that various domains in the MGE of the mouse brain provide sources of tangentially migrating cells between E12.5 and E13.5 (Ghanem et al., 2007). Alternatively, some of the dorsal and ventral MGE migrating cells could be derived from the ventral MGE and are therefore, labeled at different positions along their migratory

route.

1.18 *Dlx* Cis-Acting Regulatory Elements

Enhancers are regulatory elements that control gene expression by upregulation and the recruitment of transcriptional activators. Enhancers tend to function independent of position or orientation and are located upstream or downstream of their target genes, sometimes within hundreds or thousands of kilobases (Arnone and Davidson 1997).

Two previously identified enhancers, I56i (440bp) and I56ii (300bp), were identified in the intergenic region between *Dlx5* and *Dlx6* of zebrafish, mouse, and human (Zerucha, Stuhmer et al. 2000; Ghanem, Jarinova et al. 2003). The nucleotide sequence of these elements is shown to be >85% conserved between zebrafish, mouse, and human. These enhancers were able to target the expression of a reporter transgene to the forebrain of both mouse and zebrafish in a pattern similar to endogenous gene expression (Zerucha, Stuhmer et al. 2000). Expression of *lacZ* in these mice was seen in both the pallium and subpallium similar to the endogenous expression of *Dlx5* and *Dlx6*. The expression pattern observed resembled more closely to the *Dlx5* endogenous pattern. In zebrafish embryos, a 1.6kb fragment containing both the I56i and I56ii elements directs expression of a reporter gene that also recapitulates the endogenous *Dlx5* and *Dlx6* expression in the forebrain (Zerucha, Stuhmer et al. 2000; Ghanem, Yu et al. 2007). Results from these two experiments have lead to suggestions that these intergenic enhancers exhibit a conserved function.

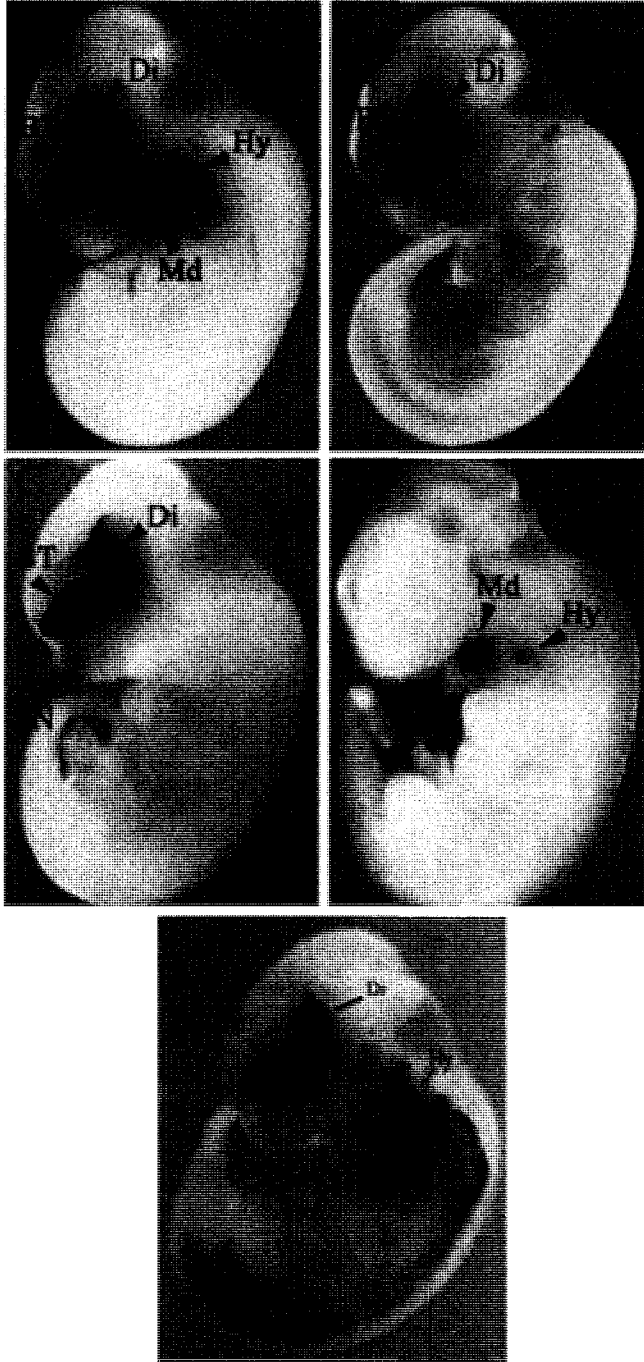
Two enhancers, I12b (400bp), I12a (550bp), were previously identified in the intergenic region of *Dlx1* and *Dlx2*, whereas one enhancer, URE2 (900bp), was identified upstream of *Dlx1* of mouse, human, zebrafish, and two species of pufferfish (Ghanem,

Jarinova et al. 2003). The I12b enhancer was able to target the expression of a reporter transgene to the forebrain of both mouse and zebrafish, whereas, the I12a construct targeted expression to the branchial arches (Zerucha et al. 2000, Ghanem et al 2003) (Figure 1.4). It has been suggested that these enhancers play a role in the regulation of *Dlx1* and *Dlx2* in adult GABAergic neurons since they are active in 66% and 93% of these cells, respectively and most of these cells contain GABA (Ghanem et al., 2007). All of these enhancers (I56i, I56ii, URE2, I12a, I12b) show little sequence similarity, however, maintain overlapping expression patterns during development (Ghanem, Jarinova et al. 2003).

Dlx gene regulation (*Dlx1*, *Dlx2*, *Dlx5*, *Dlx6*) in the forebrain development of the mouse have been studied (Liu, Ghattas et al. 1997; Eisenstat, Liu et al. 1999; Panganiban and Rubenstein 2002). It has been shown that *Dlx* expression is under the control of a dynamic and complex regulation that changes temporally and spatially during embryonic development. Furthermore, the sum of the expression patterns of the distinct enhancer elements, URE2, I12b, I56i, represents the overall patterns of *Dlx* expression pattern in the developing brain (Zerucha, Stuhmer et al. 2000; Ghanem, Yu et al. 2007). *Dlx* gene expression in the telencephalon is restricted to differentiating GABAergic cells born in the ventricular and subventricular zones of the lateral and medial ganglionic eminences (Anderson, Qiu et al. 1997; Stuhmer, Anderson et al. 2002; Stuhmer, Puellas et al. 2002).

Figure 1.4. Enhancer activity of conserved Dlx intergenic sequences in transgenic mice. (A-E) and zebrafish (F-J). (A) Mouse I56i, (B) Mouse I56ii, (C) Mouse I12b, (D) Mouse I12a, and (E) Mouse URE2, (F) head of 48hr primary transgenic zebrafish injected with the control *dlx6a-GFP* reporter plasmid, (G, H) *dlx6a-GFP* reporter plasmid that also contained a 1.4-kb *dlx5a/dlx6a* intergenic fragment containing I56i and I56 ii (I) *dlx6a-GFP* that also contained a 4.0-kb mouse *Dlx5/Dlx6* intergenic fragment that comprises I56i, (J) *dlx6a-GFP* that also contained a 2.8-kb mouse *Dlx5/Dlx6* intergenic fragment that comprises both I56i and I56ii. Abbreviations: BT, telencephalon; Di, diencephalon; Hy, second branchial arch; Md, mandibular component of the first branchial arch; I, diencephalic domain; II, telencephalic domain. Adapted from Ghanem et al. 2003.

A)



B)



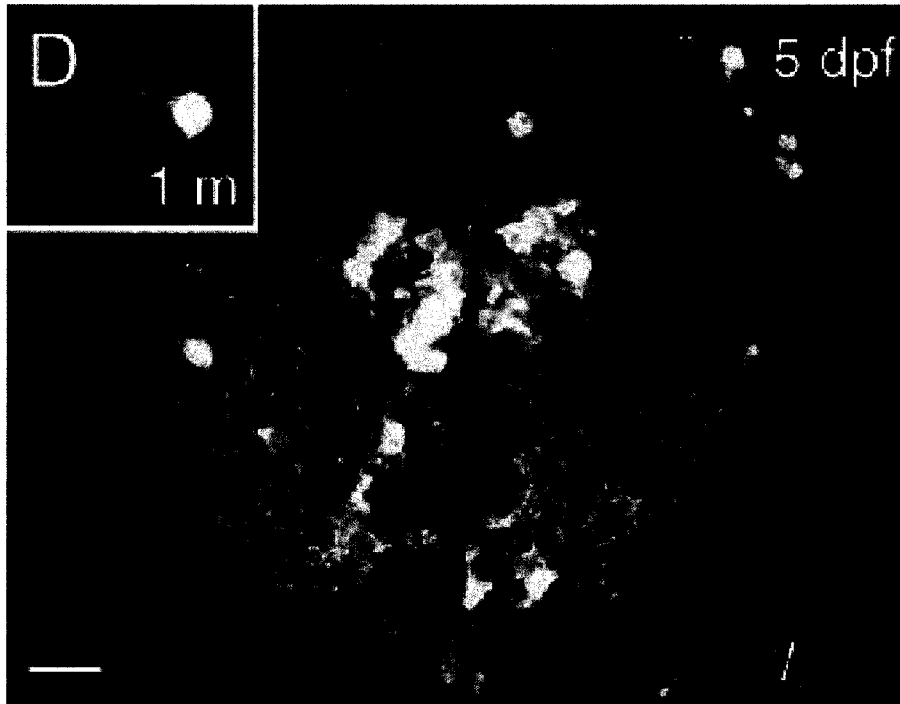
These cells then undergo radial or tangential migration to their final destinations in various regions including the piriform, cerebral cortex, hippocampus, and olfactory bulb (Anderson, Eisenstat et al. 1997). Studies using a zebrafish *dlx5/6* enhancer-lacZ reporter construct showed that >90% of X-Gal cells also expressed GABA in the adult mouse and similarly, >90% of GABA expressing cells also expressed X-Gal (Stuhmer et al. 2002). This high level of X-Gal and GABA co-expression was also seen in the hippocampus, olfactory bulb, and all parts of the amygdala. Calbindin- and calretinin- expressing cells also showed β -galactosidase activity in most of the cells. Furthermore, all parvalbumin and almost all nNOS and NPY neurons overlapped with X-Gal.

Studies have demonstrated that the distinct *Dlx* enhancer elements, URE2, I12b, and I56i define molecularly distinct subdivisions of the LGE, MGE, and CGE of the developing mouse brain (Ghanem, Yu et al. 2007). *I12b-lacZ*, and *I56i-lacZ* are expressed in the majority of neurons containing somatostatin, vasoactive-intestinal peptide, and calbindin, whereas *URE2-lacZ* is expressed in the majority of neurons containing parvalbumin, calretinin, neuronal nitric oxide synthase, and, neuro-peptide Y. Recent work using mice has suggested different spatiotemporal activities of the conserved regulatory elements URE2, I12b, and I56i in tangentially migrating cells derived from the medial ganglionic eminence and caudal ganglionic eminence between E11.5 and E13.5 (Ghanem et al., 2007). It is thought that *URE2-lacZ* expressing cells that migrate to the cortex are derived from the mMGE and mCGE due to enhancer activity in these domains at E12.5. This *URE2-lacZ* expression is detected 1d later than the *I12b-lacZ* activity and the *I56i-lacZ* activity. These results suggest that cells express various combinations of *URE2-lacZ+*, *I12b-lacZ+*, and *I56i-lacZ+*, which eventually leads to

distinct populations of migrating cells that present distinct fates once they reach their destination in the developing cortex (Ghanem, Yu et al. 2007).

It has been shown that subpopulations of interneurons migrate from the ventral telencephalon to the dorsal telencephalon, providing it with inhibitory interneurons. This phenomenon was visualized in a transgenic line of zebrafish *tg(1.4dlx5a-6a:GFP)* (Ghanem et al., 2003, Marin et al., 2008). Beginning at 3dpf, there is a ventrodorsal and rostrocaudal movement of GFP+ cells that results in an abundant number of interneurons being brought to the dorsal telencephalon. Cells in the dorsal telencephalon were monitored over the duration of 1 month with 1 GFP+ cell being detected at 3dpf, many scattered cells detected at 5dpf, and finally, >700 cells with morphology resembling dorsal telencephalic interneurons can be detected. These migrating cells would undergo rapid movements, intermittent standstills, and exploratory movements of their neurites. To identify the phenotype of these migrating cells, they were stained with GAD65 and GAD67 using fluorescent in situ hybridization combined with GFP immunohistochemistry. In the dorsal telencephalon of 5dpf and 5-month-old zebrafish, all GFP+ cells expressed GAD65/67 mRNAs, whereas, not all GAD-expressing cells were GFP+ (Figure 1.5). From this, they concluded that the *dlx5a-6a:GFP+* migrating cells are the precursors of the GABAergic neurons of the dorsal zebrafish telencephalon (Marin et al. 2008). It was also noted that the majority of these migrating cells headed for the caudal part of the telencephalon, often migrating, through the lateral regions, as if these domains were more permissive or provided cues and/or substrates for their migration. They suggest that it is probable that region specificities exist for the origin, route, and destination of zebrafish dorsal interneurons. A definitive map, however, of the

Figure 1.5. GFP co-labels with GAD in the developing zebrafish telencephalon of the transgenic line Tg(1.4kb-dlx5a/dlx6a:EGFP). Double immunohistochemistry showing GAD expressing cells (red) and GFP expressing cells (green) in the developing telencephalon of a 5 day post-fertilization zebrafish. Yellow indicates co-expression between GAD and GFP. Calibration bars = 20um. Adapted from Marin et al., 2008.



different regions of the ventral telencephalon in developing zebrafish has yet to be completed, and so therefore, they assume it to be premature to interpret the behavior of zebrafish migrating interneurons solely on the basis of their origin (Marin et al., 2008).

1.19 Objectives of this Project

As described above, previous studies have shown that the *cis*-acting regulatory elements I12b, I56i, and I56ii give rise to distinct types of interneurons with a set migratory route in the developing mouse brain. It is hypothesized that the enhancers I12a, I12b, I56i, and I56ii are differentially active in interneuron precursors in the developing telencephalon of the zebrafish and that these interneuron precursors give rise to distinct subtypes of interneurons. These interneuron precursors migrate from the ventral pallium in a ventral to dorsal manner where they populate the dorsal pallium. The goal of this project is to analyze the differential activity of the I12a/b regulatory elements of the *dlx1a/2a* locus and the I56i/ii regulatory elements of the *dlx5a/6a* locus in the developing zebrafish telencephalon and ultimately, elucidate a more thorough understanding of *Dlx* regulation.

In order to achieve this goal, several objectives were devised:

- Identify in which cells the *dlx* enhancers I12a/b, and I56i/ii are active in the developing zebrafish forebrain using transgenic zebrafish
- Track the migration pattern of the enhancer-active cells and determine the differential activity between the different enhancers
- Investigate whether enhancers contribute to defining interneuron precursors in forebrain development using GAD65/67 immunohistochemistry
- Investigate whether enhancers contribute to defining distinct subtypes of interneurons in zebrafish forebrain development using calretinin immunohistochemistry

2. METHODS

2.1. Histology

48hpf, 72hpf, and 96hpf zebrafish embryos were anesthetized in 3% Tricaine and fixed for a minimum of 4 hours up to 24 hours in 4% paraformaldehyde/PBS at room temperature. Zebrafish were then equilibrated in 30% sucrose/1X PBS overnight at 4°C prior to sectioning. Zebrafish were oriented and embedded in Tissue Freezing Medium (Triangle Biomedical Sciences) and cryosectioned at 10µm using a cryostat (Leica CM1850) at -20°C. Sections were placed on Superfrost®/Plus slides (Fisher Scientific).

2.2 Immunohistochemistry

48hpf, 72hpf, 96hpf, 7dpf, and 14dpf sections of the zebrafish telencephalon were washed 3 X 5 min each with 1X PBS to remove cryoprotectant. Sections were preincubated in a blocking solution of 1% bovine serum albumin (BSA) and 0.5% BSA, for at least 30 min at room temperature. The solution was then exchanged with a fresh aliquot containing the primary antibody overnight at room temperature, followed by 3 X 10 min washes in 1X PBST. Sections were then incubated in the other primary antibody overnight at room temperature, followed by 3 X 10 min washes in 1X PBST. Sections were incubated with the secondary antibody in 1X PBS for at least 2h at room temperature.

The following primary antibodies were used: mouse monoclonal anti-calretinin (1:1000 Swant 6B3), mouse monoclonal anti-parvalbumin (1:1000 Swant 235), mouse monoclonal glutamic acid decarboxylase (Gad65/67) (1:1000 Biomol® International GC

3108), rabbit polyclonal anti-green fluorescent protein (1:1000 Invitrogen™ A-11122), rat monoclonal anti-somatostatin (1:100 Chemicon International MAB354).

The following secondary antibodies were used: Alexa Fluor 594® goat anti-rabbit IgG (H+L) (1:300 Invitrogen™ A11012), Alexa Fluor 48 goat anti-mouse IgG (H+L) (1:300 Invitrogen™ A11001, Alexa Fluor 48 goat anti-rabbit IgG (H+L) (1:300 Invitrogen™ A11008), Alexa Fluor 594® goat anti-mouse IgG (H+L) (1:300 Invitrogen™ A11005), Alexa Fluor 594® goat anti-rat IgG (H+L) (1:300 Invitrogen A11007)™

2.3 Immunohistochemistry Optimization

48hpf, 72hpf, 96hpf, 7dpf, 14dpf, 30dpf, 1-2ypf zebrafish were sectioned and immunohistochemistry performed on each stage with each antibody and various block and wash solutions.

Various dilutions tested during optimization include:

Mouse anti-calretinin: 1:300, 1:500, 1:1000, 1:2000, and 1:3000

Rabbit anti-calretinin: 1:1000, 1:2500

Mouse anti-parvalbumin: 1:300, 1:500, 1:500, 1:1000, 1:2500

Rabbit anti-parvalbumin: 1:1000, 1:2500, 1:4000

Rat anti-somatostatin: 1:50, 1:100, 1:300, 1:500, 1:1000, 1:2000, 1:3000

Rabbit anti-NPY: 1:1000, 1:2500

Rabbit anti-nNOS: 1:100, 1:500, 1:1000

Rabbit anti-GABA: 1:200, 1:500, 1:1000, 1:3000

Mouse anti-Gad65/67: 1:300, 1:500, 1:1000, 1:2000

Rabbit anti-VIP: 1:400

Block solutions tested during optimization include:

- 1% BSA, 5% goat serum, 0.1M PBS or 0.5% Tween-20, or 0.1M PBST, 0.3-0.5% Triton-X
- 1%BSA in 0.5%PBS

Wash solutions tested during optimization include:

- 0.5% Triton-X, 0.5% PBST
- 0.5% PBST
- 1% PBST
- 1% Triton-X100 in PBS

Section thickness tested during optimization include:

- 5 μ m, 8 μ m, 10 μ m, 12 μ m, 15 μ m, 20 μ m, 30 μ m

2.4 Transgenic Zebrafish

Transgenic zebrafish were generated with a reporter gene driven by intergenic fragments from *dlx1a/2a* and *dlx5a/6a*. The *Tg(6kb-dlx1a/dlx2a:EGFP)* line was generated using 6kb of the *dlx1a/2a* intergenic region, a β -globin minimal promoter and EGFP (MacDonald *et al.*, unpublished observations). *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line was generated using 1.4kb of the *dlx5a/6a* intergenic region, 2.5kb of the *dlx6a* promoter, and EGFP (Ghanem *et al.*, 2003).

3. RESULTS

3.1 Detailed characterization of enhancer activity in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* zebrafish brain via immunohistochemistry

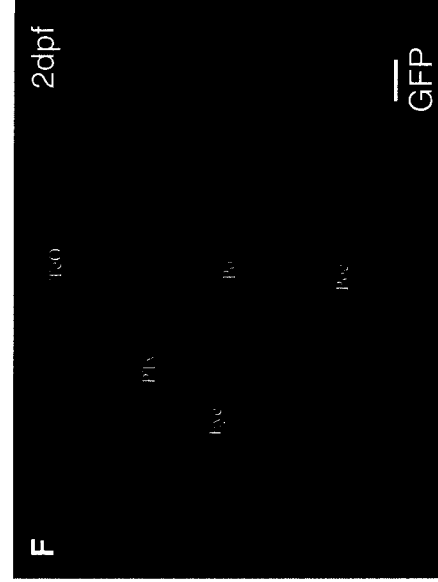
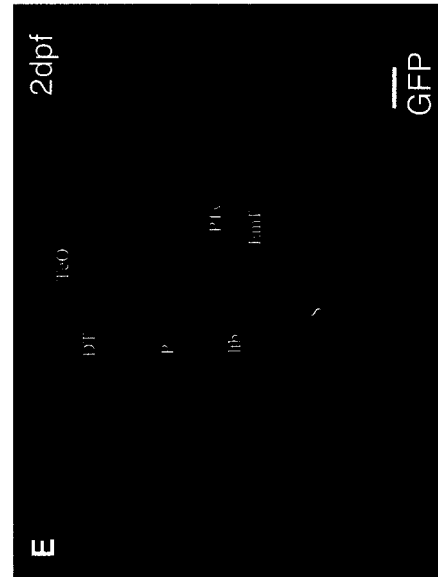
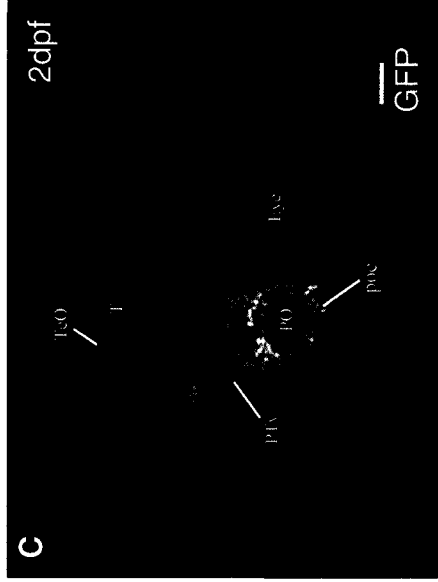
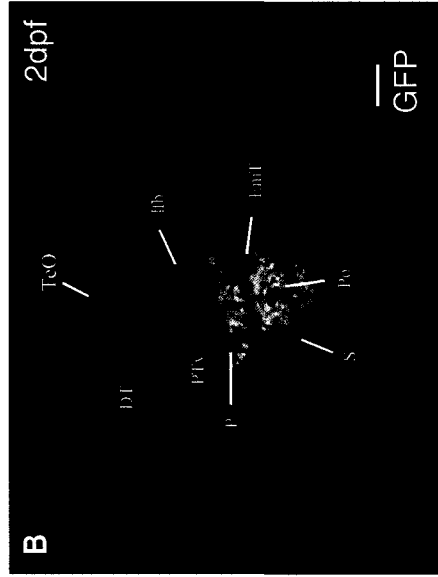
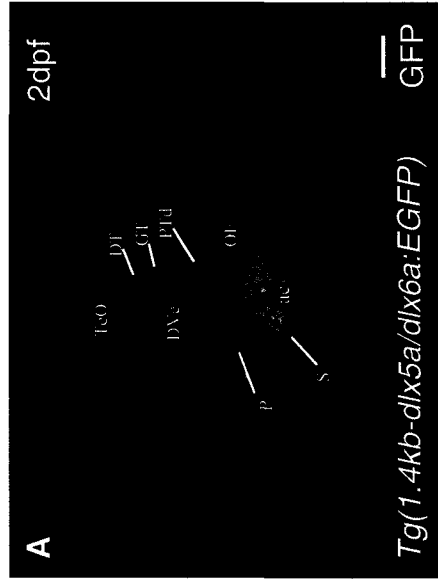
Studies have demonstrated that the *cis*-acting regulatory elements I56i and I56ii are active in distinct subtypes of interneurons in the zebrafish brain. A transgenic line, *Tg(1.4kb-dlx5a/dlx6a:EGFP)*, has previously been generated to track the migratory route of these neurons through the zebrafish brain. Some characterization of this migratory route has been performed in past studies (Zerucha et al., 2000; Ghanem et al., 2003; Mione et al., 2006) yet in the present study, a more detailed characterization of the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line has been performed using a GFP antibody to detect cells expressing GFP, indicating the cells in which the enhancers are active. Embryonic zebrafish were fixed and cryosectioned preceding immunohistochemistry, followed by fluorescence microscopy observation. This detailed analysis has been used to compare the differential enhancer activity with the newly generated *Tg(6kb-dlx1a/dlx2a:EGFP)* line of zebrafish (MacDonald *et al.*, unpublished observations), in which two other *cis*-acting regulatory elements I12a and I12b are tested.

3.1.1 Expression of *Tg(1.4kb-dlx5a/dlx6a:EGFP)* in the 2dpf zebrafish brain

At 2dpf, there is an abundance of cells expressing GFP covering most of the subpallium from the medio-lateral area of the olfactory epithelium (OE) in towards the lateral edges of the anterior commissure (ac) but not reaching the ac (Figure 3.1A of Panel A-F). Lateral to this is GFP expression that appears to be expressed from an abundant number of GFP+ structures, more caudally. In the pallium, there are a few cells

Figure 3.1 Detailed characterization of enhancer activity in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain via immunohistochemistry at 2dpf. (A-C) 2dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10µm) of the zebrafish brain A) anti-GFP rostral B) anti-GFP medial C) anti-GFP caudal (D-F) 2dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10µm) of the zebrafish brain D) anti-GFP rostral E) anti-GFP medial F) anti-GFP caudal. Green structures are GFP+.

ac: anterior commissure, DT: dorsal thalamus, DVe: diencephalic ventricle, DT: dorsal thalamus, EmT: eminentia thalami, GT: griseum tectale, lfb: lateral forebrain bundle, OE: olfactory epithelium, P: pallium, po: preoptic region, poc: postoptic commissure, PTD: dorsal part of posterior tuberculum, PTv: ventral part of posterior tuberculum, S: subpallium, TeO: tectum opticum. Calibration bar = 20µm



(7-10) expressing GFP in a linear fashion spreading medially from the dorsal part of the posterior tuberculum (PTd) near the diencephalic ventricle (DVe) to the inner edges of the eyes. The number of cells increases and scatters medially towards the dorsal thalamus (DT) and PTd (Figure 3.1A). Rostrally, a pair of GFP expressing cells is located laterally below the optic tectum (TeO) above the griseum tectale (GT) on both the right and the left sides of the brain that disappear and are replaced by more scattered cells caudally (Figure 3.1A).

There are scattered GFP+ cells throughout the DT. GFP+ cells are in abundance in the preoptic region (PO) with GFP+ structures lateral to the cluster of cells spreading out to the inner region of the eyes and then up towards the dorsal region of the brain (Figure 3.1B). There is an abundance of GFP+ cells located at the ventral part of the posterior tuberculum (PTv) that extend laterally and dorsally with GFP+ structures, resembling axon tracts, extending dorsally towards the TeO (Figure 3.1B).

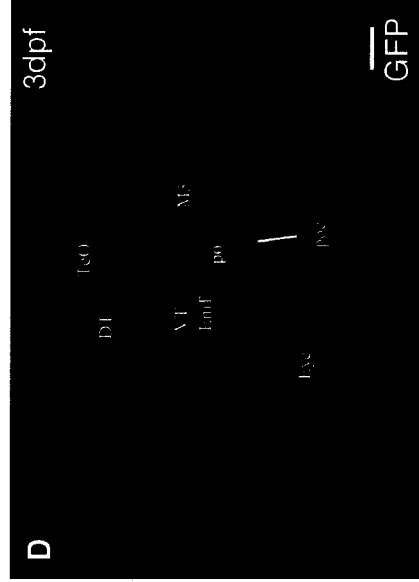
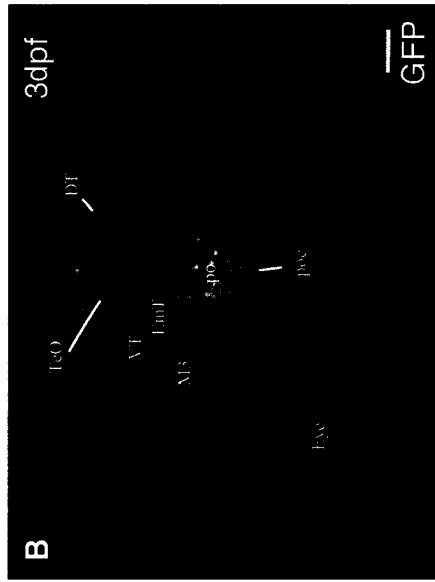
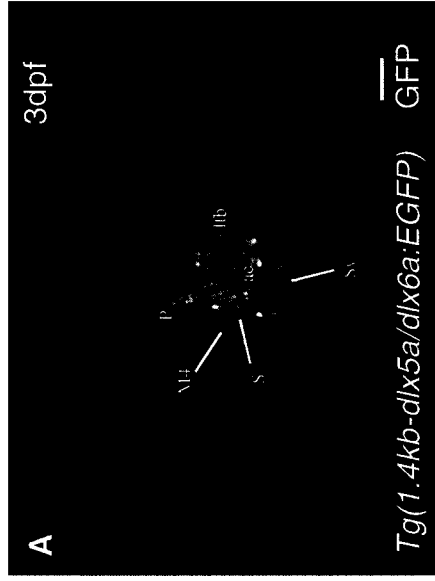
The TeO and midbrain tegmentum (T) exhibit several scattered GFP+ cells (Figure 3.1C). Ventrally to this, between the T and PTv, is a horizontal stripe of GFP+ axon tracts and cells that extend from the inner edge of one eye to the next. The lateral edges of these GFP+ structures curve slightly dorsally towards the TeO. There is an abundance of GFP+ cells from the medial base of the optic postoptic commissure (poc) both laterally and dorsally towards the T in a “U” shaped manner (Figure 3.1C). There is a lack of cells in between this “U shaped” pattern, however, at the dorsal area of these cells are another group of GFP+ cells extending laterally towards the midline until they join (Figure 3.1C).

3.1.2 Expression of *Tg(1.4kb-dlx5a/dlx6a:EGFP)* in the 3dpf zebrafish brain

At 3dpf, there is an abundance of GFP+ cells covering the rostral portion of the brain from the ventral division of the subpallium (Sv) out to the inner edges of the eyes and OE all the way up to the pallium (Figure 3.2A of Panel A-D). Several (approximately 5) of the GFP+ cells at the lateral edges of the telencephalic migrated area (M4) appear to be migrating in a ventral to dorsal manner with axon tracts extending and trailing ventrally. At this location in the brain, there are only several (approximately 5) GFP+ cells reaching the pallium (Figure 3.2A).

Further caudally, there are almost no GFP+ cells in the pallium. The few cells that are present, however, appear to form horizontal clusters (approximately 50 cells) that extend medially from the DT out to the migrated area of the eminentia thalami (EmT) (M3) (Figure 3.2B). There are two small clusters of cells in the outer regions near the lateral forebrain bundle (lfb). There are several GFP+ cells (approximately 7) located dorsally in the TeO. The number of GFP+ cells, once again, increases more caudally. There is an abundance of GFP+ cells in the ventral region of the Po that extends laterally towards the M3 and lfb up dorsally towards the migrated pretectal area (M1) and TeO. There are GFP+ cells that form a horizontal stripe along the postoptic commissure (poc) and connect to the dorsally migrating cells at the lateral edges. There are many (>50) GFP+ cells that occupy the TeO with axon tracts that extend ventrally as well as dorsally towards the dorsal TeO. These GFP+ cells appear to have migrated in a ventral to dorsal manner (Figure 3.2B).

Figure 3.2 Detailed characterization of enhancer activity in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain via immunohistochemistry at 3dpf. (A-B) 3dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. A) anti-GFP rostral B) anti-GFP caudal (C-D) 3dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain C) anti-GFP rostral D) anti-GFP caudal. Green structures are GFP+. ac: anterior commissure, DT: dorsal thalamus, DVe: diencephalic ventricle, DT: dorsal thalamus, EmT: eminentia thalami, lfb: lateral forebrain bundle, M3: migrated area of EmT, M4: telencephalic migrated area, OE: olfactory epithelium, P: pallium, po: preoptic region, poc: postoptic commissure, S: subpallium, Sv: ventral region of the subpallium, TeO: tectum opticum, VT: ventral thalamus. Calibration bar = 20 μ m



3.1.3 Expression of *Tg(1.4kb-dlx5a/dlx6a:EGFP)* in the 4dpf zebrafish brain

At 4dpf, the most rostral part of the brain shows an abundance of GFP+ cells covering the surface of the subpallium out to the OE and up to the pallium and meet medially at the ac. The cells gradually exhibit a more uniform pattern at the ac up to the dorsal division of the subpallium (Sd) at the M4 (Figure 3.3A of Panel A-D).

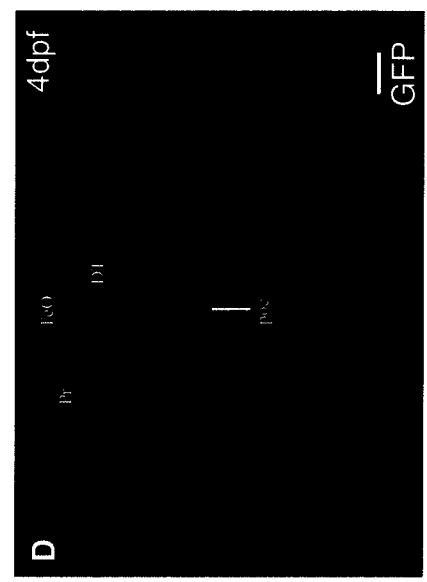
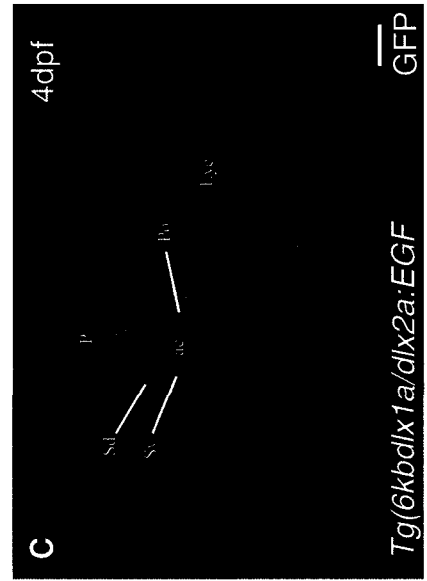
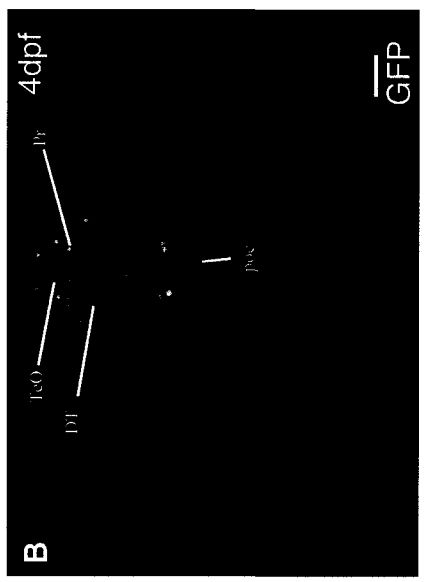
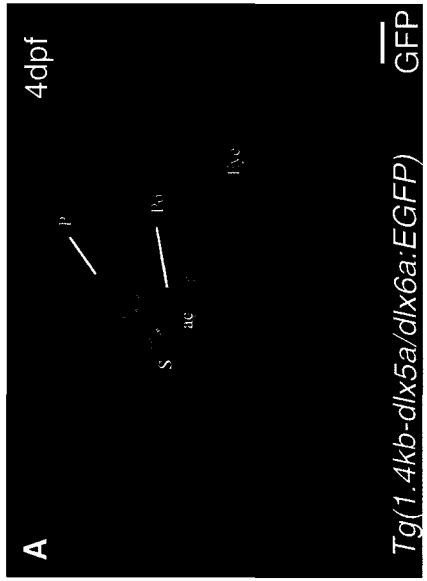
Towards the midbrain the dorsal GFP+ cells in the pallium decrease in number. The GFP+ cells only reach dorsally at the Sd and do not occupy the pallium. The central GFP+ cells cluster and are accentuated with a uniform expression of GFP+ structures at the M3 and lfb.

Caudally, the central GFP+ cells reach the lfb and start to curve laterally and dorsally, where several (approximately 2) cells can be seen in the TeO (Figure 3.3B). These dorsally located GFP+ cells increase in number more caudally until there are many (>80) GFP+ cells located in the TeO. These cells axon tracts extend from the lateral regions of the TeO and inwards medially towards the centre region of TeO (Figure 3.3B).

3.2 Detailed characterization of enhancer activity in the *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain via immunohistochemistry

It has been shown that the *cis*-acting regulatory element I12b is active in specific subtypes of interneurons in the developing mouse brain (Stuhmer, Anderson et al. 2002; Stuhmer, Puelles et al. 2002; Ghanem, Yu et al. 2008). These interneurons tangentially migrate from the ventral telencephalon to the developing cortex. A transgenic line of zebrafish *Tg(6kb-dlx1a/dlx2a:EGFP)* (MacDonald *et al.*, unpublished observations) has

Figure 3.3 Detailed characterization of enhancer activity in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain via immunohistochemistry at 4dpf. (A-B) 4dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. A) anti-GFP rostral B) anti-GFP caudal (C-D) 4dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain C) anti-GFP rostral D) anti-GFP caudal. Green structures are GFP+. ac: anterior commissure, DT: dorsal thalamus, lfb: lateral forebrain bundle, P: pallium, po: preoptic region, poc: postoptic commissure, Pr: pretectum, S: subpallium, Sd: dorsal region of the subpallium, Sv: ventral region of the subpallium, TeO: tectum opticum. Calibration bar = 20 μ m



recently been generated to track the migratory route of cells in which the *Dlx1a/2a* CREs target reporter transgene expression in the developing zebrafish brain. Embryonic zebrafish were fixed and cryosectioned, preceding immunohistochemistry and observed via fluorescence microscopy. In this study, a detailed characterization of the migrating neurons has been made and a comparison of differential activity between the *Tg(6kb-dlx1a/dlx2a:EGFP)* line and *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line performed early in development.

3.2.1 Expression of *Tg(6kb-dlx1a/dlx2a:EGFP)* in the 2dpf zebrafish brain

At 2dpf, there is an abundance of cells expressing GFP covering most of the subpallim from the lateral area of the OE in towards the lateral edges of the ac. These cells cluster more centrally surrounding the ac leaving the lateral edges curving up very slightly towards the dorsal portion of the brain. There are approximately 20 GFP+ cells above this pattern of cells on both the left and the right side that spans horizontally from the ac to the inner edges of the eyes. (Figure 3.1D).

The number of the GFP+ cells decrease caudally in the brain (Figure 3.1E). There are several GFP+ cells following a circular pattern at the Po that form small clusters of approximately 20 GFP+ cells each, on both lateral edges of this circular pattern close to the inner eye at the PTv and also at the ventral area near the poc, unlike the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* zebrafish where there are many more GFP+ cells (Figure 3.1F).

The number of GFP+ cells decreases both in the dorsal region and caudal regions of the developing zebrafish brain in the *Tg(6kb-dlx1a/dlx2a:EGFP)* line, unlike the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line in which there is an abundance of GFP+ cells.

Furthermore, unlike the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* zebrafish, where there are GFP+ cells in the pallium, the *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish exhibit no GFP+ cells in the pallium at any point at 2dpf (Figure 3.1A-F).

3.2.2 Expression of *Tg(6kb-dlx1a/dlx2a:EGFP)* in the 3dpf zebrafish brain

At 3dpf, rostrally, there is an abundance of GFP+ cells covering the Sd and part of the Sv but not quite extending dorsally to the pallium, like what is observed in the *Tg(6kb-dlx1a/dlx2a:EGFP)* line. These cells meet at the ac and extend out towards the M4. The GFP+ cells merge to become two horizontal stripes of GFP+ cells extending medially from the VT across the EmT out towards the inner edges of the eyes (Figure 3.2D).

More caudally, these two stripes of cells are absent and there is a large cluster of cells extending from the Po up to the DT and do not touch the lateral edges of the brain. This large cluster of cells eventually decreases in size to the point where there are only a few cells (approximately 10) (Figure 3.2E). This is unlike the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line in which GFP+ cells occupy a large portion of both the ventral and dorsal caudal region of the brain (Figure 3.2 E, F).

Unlike the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* zebrafish, the *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish do not show any GFP+ cells in the dorsal region of the brain at any point at 3dpf and the GFP+ cell number decreases caudally (Figure 3.2A-F).

3.2.3 Expression of *Tg(6kb-dlx1a/dlx2a:EGFP)* in the 4dpf zebrafish brain

At 4dpf, there is an abundance of GFP+ cells covering the Sv up to the Sd and laterally to the OE. There are also several (approximately 10) GFP+ cells occupying the pallium (Figure 3.3C). More caudally, these cells in the pallium are not present and the GFP+ cells occupying the more ventral portion of the brain decrease in number. This is different than the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line, in which the number of GFP+ cells in the pallium is larger and persistent further into the medial portion of the brain (Figure 3.3A, C).

Between the rostral and caudal regions of the brain, the GFP+ cell number increases at the Po and extends up to the ventral portion of the pallium. There is a uniform GFP+ pattern resembling axon tracts that extends in a “U” shaped formation medially from the Po and laterally towards the lfb and then dorsally towards the VT.

Caudally, there are very little GFP positive cells and the “U” shaped uniform pattern is no longer present more caudally (Figure 3.3D). There are GFP+ cells occupying the ventral region at the level of the poc (Figure 3.3D). In contrast to the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* zebrafish, there appear to be no GFP+ cells in the dorsal pallium towards the caudal part of the *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain (Figure 3.3B, D).

Therefore, at 4dpf, the *Tg(6kb-dlx1a/dlx2a:EGFP)* line shows a much smaller number of GFP+ cells in the dorsal region of the brain (0-10) in comparison to the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line (2->80).

3.3 Detailed characterization of GAD65/67 in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* zebrafish and the *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain via immunohistochemistry

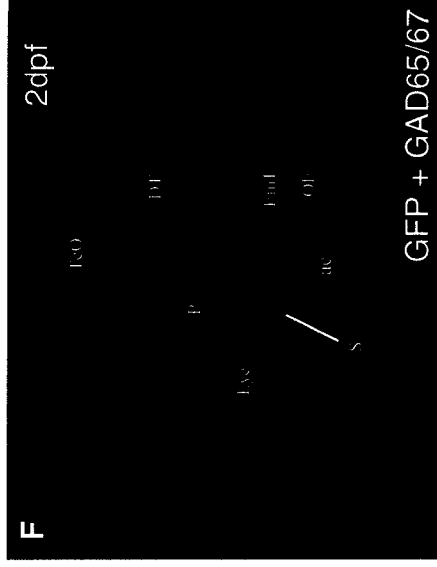
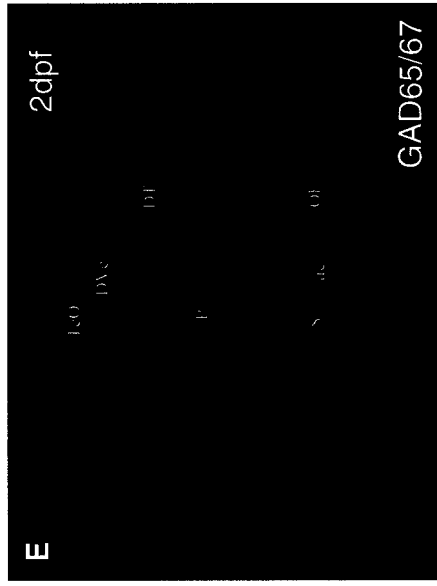
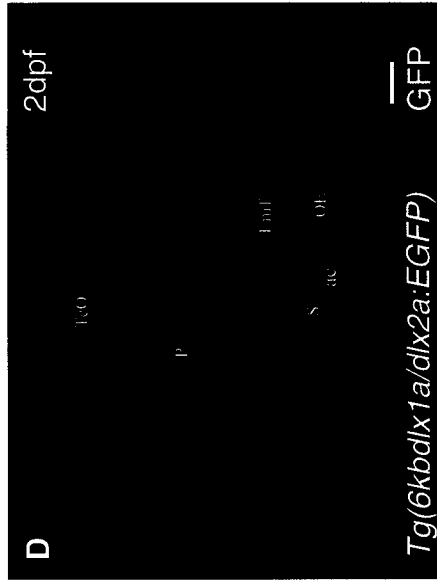
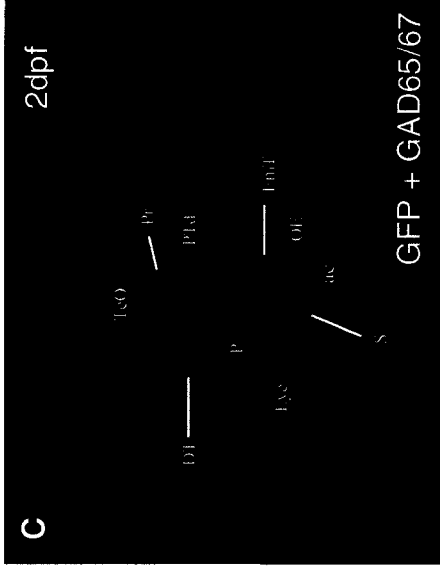
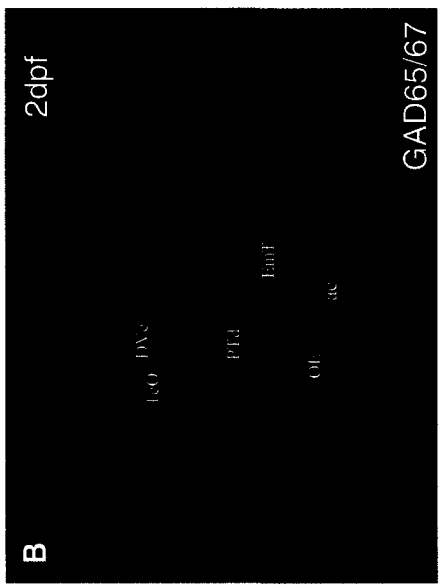
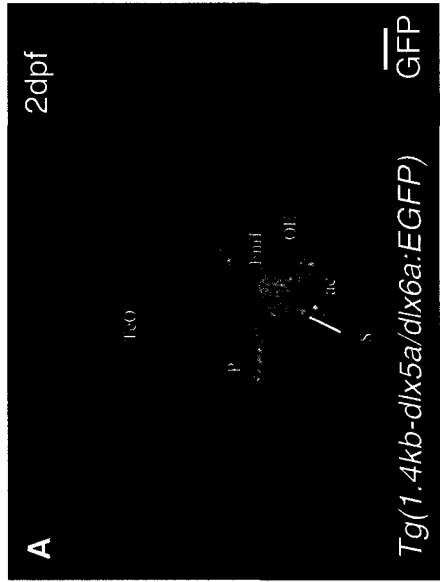
It has been previously shown that the *Dlx* genes give rise to GABAergic interneurons in the developing mouse brain. These interneurons have also been shown to migrate from the ventral telencephalon to the developing cortex during the early stages of development (Stuhmer, Puelles et al. 2002; Ghanem, Yu et al. 2007; Ghanem, Yu et al. 2008). Previously, GAD65 and GAD67 expression has been observed and characterized in the developing zebrafish brain (Mueller et al., 2006). In order to determine whether the *Dlx* enhancers I56i/ii and/or I12a/b are active in these GAD65/67 expressing interneurons, immunohistochemistry using GAD65/67 was performed and observed via fluorescence microscopy and a description completed.

3.3.1 2dpf

At 2dpf, there is an abundance of cells expressing GAD65/67 covering most of the subpallium from the medio-lateral area of the OE in towards the lateral edges of the ac but not reaching the ac. In the pallium, there are a few cells (7-10) expressing GAD65/67 in a linear fashion spreading medially from the PTd near the DVe to the inner edges of the eyes. These cells rise dorsally and then curve inwards towards the DT; they also increase in number more caudally (Figure 3.4B, E of Panel A-F)).

There are scattered GAD65/67+ cells throughout the DT. GAD65/67GFP+ cells are in abundance in the PO with a more homogeneous pattern rather than individual cellular conformation lateral to the cluster of cells spreading out to the inner region of the

Figure 3.4 The *dlxa5/6a* and *dlx1a/2a* CREs target reporter transgenes to Gad65/67 expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 2dpf. (A-C) 2dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. A) anti-GFP B) anti-GAD65/67 C) merged images of both GFP and GAD65/67 (D-F) 2dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain D) anti-GFP E) anti GAD65/67 F) merged images of both anti-GFP and GAD65/67. Green structures are GFP+, red structures are GAD65/67+ and yellow structures are both GFP+ and GAD65/67+. ac: anterior commissure, DT: dorsal thalamus, DVe: diencephalic ventricle, DT: dorsal thalamus, EmT: eminentia thalami, OE: olfactory epithelium, P: pallium, po: preoptic region, poc: postoptic commissure, PTd: dorsal part of posterior tuberculum, PTV: ventral part of posterior tuberculum, S: subpallium, TeO: tectum opticum. Calibration bar = 20 μ m



eyes and then up towards the dorsal part of the brain (Figure 3.4B, E). There is a GAD65/67 expressing region located at the PTv that extends laterally and then dorsally towards the optic TeO.

Ventrally to this, between the T and PTv, is a horizontal stripe-like region of GAD65/67 expression that extends from the inner edge of one eye to the next. The lateral edges of this GAD65/67 pattern curves slightly dorsally towards the TeO. There is GAD65/67 expression pattern beginning from the medial base of the oc extending both laterally and dorsally towards the T in a “U” shaped manner. The ventral portion of this pattern contains a cluster of cells, whereas, the lateral edges appear to contain a more homogeneous expression pattern. There is a lack of cells in between this “U shaped” pattern, however, at the dorsal area of this pattern is a group of GAD65/67+ cells extending laterally to medially until they join. Gad65/67+ cells form a circular pattern at the around the po at the rostral and intermediate hypothalamus (Hr/Hi) (Figure 3.5H, K of Panel G-L).

3.3.2 3dpf and 4dpf

Rostrally, Gad65/67+ cells uniformly cover the subpallium out to the OE and up to the olfactory bulbs (OB). The Gad65/67+ cells do not quite reach the pallium at the most rostral point but start to move upwards in the midbrain (Figure 3.6B, E of Panel A-F). There are two uniform stripes of Gad65/67+ cells extending centrally from the EmT and spread horizontally to the M3 and lfb and begin to curve dorsally towards the VT (Figure 3.6E).

Figure 3.5 The *dlx5a/6a* and *dlx1a/2a* CREs target reporter transgenes to GAD65/67 expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 2dpf. (G-I) 2dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. G) anti-GFP H) anti-GAD65/67 I) merged images of both GFP and GAD65/67 (J-L) 2dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain J) anti-GFP K) anti-GAD65/67 L) merged images of both anti-GFP and GAD65/67. Green structures are GFP+, red structures are GAD65/67+ and yellow structures are both GFP+ and GAD65/67+. Hi: intermediate hypothalamus, Hr: rostral hypothalamus, N: region of the nucleus of the medial longitudinal fascicle, P: pallium, po: preoptic region, poc: postoptic commissure, PTd: dorsal part of posterior tuberculum, PTv: ventral part of posterior tuberculum, S: subpallium, Sd: dorsal region of the subpallium, Sv: ventral region of the subpallium, T: midbrain tegmentum, TeO: tectum opticum. Calibration bar = 20 μ m

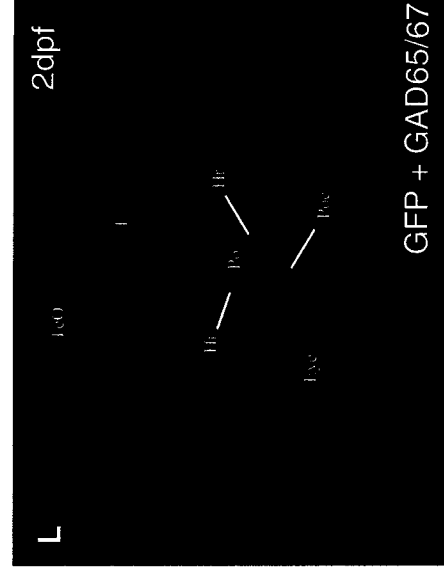
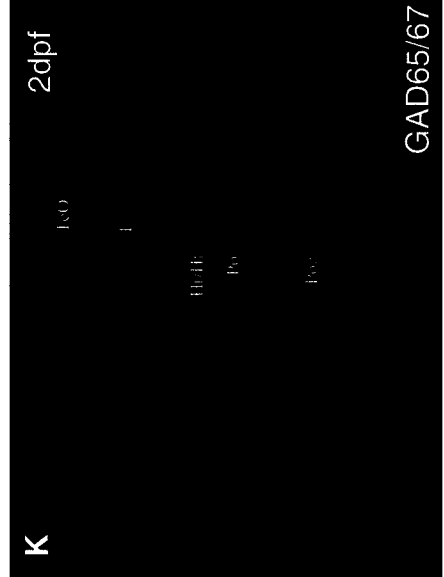
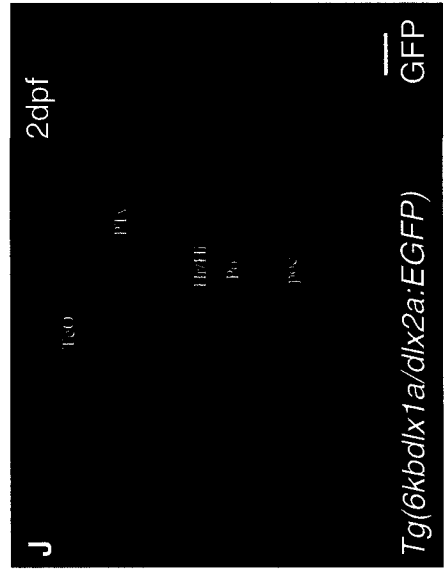
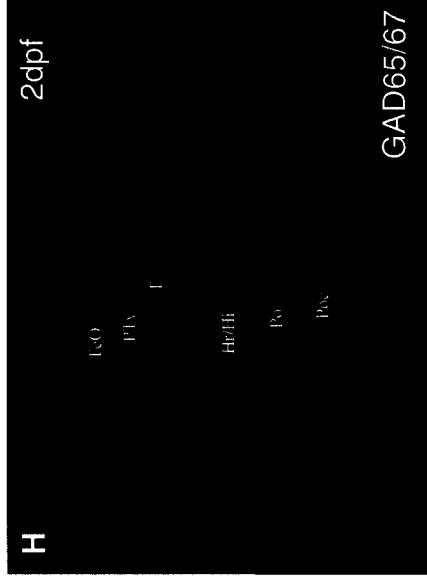
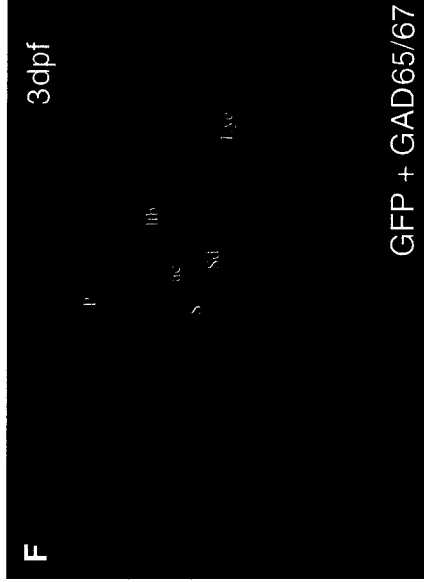
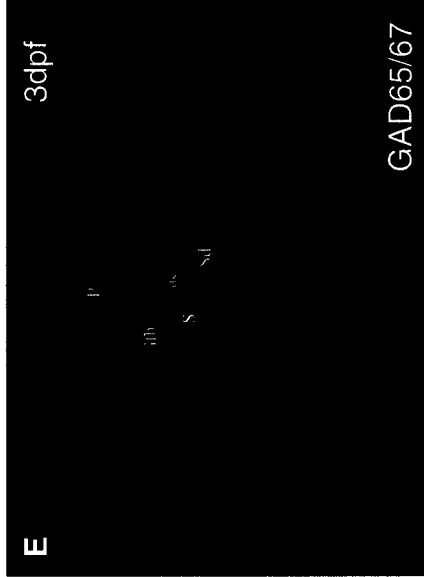
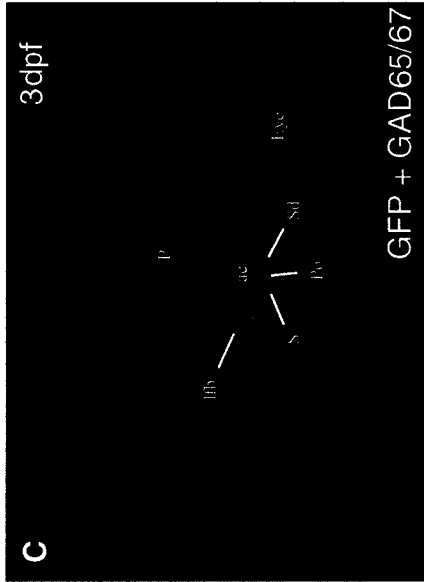
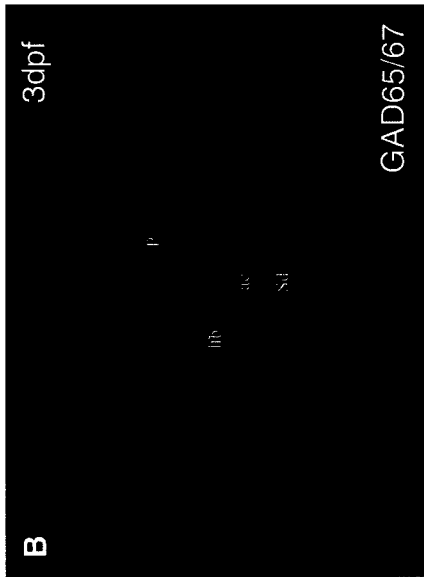


Figure 3.6 The *dlx5a/6a* and *dlx1a/2a* CREs target reporter transgenes to Gad65/67 expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 3dpf. (A-C) 3dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. A) anti-GFP B) anti-GAD65/67 C) merged images of both GFP and GAD65/67 (D-F) 3dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain D) anti-GFP E) anti GAD65/67 F) merged images of both anti-GFP and GAD65/67. Green structures are GFP+, red structures are GAD65/67+ and yellow structures are both GFP+ and GAD65/67+. Ac: anterior commissure, lfb: lateral forebrain bundle, P: pallium, po: preoptic region, S: subpallium, Sd: dorsal region of the subpallium. Calibration bar = 20 μ m



Caudally, the Gad65/67+ cells spread in a uniform manner from the base of the poc and form a “U” shaped pattern that spreads laterally towards the inner edges of the eyes and up towards the TeO (Figure 3.7H, K of Panel G-L).. There are two uniform bands lining the most dorsal outer edges of the TeO (Figure 3.7H, K).

3.4 The *dlx5a/6a* CREs target transgene expression to Gad65/67 expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* zebrafish brain

It has previously been shown that the *Dlx* gene enhancers, I56i, and I12b are active in GABAergic interneurons that migrate tangentially from the ventral telencephalon to the pallium in the developing mouse brain (Stuhmer, Puelles et al. 2002; Ghanem, Yu et al. 2007). In order to determine whether the two intergenic fragments containing the *Dlx5a/6a* and *Dlx1a/2a* CREs target reporter transgene expression to GABAergic interneurons in the developing zebrafish brain, GAD65/67 and GFP immunohistochemistry was performed and co-expression observed via fluorescent microscopy. The two transgenic lines were then compared for their patterns of co-expression.

3.4.1 Expression of GAD65/67 and *Tg(1.4kb-dlx5a/dlx6a:EGFP)* in the 2dpf zebrafish brain

At 2dpf, most rostrally, there is a high incidence of GFP and GAD65/67 co-expression from the Sv up to the ventral division of the pallium (Pv) in two large clusters of cells (>100) spreading lateral to the EmT but not quite reaching the ac (Figure 3.4C). Above these two clusters are two horizontal stripes of GFP+ cells that are also

GAD65/67+ extending from the ac out to the inner edges of the eyes, close to the EmT (Figure 3.4C). Above this are two stripes of GAD65/67+ and GFP- cells curving from the lateral edges of these two horizontal stripes near the eyes and curving dorsally and medially towards the pretectum (Pr). Virtually all the GFP+ cells express GAD65/67 but certain areas of GAD65/67+ cells do not express GFP (Figure 3.4C).

All of the previous GFP+ structures that are also GAD65/67+ expand so that the ventrally located structures cover the Po and meet almost right to the ac, expand laterally to the EmT and dorsally towards the pallium. The two horizontal stripes expand thicker vertically and appear to send axon tracts dorsally in a curving manner up the lateral edges of the inner eyes towards the Pr. There are two stripes of GAD65/67+ cells that are also GFP- expanding from the lateral edges, occupying the dorsal region near the Pr. There are several (>40) GFP+ and GAD65/67- cells scattered along the DT. There are several (>30) GAD65/67+ and GFP- cells scattered along the PTd. Most GFP+ cells express GAD65/67 but not all GAD65/67 cells express GFP in this area.

At the midbrain, there are GFP+ cells that are also GAD65/67+ expanding from the ventral-most region of the poc that curves dorsally in a “U” shaped fashion meeting in a horizontal stripe at the PTv/PTd. All of the GFP+ cells are GAD65/67+ and all of the GAD65/67 cells are GFP+ in this region. There is a horizontal stripe of cells spreading from the inner edges of each eye meeting at the centre near the region of the nucleus of the medial longitudinal fascicle (N). There are approximately 10 GFP+ and GAD65/67- cells occupying the area near the N and several (>6) GFP+ and GAD65/67- cells occupying the TeO.

Most caudally, there are GFP+ cells that are GAD65/67- (>50) lining the ventral most portion of the poc (Figure 3.5I). Directly above this is a circular “O” shape of GFP+ cells that are also GAD65/67+ spanning from the Hr/Hi out to the inner edges of the eyes and meeting at the PTv (Figure 3.5I). There are no GFP+ or GAD65/67+ cells at the inner portion of this circle. Near the N on each side, there are GFP+ and GAD65/67- cells (>15) with axon tracts extending ventrally that are also GFP+ and GAD65/67- (Figure 3.5I). The TeO has several scattered GFP+ cells that appear to be moving in a ventral to dorsal manner. Most of the cells located ventrally appear to be GFP+ and GAD65/67+ whereas the dorsal cells appear to be GFP+ and GAD65/67- (Figure 3.5I).

3.4.2 Expression of GAD65/67 and *Tg(1.4kb-dlx5a/dlx6a:EGFP)* in the 3dpf zebrafish brain

At 3dpf, most rostrally, virtually all of the GFP+ cells expanding from the base of the subpallium to the top of the pallium are GAD65/67+ with the exception of the cells lining near the ac, which are GFP+ but GAD65/67- (Figure 3.6C). There are GFP+ cells that are also GAD65/67+ near the lfb (Figure 3.6C). This observation continues all the way through to the midbrain. The Po out to the lfb and up to the Emt contains >100 GFP+ and GAD65/67+ cells. There are also structures at the lateral edges resembling axon tracts that are GFP+ and GAD65/67+ (Figure 3.6C).

Towards the midbrain, there are several GFP+ and GAD65/67- cells (>15) occupying the dorsal region of the brain around the Sd and pallium, this continues to the Tve. In this region, there are also many (>20) GFP+ and GAD65/67+ cells that appear to migrate in a ventral to dorsal manner along the marginal zone of the TeO towards the midline (Figure 3.7I). More ventrally, there is almost a high incidence of GFP+ and

GAD65/67+ cells in a “U” shaped pattern spanning from the base of the Po out to the lateral edges of the eyes near the lfb and up towards the M3 (Figure 3.7I). Directly in the centre of the “U” is a cluster of GFP+ and GAD65/67- cells. The outer lateral edges of the TeO shows a virtual 100% GFP+ and GAD65/67+ co-expression pattern in structures resembling cells and axon tracts. These axon tracts appear to be projecting towards the central TeO (Figure 3.7L).

It appears that most of the GFP+ cells and axon tracts are GAD65/67+ and most of the GAD65/67+ structures are GFP+ in both the dorsal and ventral regions (Figure 3.6C, Figure 3.7I).

3.4.3 Expression of GAD65/67 and *Tg(1.4kb-dlx5a/dlx6a:EGFP)* in the 4dpf zebrafish brain

At 4dpf, rostrally, there is an abundance of GFP+ cells covering the surface of the OB and pallium (Figure 3.8A). These structures are virtually all GFP+ and GAD65/67+ in the more dorsal region yet only a few cells (approximately 10) co-express with GAD65/67 in the subpallium (Figure 3.8C).

At the midbrain, all the GFP+ cells and axon tracts are GAD65/67+ with the exception of >10 GFP+ and GAD65/67- cells in the central region of the pallium and near the EmT. All the GAD65/67+ including cells are GFP+ from the base of the Poc out to the eyes and up to the pallium (Figure 3.8C). Slightly further caudal, there are GFP+ and GAD65/67- cells lining the lateral edges of the eyes and at the poc.

Figure 3.7 The *dlx5a/6a* and *dlx1a/2a* CREs target reporter transgenes to Gad65/67 expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 3dpf. (G-I) 3dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. G) anti-GFP H) anti-GAD65/67 I) merged images of both GFP and GAD65/67 (J-L) 3dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain J) anti-GFP K) anti GAD65/67 L) merged images of both anti-GFP and GAD65/67. Green structures are GFP+, red structures are GAD65/67+ and yellow structures are both GFP+ and GAD65/67+. DT: dorsal thalamus, EmT: eminentia thalami, lfb: lateral forebrain bundle, po: preoptic region, pos: postoptic commissure, M3: migrated area of EmT, TeO: tectum opticum. Calibration bar = 20 μ m

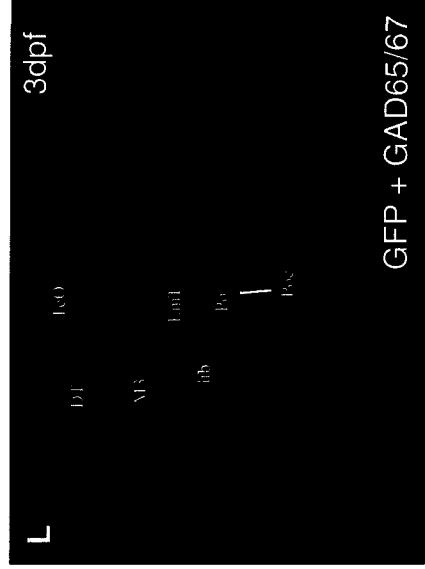
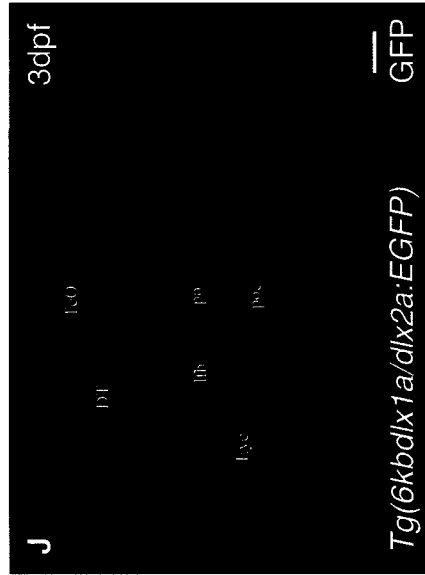
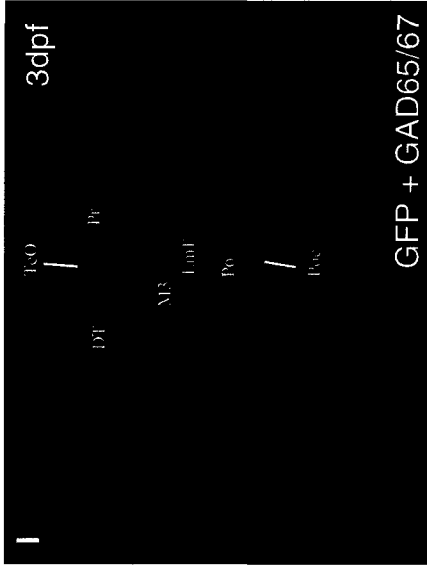
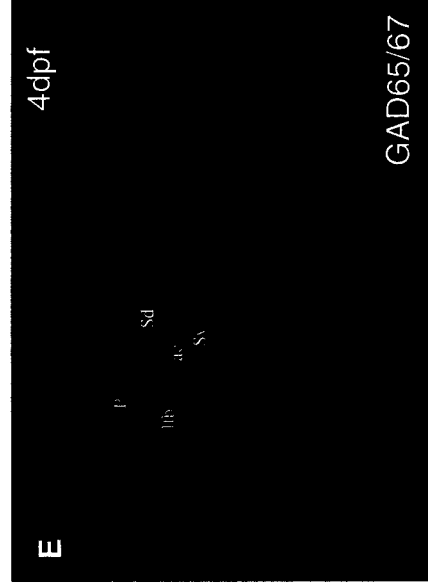
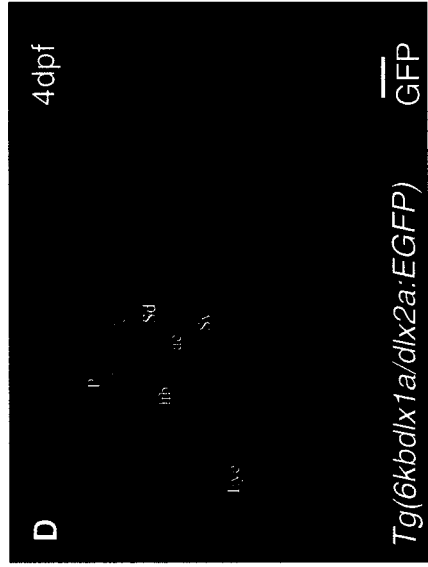
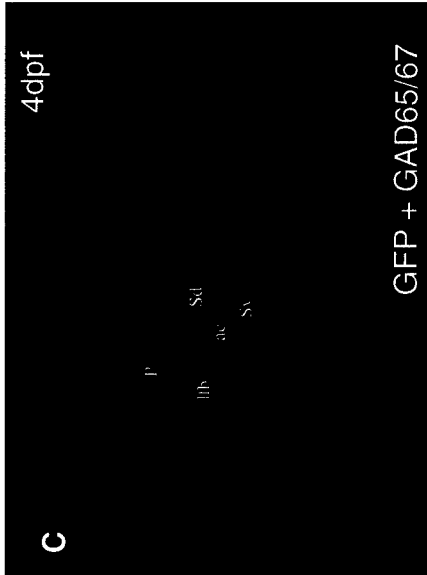
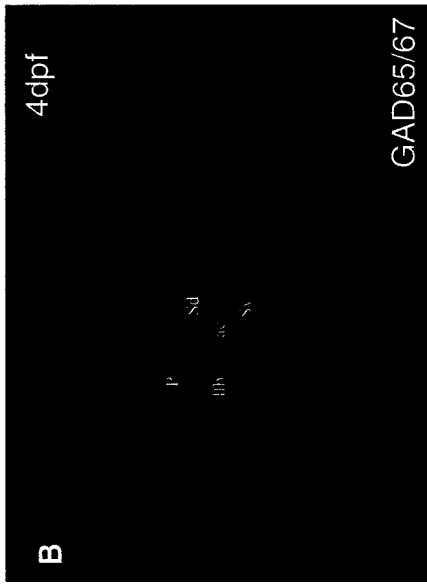
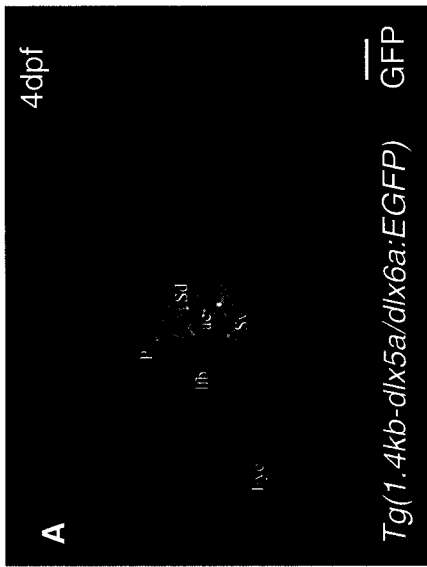


Figure 3.8 The *dlx5a/6a* and *dlx1a/2a* CREs target reporter transgenes to *Gad65/67* expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 4dpf. (A-C) 4dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. A) anti-GFP B) anti-GAD65/67 C) merged images of both GFP and GAD65/67 (D-F) 4dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain D) anti-GFP E) anti GAD65/67 F) merged images of both anti-GFP and GAD65/67. Green structures are GFP+, red structures are GAD65/67+ and yellow structures are both GFP+ and GAD65/67+. ac: anterior commissure, lfb: lateral forebrain bundle, P: pallium, Sd: dorsal region of the subpallium, Dv: ventral region of the subpallium. Calibration bar = 20 μ m



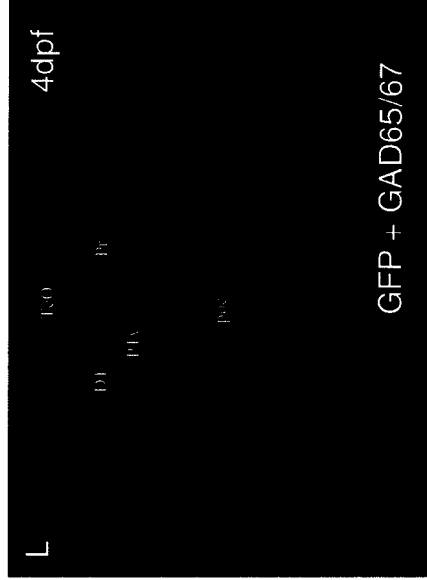
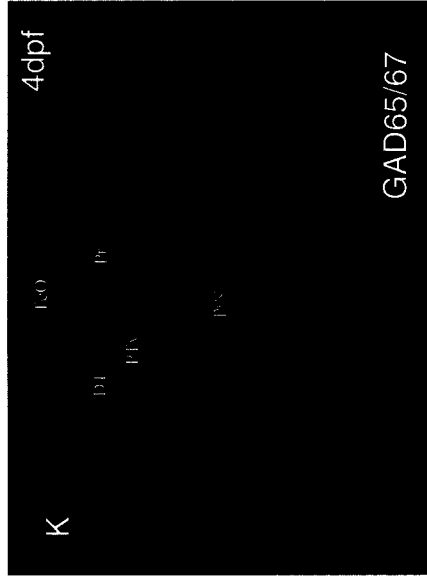
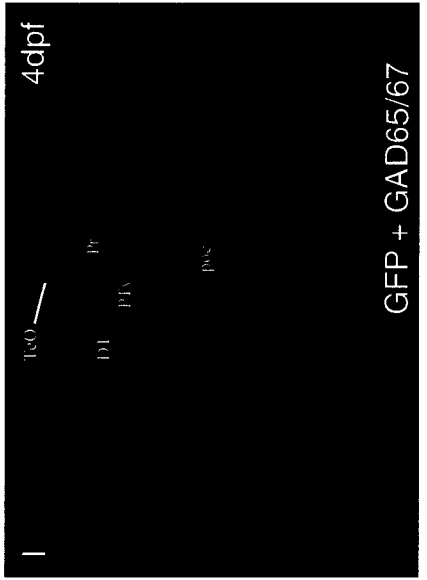
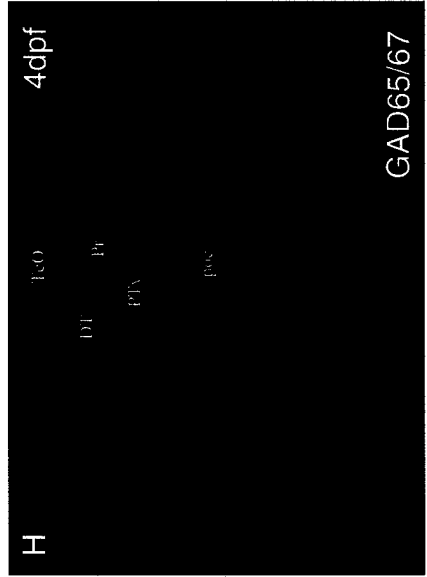
Caudally, most of the GAD65/67+ structures are GFP+ but there are many GFP+ cells that do not co-express with GAD65/67 (Figure 3.9I). The cells are located at the centre near the prethalamus (VT) and at the base of the poc. These subtypes of cells are also seen in the TeO and the Pr and DT (Figure 3.9I).

3.5 The *dlx1a/2a* CREs target transgene expression to *Gad65/67* expressing cells in the *Tg(6kbdlx1a/dlx2a:EGFP)* zebrafish brain

3.5.1 Expression of GAD65/67 and *Tg(6kbdlx1a/dlx2a:EGFP)* in the 2dpf zebrafish brain

At 2dpf, most rostrally, there are several GFP+ and GAD65/67+ cells in the subpallium near the ac. There is also an abundance of GFP+ and GAD65/67+ cells covering the Sv out to the inner edges of the OE and meeting at the ac up until the Pv (Figure 3.4F). This is similar to the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* where virtually all of the GFP positive cells are GAD65/67 positive as well (Figure 3.4C). Above these structures are two horizontal stripes (>100 cells) of GFP+ and GAD65/67- cells stretching from the central pallium near the ac out to the inner edges of the eyes (Figure 3.4F). From the outermost region of these stripes are two uniform stripes of GAD65/67+ and GFP- structures curving dorsally towards the DT. Further caudal to this, the outer GAD65/67+ stripes expand dorsally and laterally and remain GFP-. The cells in the Sv meeting at the ac are almost all GFP+ and GAD65/67+. It must be noted that there are

Figure 3.9 The *dlx5a/6a* and *dlx1a/2a* CREs target reporter transgenes to GAD65/67 expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 4dpf. (G-I) 4dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. G) anti-GFP H) anti-GAD65/67 I) merged images of both GFP and GAD65/67 (J-L) 4dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain J) anti-GFP K) anti GAD65/67 L) merged images of both anti-GFP and GAD65/67. Green structures are GFP+, red structures are GAD65/67+ and yellow structures are both GFP+ and GAD65/67+. DT: dorsal thalamus, poc: preoptic commissure, Pr: pretectum, PTV: ventral part of posterior tuberculum, TeO: tectum opticum. Calibration bar = 20 μ m



many GFP+ cells in the dorsal region of the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line and none observed in the *Tg(6kbdlx1a/dlx2a:EGFP)* (Figure 3.4A, D).

Caudally, there are 3 clusters of GFP+ cells, the lateral clusters containing >50 cells and the ventral cluster >15 (Figure 3.5L). The lateral clusters lie at the inner edges of the eyes on the outer circle of the Po. These clusters appear to contain a faint expression of GAD65/67. The ventral cluster is GFP+ and GAD65/67-. There are GFP+ and GAD65/67+ structures resembling axon tracts that form a circular “O” shaped pattern that connect to each of the GFP+ clusters (Figure 3.5L). In this region of the *Tg(6kbdlx1a/dlx2a:EGFP)* line, most of the cells that are GFP+ are GAD65/67+ and most of the GAD65/67+ cells are GFP+ except for the ventral-most cluster of cells. In the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line, there are many more GFP+ that are also GAD65/67+ as well as many more GFP+ cells occupying the TeO (Figure 3.5I).

Overall, there are few GFP+ and GAD65/67+ cells in the dorsal region of the brain in the *Tg(6kbdlx1a/dlx2a:EGFP)* line and the number of GFP+ cells decreases caudally, whereas there is a much larger number of GFP+ and GAD65/67+ cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line and the number of GFP+ cells caudally is ample (Figure 3.4C, D, Figure 3.5I,L).

3.5.2 Expression of GAD65/67 and *Tg(6kbdlx1a/dlx2a:EGFP)* in the 3dpf zebrafish brain

At 3dpf, rostrally, there is a high incidence of co-expression between GFP and GAD65/67 extending from the subpallium, out to the OR and up to the pallium (Figure 3.6F). This is a similar pattern seen in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line. This pattern continues as a “U” shaped pattern that extends from the base of the Po out to the M3 and

up towards the pallium. There are GFP+ and GAD65/67+ cells occupying the centre of this “U” shape at the Sd yet there are no cells occupying the dorsal region near the pallium (Figure 3.6F).

There is a high incidence of co-expression between GFP and GAD65/67 at the Po up to the EmT, however, at the base of the Po, there are GFP+ and GAD65/67- cells (>15) in a cluster. Caudally, the number of GFP+ cells significantly decreases (approximately 12) and they occupy the ventral region near the poc (Figure 3.7L). This is unlike the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line in which there are many cells in the caudal region of the brain, including the pallium (Figure 3.7I).

The *Tg(6kbdlx1a/dlx2a:EGFP)* line and the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line have similar GFP and GAD65/67 co-expression patterns rostrally, yet caudally the *Tg(6kbdlx1a/dlx2a:EGFP)* line shows a significant decrease in GFP+ throughout the brain as well as no GFP+ cells in the dorsal region of the pallium (Figure 3.6C, F, Figure 3.7 I,L). This is unlike the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line in which there are many GFP+ and GAD65/67 cells and axon tracts occupying the caudal region of the brain and the pallium (Figure 3.7I).

3.5.3 Expression of GAD65/67 and *Tg(6kbdlx1a/dlx2a:EGFP)* in the 4dpf zebrafish brain

At 4dpf, rostrally, there is a high incidence co-expression between GFP+ and GAD65/67+ cells (Figure 3.8F). These structures stretch along the areas from the subpallium out to the OE and up to the pallium. This co-expression continues to the ac out to the M4 and up to the pallium, leaving just several (2-3) GFP+ and GAD65/67- cells in the latero-dorso pallium. There appear to be no GFP+ and GAD65/67+ cells

occupying the the pallium (P), or TVe (Figure 3.8F). GFP+ and GAD65/67+ cells appear to migrate in a ventral to dorsal manner along the lateral edges of the M4 region. This is a similar co-expression pattern as seen in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line (Figure 3.8C).

At the midbrain and further caudally, there is a low occurrence of co-expression between GAD65/67 and GFP from the Po up to the TeO (Figure 3.9L). The same co-expression is seen at the lateral edges of these structures but appears as a more uniform pattern spanning on each side from the lateral edges of the poc up the lfb to the M3 near the TeO. This is unlike the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line in which there are many GFP+ and GAD65/67+ cells occupying the dorsal region of the brain (Figure 3.9I).

It appears that the co-expression patterns of GFP and GAD65/67 positive cells in the *Tg(6kbdlx1a/dlx2a:EGFP)* line resembles the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line from the rostral portion of the brain to the midbrain, however, these expression patterns are different from the midbrain to the caudal region (Figure 3.8C, F, Figure 3.9I, L). The *Tg(6kbdlx1a/dlx2a:EGFP)* line has no GFP+ cells occupying the dorsal region of the brain, whereas the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line has many cells occupying this region (Figure 3.8A, D, Figure 3.9G, J).

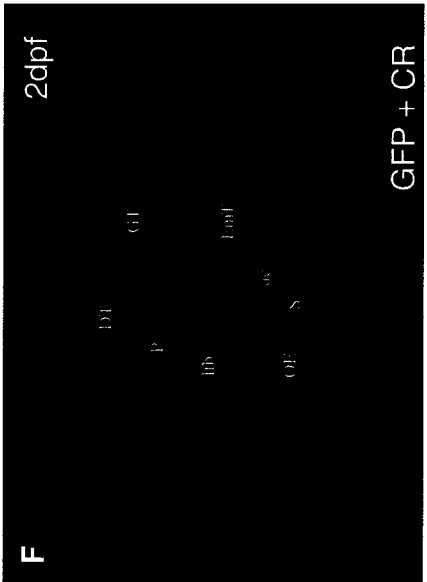
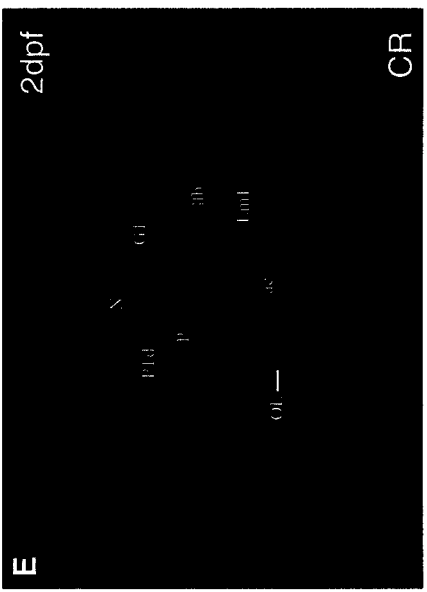
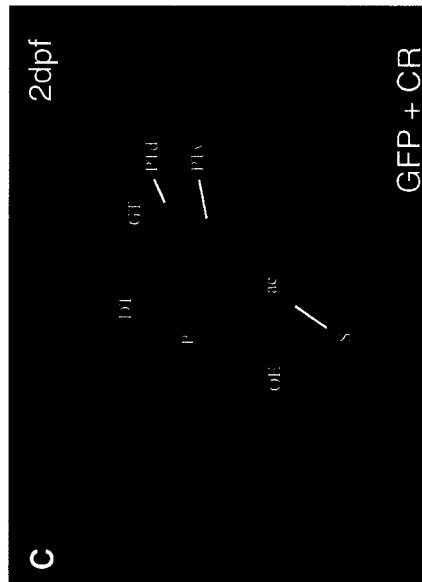
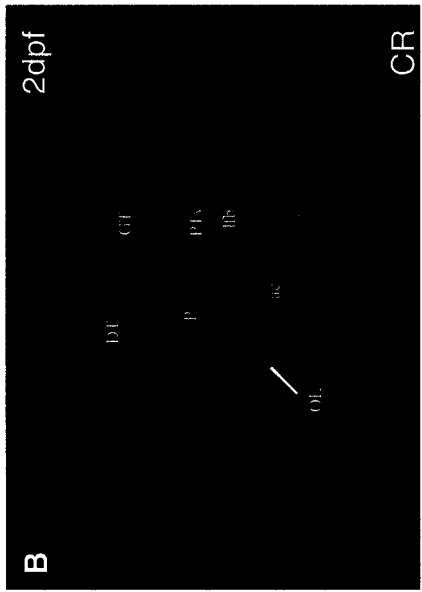
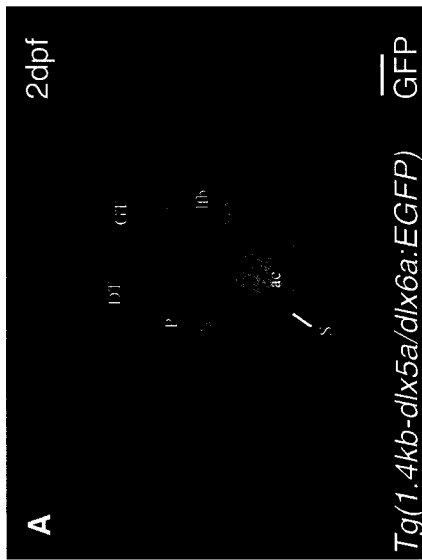
3.6 Detailed characterization of Calretinin in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* zebrafish and the *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain via immunohistochemistry

It is known that interneurons can express various neurotransmitters including GABA and calretinin. There may be many parameters to distinguish one subtype of interneuron from another; therefore, a marker such as calretinin is used in tandem with a marker such as GABA to identify one particular subtype of interneuron. In mice, it has been shown that the *Dlx* enhancer elements, URE2, I12b, and I56i are active in various subtypes of interneurons, with calretinin defining a major subtype (Anderson, Eisenstat et al. 1997; Stuhmer, Anderson et al. 2002; Stuhmer, Puelles et al. 2002). A characterization of calretinin has been performed on the adult zebrafish brain but little on the developing zebrafish brain (Castro et al., 2006). In this study, calretinin immunohistochemistry was performed followed by fluorescence microscopy to determine whether the *Dlx5a/6a* and *Dlx1a/2a* CREs target transgene expression to cells that also express calretinin. A description of the calretinin marker has been completed. If the cells are GFP+, GAD65/67+ and CR+, we can determine that the cells, in which the enhancer(s) is (are) active are GABAergic interneurons of a calretinin-expressing subtype.

3.6.1 2dpf

At 2dpf, rostrally, CR+ cells (>75) are scattered throughout the subpallium and extend up towards the pallium at the lateral edges of the DVe. There is also an abundance of CR+ cells occupying the OE (Figure 3.10B, E of Panel A-F). There are several (>10) CR+ cells in the DT and an abundance in the subpallium. There are CR+ cells spanning

Figure 3.10 The *dlx5a/6a* and *dlx1a/2a* CREs target reporter transgenes to calretinin expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 2dpf. (A-C) 2dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. B) anti-GFP B) anti-CR C) merged images of both GFP and CR (D-F) 2dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain D) anti-GFP E) anti-CR F) merged images of both GFP and CR. Green structures are GFP+, red structures are CR+ and yellow structures are both GFP+ and CR+. ac: anterior commissure, DT: dorsal thalamus, GT: griseum tectale, lfb: lateral forebrain bundle, N: region of the nucleus of medial longitudinal fascicle, OE: olfactory epithelium, P: pallium, PTd: dorsal region of posterior tuberculum, PTv: ventral region of posterior tuberculum, S: subpallium. Calibration Bar = 20 μ m



two horizontal stripes from the outer portion of the DT up towards the GT (Figure 3.10B, E).

At the midbrain, the number of CR+ cells decreases. There are several CR+ cells (>20) occupying a circular “O” shaped pattern from the lateral edges of the subpallium out to the lateral edges of the pallium near the eyes. Above these cells are CR+ cells (>20) lining the inner edges of the eyes dorsally towards the PTd and in towards the DT. There are several (approximately 15) CR+ cells near the ac and CR+ cells (>20) occupying the N.

Caudally, there are >20 CR+ cells, occupying the PTv and the Hr/Hi region of the brain (Figure 3.11H, K). There are also CR+ structures resembling axon tracts occupying this area. There are >6 CR+ cells scattered in the central dorsal region of the TeO that appear to migrate in a ventral to dorsal manner (Figure 3.11H, K).

3.6.2 3dpf

At 3dpf, rostrally, there are two large clusters of CR+ cells in each of the OE regions. Next to these cells are many (>100) CR+ cells spanning across the Sv, out to the OR and up to the pallium (Figure 3.12 B, E of Panel A-F). This continues medially until there is a “U” shaped uniform CR+ pattern spanning from the base of the ac out to the lateral inner edges of the eyes at the M4 and up towards the pallium (Figure 3.12E).

More caudally, the number of CR+ cells increases significantly (>250), occupying the brain from the po out to the lfb and up towards the DT (Figure 3.13H, K of Panel G-L). There are two horizontal stripes of CR+ cells extending from the inner PTv/PTd region out to the lateral edges near the lfb. It also appears at the inner lateral edges of the

Figure 3.11 The *dlxa5/6a* and *dlx1a/2a* CREs target reporter transgenes to calretinin expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain at 2dpf. (A-C) 2dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. A) anti-GFP B) anti-CR C) merged images of both anti-GFP and CR (D-F) 2dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain D) anti-GFP E) anti-CR F) merged images of both anti-GFP and CR. Green structures are GFP+, red structures are CR+ and yellow structures are both GFP+ and CR+. lfb: lateral forebrain bundle, Hi: intermediate hypothalamus, Hr: rostral hypothalamus, N: region of the nucleus of medial longitudinal fascicle, Ptd: dorsal part of posterior tuberculum, PTV: ventral part of posterior tuberculum, TeO: tectum opticum. Calibration Bar = 20 μ m

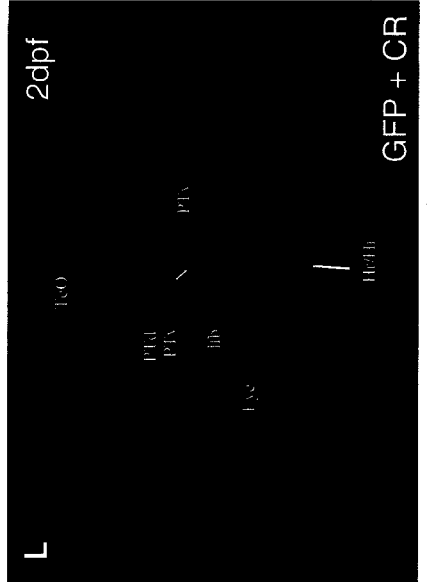
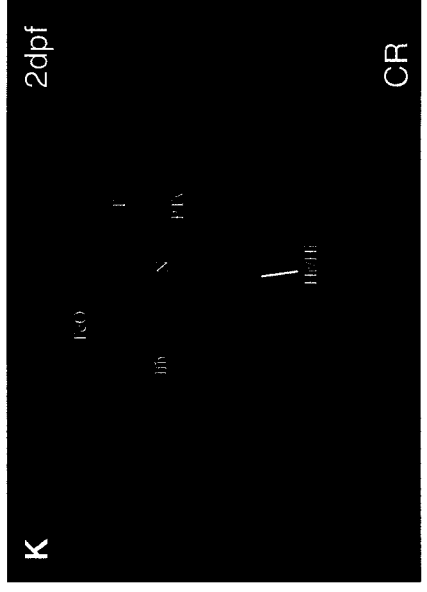
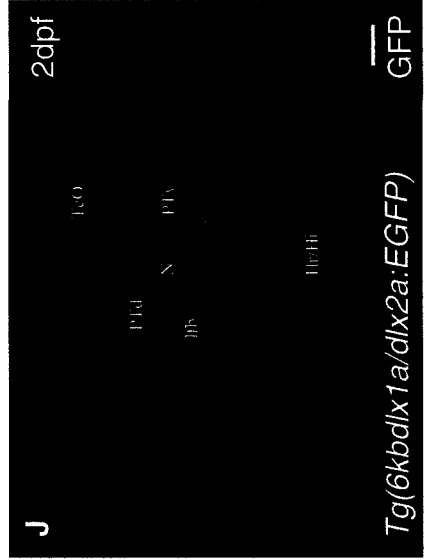
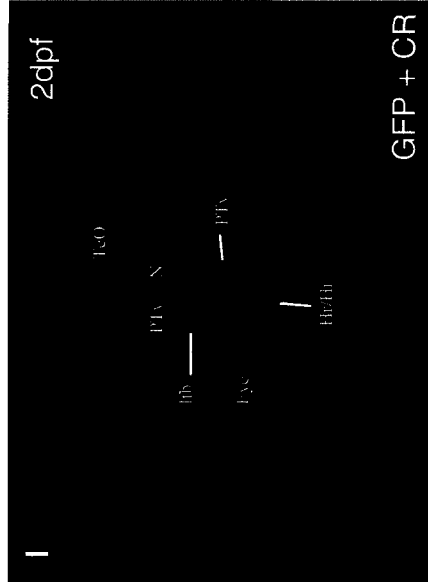
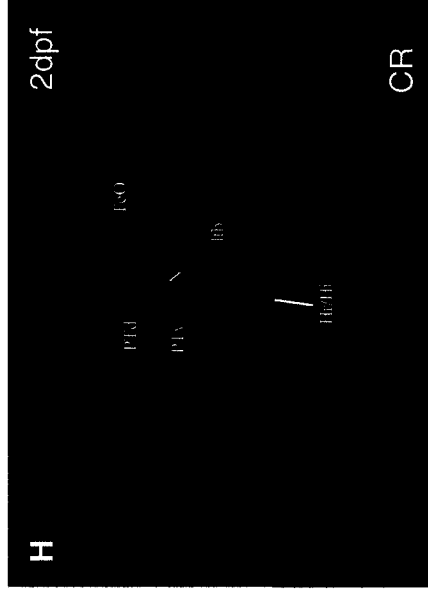
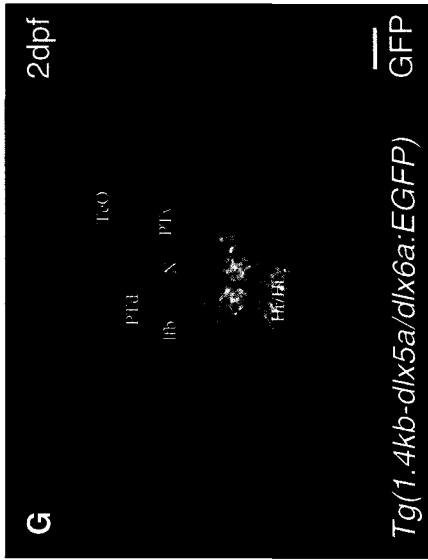
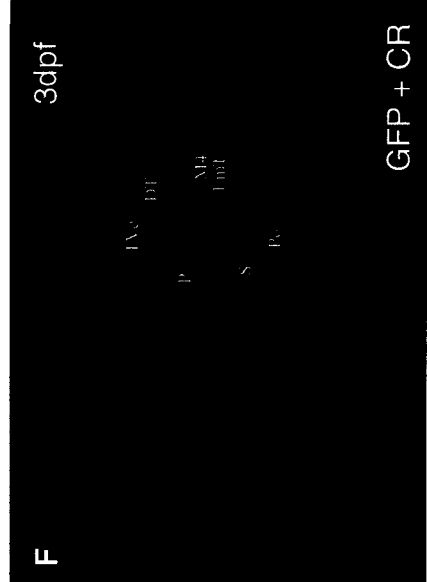
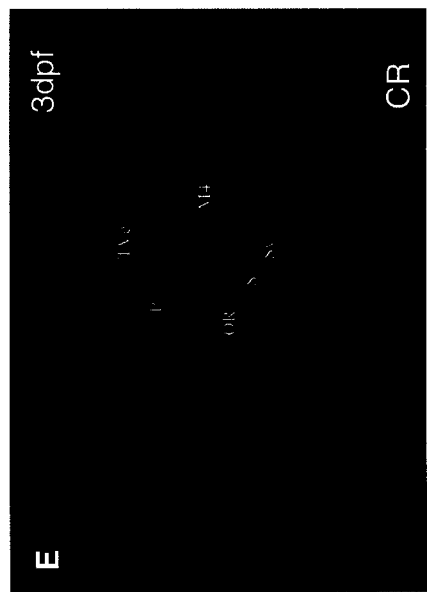
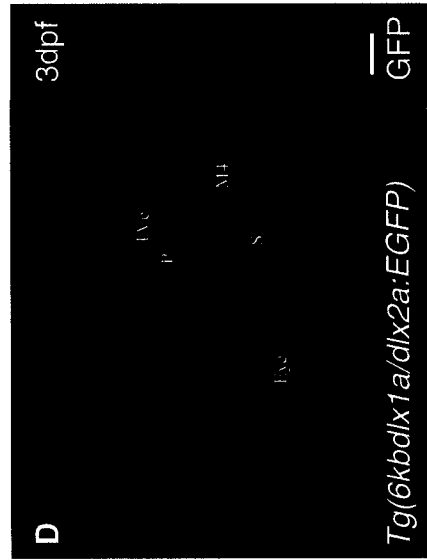
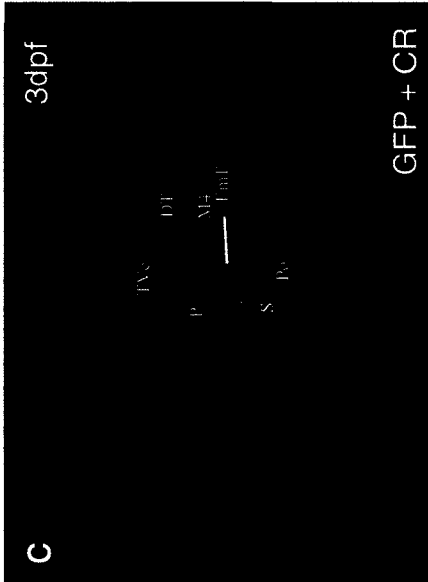
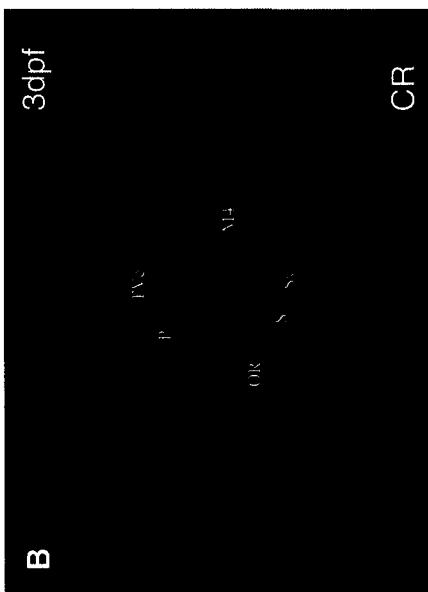
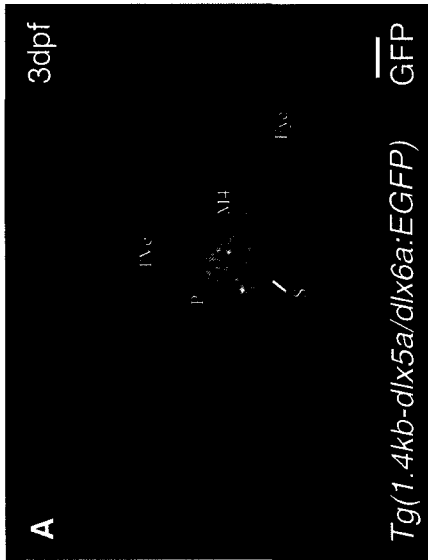


Figure 3.12 The *dlx5a/6a* and *dlx1a/2a* CREs target reporter transgenes to calretinin expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 3dpf. (A-C) 3dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain. A) anti-GFP B) anti-CR C) merged images of both anti-GFP and CR (D-F) 3dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain D) anti-GFP E) anti-CR F) merged images of both anti-GFP and CR. Green structures are GFP+, red structures are CR+ and yellow structures are both GFP+ and CR+. DT: dorsal thalamus, EMT: eminentia thalami, M4: telencephalic migrated area, OR: optic region, P: pallium, po: preoptic region, poc: postoptic commissure, S: subpallium, Sv: ventral region of the subpallium, Tve: telencephalic ventricle. Calibration Bar = 20 μ m



eyes, near the M3, that the cells are migrating in a ventral to dorsal manner to the most dorsal part of the brain and then curve medially towards the DT (Figure 3.13H, K) .

3.6.3 4dpf

At 4dpf, rostrally, there is an abundance of CR+ cells extending from the base of the subpallium out to the OE and up to the TVe and pallium (Figure 3.14B, E of Panel A-F). This pattern continues towards the midbrain. There is an abundance of CR+ cells occupying the M4 that appear to migrate in a ventral to dorsal manner up towards the TVe. There many CR+ cells (>100) occupying a more central region near the Sd in a ventral to dorsal manner (Figure 3.14B, E).

Caudally, the number of CR+ cells increases significantly (>250) in the dorsal region of the brain (Figure 3.15H, K of Panel G-L). At the lateral edges of the pallium, there is an abundance of CR+ cells that are near the dorsal inner edge of the eyes along the dorsal portion of the brain, medially towards the epiphysis (E). From the bottom of these two groups of cells are two horizontal stripes of CR+ cells spanning from the lateral edges of the brain near the M3 and inwards towards the VT. There are many CR+ (>100) cells occupying the dorsal region of the brain (Figure 3.15H, K).

3.7 The *dlx5a/6a* CREs target transgene expression to interneurons of the calretinin subtype in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* zebrafish brain

It has been shown that calretinin marks a major subtype of GABAergic interneurons in the mouse brain (Stuhmer, Anderson et al. 2002; Stuhmer, Puelles et al. 2002; Ghanem, Jarinova et al. 2003; Ghanem, Yu et al. 2007; Ghanem, Yu et al. 2008)

Figure 3.13 The *dlx5a/6a* and *dlx1a/2a* CREs target reporter transgenes to calretinin expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 3dpf. (G-I) 3dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain G) anti-GFP H) anti-CR I) merged images of both anti-GFP and CR (J-L) 3dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain J) anti-GFP K) anti-CR L) merged images of both anti-GFP and CR. Green structures are GFP+, red structures are CR+ and yellow structures are both GFP+ and CR+. DT: dorsal thalamus, EmT: eminentia thalami, Hi: intermediate hypothalamus, Hr: rostral hypothalamus, lfb: lateral forebrain bundle, M3: migrated area of EmT, po: preoptic region, PTd: dorsal part of posterior tuberculum, PTv: ventral part of posterior tuberculum, po: preoptic region, poc: postoptic commissure, TeO: tectum. Calibration Bar = 20 μ m

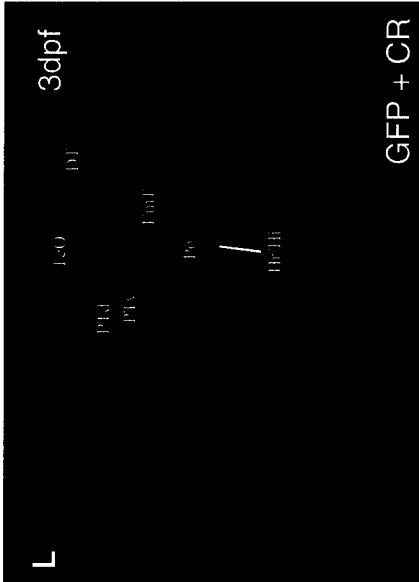
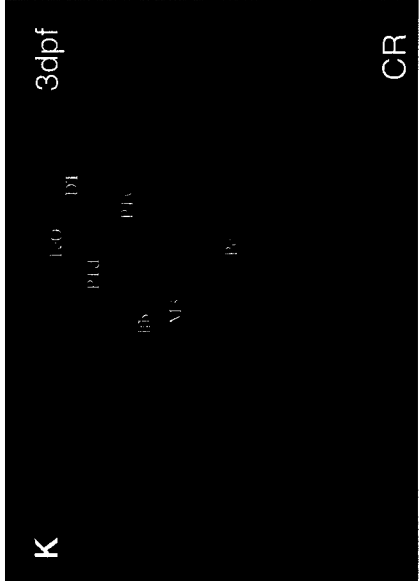
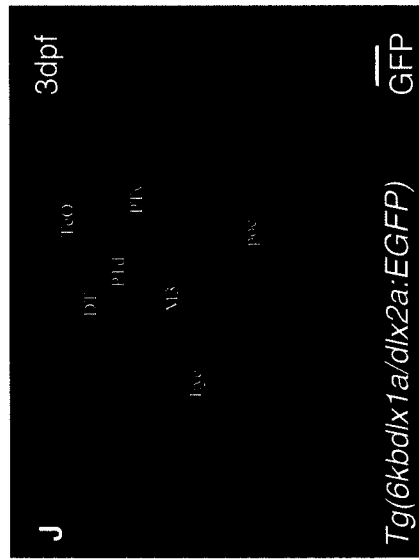
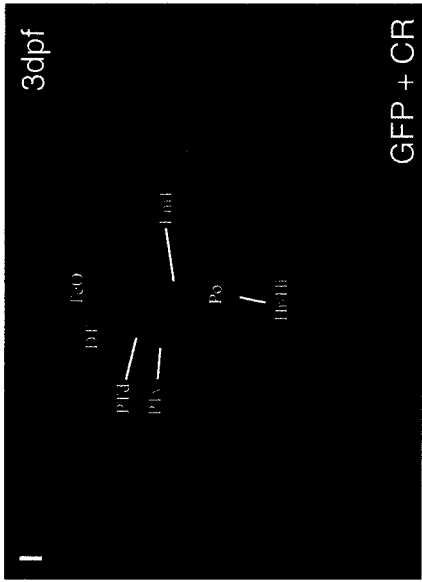
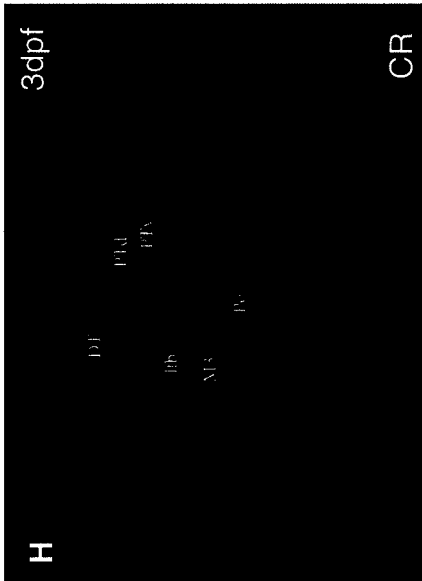
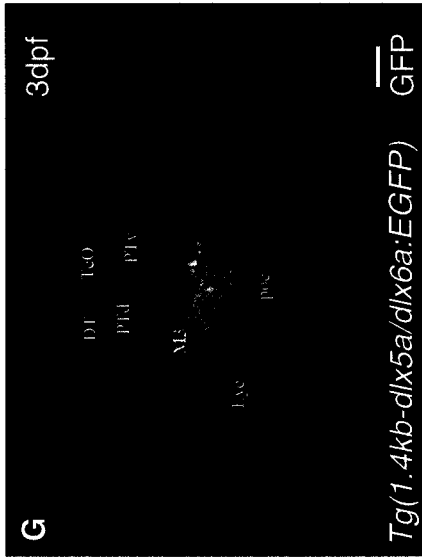


Figure 3.14 The *dlx5a/6a* and *dlx1a/2a* CREs target reporter transgenes to calretinin expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 4dpf. (A-C) 4dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain A) anti-GFP B) anti-CR C) merged images of both anti-GFP and CR (D-F) 4dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain D) anti-GFP E) anti-CR F) merged images of both anti-GFP and CR. Green structures are GFP+, red structures are CR+ and yellow structures are both GFP+ and CR+. ac: anterior commissure, P: pallium, Sd: dorsal region of the subpallium, Sv: ventral region of the subpallium. Calibration Bar = 20 μ m

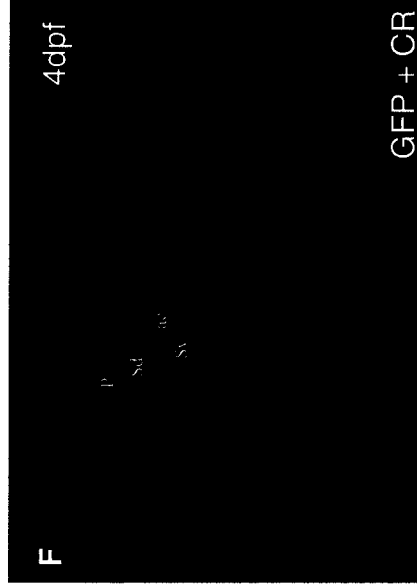
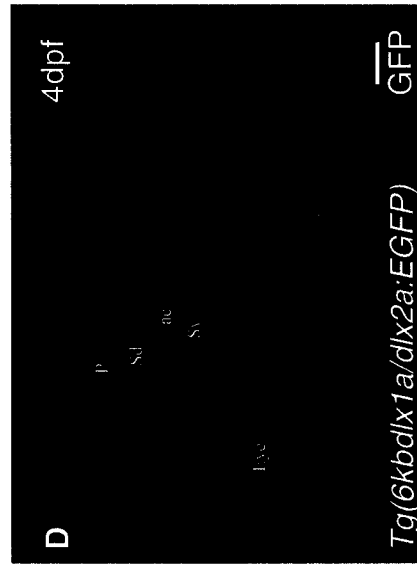
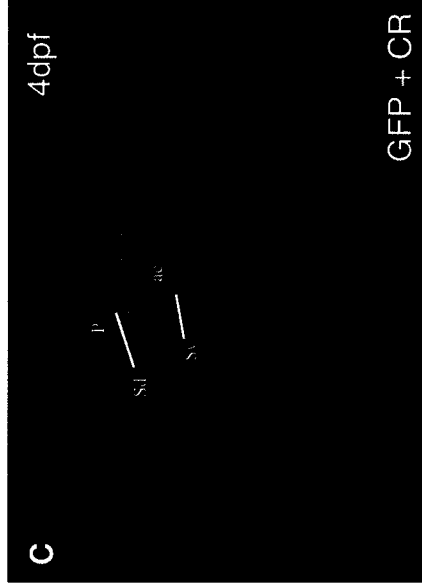
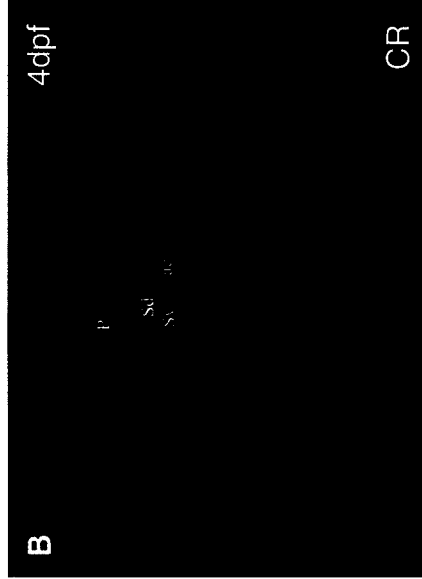
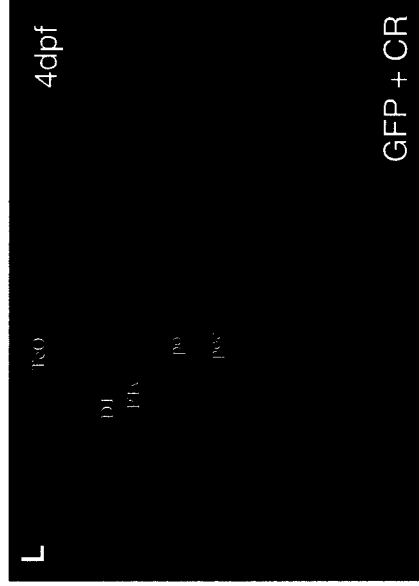
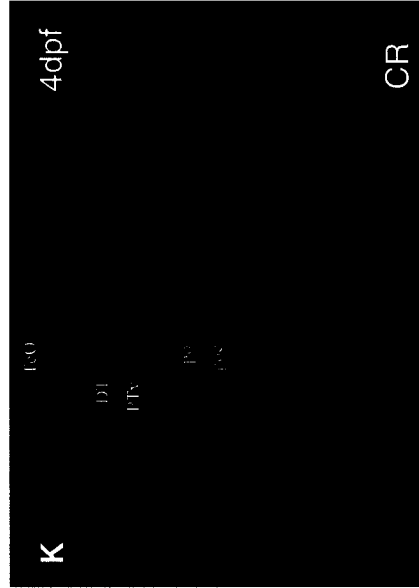
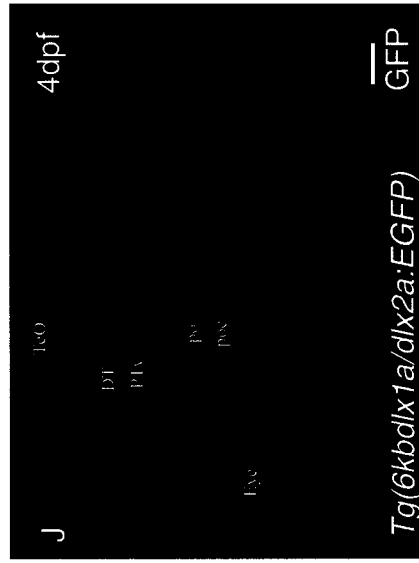
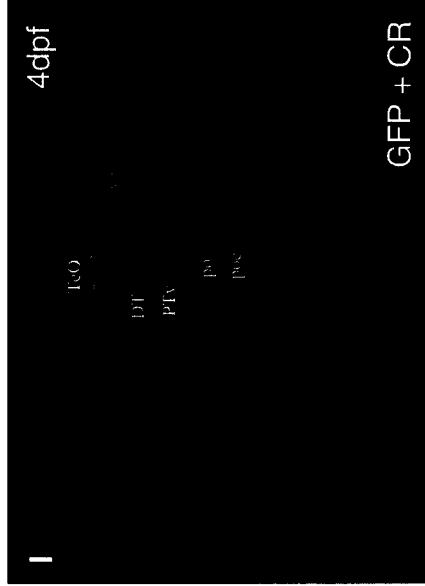
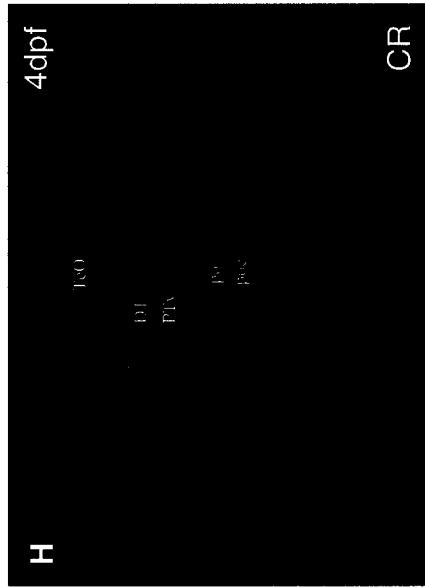


Figure 3.15 The *dlx5a/6a* and *dlx1a/2a* CREs target reporter transgenes to calretinin expressing cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)* zebrafish brain, respectively, at 4dpf. (G-I) 4dpf *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line sections (10 μ m) of the zebrafish brain G) anti-GFP H) anti-CR I) merged images of both anti-GFP and CR (J-L) 4dpf *Tg(6kbdlx1a/dlx2a:EGFP)* line sections (10 μ m) of the zebrafish brain J) anti-GFP K) anti-CR L) merged images of both anti-GFP and CR. Green structures are GFP+, red structures are CR+ and yellow structures are both GFP+ and CR+. DT: dorsal thalamus, po: preoptic region, poc: postoptic commissure, PTd: dorsal part of posterior tuberculum PTv: ventral part of posterior tuberculum, TeO: tectum opticum. Calibration Bar = 20 μ m



The enhancers I12b and I56i have also been shown to be active in the majority of CR+ GABAergic interneurons, >93% and >90%, respectively. In order to determine whether the enhancers I56i, I56ii, I12a, and I12b are active in CR+ GABAergic cells in the developing zebrafish brain, immunohistochemistry has been performed on both lines of transgenic zebrafish and analyzed via fluorescent microscopy. A comparison between both lines was then performed.

3.7.1 Expression of CR and *Tg(1.4kb-dlx5a/dlx6a:EGFP)* in the 2dpf zebrafish brain

At 2dpf, rostrally, the majority of the GFP+ cells spanning from the base of the subpallium out to the OE and up to the pallium are CR- (Figure 3.10C). There are, however, >15 GFP+ and CR+ cells scattered in this region. Dorsally, above the eyes are several (approximately 2-3) GFP+ and CR+ cells near the lateral edges close to the DT along with small clusters of GFP- and CR+ cells (Figure 3.10C). There are a few GFP+ and CR+ cells at the lateral-most edges of the DT in the dorsal region. Slightly further caudal, the number of cells in the subpallium to pallium region co-expressing GFP and CR increases substantially with >100 cells scattered in this area. Above this region are two horizontal stripes of cells with a high incidence of GFP and CR co-expression spreading from the centre of the pallium lateral towards the edges of the eyes. Above these bands of cells are two clusters of CR+ and GFP- cells near the DT (Figure 3.10C). There are also several (>20) CR+ and GFP- cells scattered throughout the subpallium and the pallium (Figure 3.10C).

At the midbrain, there are >20 GFP- and CR+ cells scattered around the region of the PTd and PTv. More ventrally are >20 CR+ cells in a circular “O” shape near the EmT

around the Po. Approximately 10 of these cells are GFP+ and CR+ and the other 10 are GFP- and CR+. The cells near the dorsal region of the brain at the lateral edges of the DT increase in number as two clusters appear to migrate in a ventral to dorsal manner. About 50% of these cells are GFP+ and CR+ and the other 50 % are GFP- and CR+.

Further caudal, there are >15 CR+ cells lining the top of the Hr/Hi region near the lateral edges of the PTv (Figure 3.11IC). All of these cells appear to be CR+ and GFP+. There are >20 GFP+ and CR+ cells lining the base of the T in a horizontal scattered stripe reaching laterally near the inner edges of the eyes. Approximately 6 CR+ and GFP- cells occupy the TeO and appear to follow a ventral to dorsal migration (Figure 3.11I).

Most of the GFP+ cells are CR-, yet most of the CR+ cells are GFP+ (Figure 3.10A, C, Figure 3.11G, I). The CR+ cells in the dorsal region of the brain are mostly GFP- and the CR+ cells in the ventral region including the subpallium are GFP+ at 2dpf.

3.7.2 Expression of CR and *Tg(1.4kb-dlx5a/dlx6a:EGFP)* in the 3dpf zebrafish brain

At 3dpf, rostrally, there are many (>50) cells that co-express GFP and CR scattered around the region of the subpallium, out laterally to the OE and up dorsally to the pallium (Figure 3.12C). Most of the CR+ cells are GFP+ and there are many GFP+ cells that are CR-. There are many (>100) CR+ cells that are GFP-, clustered in the OE. GFP+ and CR+ cells are scattered from the subpallium up to the pallium (Figure 3.12C).

At the midbrain, there are many GFP+ and CR+ cells covering from the base of the Po out towards the inner edges of the eyes, up to the PTd and DT, but not quite reaching the TeO. At the base of the Po, there are many GFP+ and CR+ structures resembling cells and axon tracts that form a “U” shaped pattern spanning out laterally to

the inner edges of the eyes near the EmT, up dorsally along the lfb, and stopping near the PTd.

Caudally, the number of CR+ cells increases significantly (>200) (Figure 3.13H). There is significant co-expression of GFP and CR in the ventral region of the brain at the Hr/Hi, out to the eyes, up dorsally towards the PTv and up to the T (Figure 3.13I). There are also several GFP- and CR+ cells (>20) in the ventral region near the inner edges of the eyes as well as two horizontal stripes of cells spanning from the centre region of the T and branching out towards the basal optic tectum (b) in which the cell number increases further caudally. There are approximately 7-10 GFP+ and CR+ cells occupying the centre, dorsal region of the TeO as well as the lateral edges near the b and increases further caudally (>20) (Figure 3.13I). The number of CR+ and GFP- cells in this region also increases (>50) further caudally (Figure 3.13I).

There is some co-expression of GFP and CR in the ventral region of the rostral, medial, and caudal portion of the brain at 3dpf. There are also many cells occupying the dorsal region of the brain that co-express CR and GFP at 3dpf (Figure 3.12A, C, Figure 3.13 G, I).

3.7.3 Expression of CR and *Tg(1.4kb-dlx5a/dlx6a:EGFP)* in the 4dpf zebrafish brain

At 4dpf, rostrally, virtually all the CR+ cells are GFP+ from the Sv out laterally to the OE and up dorsally to the pallium (Figure 3.14C). There are GFP+ and CR- cells in this region as well, but it must be noted that the number of GFP+ cells greatly outnumber the number of CR+ cells. The CR+ cells that are scattered around the PTv

out laterally to the DT and up to the Pr show a high co-expression of GFP. (Figure 3.14C).

At the midbrain, there are scattered CR+ cells that spread from the poc, dorsally through the PTv, DT and Pr all the way to the TeO. These cells are virtually all GFP+. Near the TeO, there are >20 GFP+ and CR+ cells that appear to migrate in a ventral to dorsal manner with the axons extending to the lateral edges of the TeO. This can be seen by the axon tracts that are GFP+ and CR+ migrating from the poc, out to the inner edges of the eyes and up dorsally towards the TeO and inwards where the cells lie.

Caudally, the number of GFP+ cells occupying the most dorsal region and lateral edges of the TeO increases significantly (Figure 3.15G). Virtually all of these GFP+ structures are CR+ (Figure 3.15I). There are GFP+ and CR+ cells lining the ventral region of the poc and Hr in a “U” shaped pattern that stretches out to the inner edges of the eyes and up dorsally towards the TeO. Most of these cells and axon tracts appear to be migrating in a ventral to dorsal manner as well as towards the midline of the brain near the Pr from the lateral-most edges of the TeO (Figure 3.15I).

3.8 The *dlx1a/2a* CREs target transgene expression to interneurons of the calretinin subtype in the *Tg(6kbdlx1a/dlx2a:EGFP)* zebrafish brain

In this study, observations show that the *dlx1a/2a* CREs target very minimal reporter transgene expression to the dorsal region of the developing zebrafish brain between 2dpf and 4dpf. Since observations from the anatomical description of calretinin, as seen above, indicates that the majority of CR+ cells appear from the midline to the dorsal region of the brain, there is expected to be little co-expression between GFP and

CR in the *Tg(6kbdlx1a/dlx2a:EGFP)* line between 2dpf and 4dpf. Below is a summary of expression patterns observed in the *Tg(6kbdlx1a/dlx2a:EGFP)* line in comparison to what is observed in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line.

3.8.1 Expression of CR and *Tg(6kbdlx1a/dlx2a:EGFP)* in the 2dpf zebrafish brain

At 2dpf, rostrally, the GFP+ cells occupying the subpallium out to the inner edges of the eyes and up to the pallium are CR- (Figure 3.10F). There are two horizontal stripes of CR+ cells that are all GFP- (>50) extending from the centre of the PTd and branch out laterally and curve up slightly dorsally along the lfb (Figure 3.10F). This continues into the midbrain. This observation is different than the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line in which there are cells that co-express GFP and Cr in the subpallium out laterally to the inner edges of the eyes and appear to migrate in ventral to dorsal manner from the subpallium to the pallium (Figure 3.10C, F).

From the midbrain to the caudal portion of the brain, the CR pattern is consistent with the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line (Figure 3.11I, L). There are no GFP+ cells in the dorsal region of the brain at the level of the TeO and there is very little overlap between the CR+ and GFP+ cells at any point (Figure 3.11L). There is slight overlap between GFP and CR near the region of the PTv and PTd out towards the lfb (Figure 3.11I).

The *Tg(6kbdlx1a/dlx2a:EGFP)* line shows little co-expression between GFP and CR through most parts of the 2dpf brain, whereas the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line shows a high incidence of co-expression through the rostral, medial, and caudal portions of the 2dpf brain.

3.8.2 Expression of CR and *Tg(6kbdlx1a/dlx2a:EGFP)* in the 3dpf zebrafish brain

At 3dpf, rostrally, there are approximately 50 GFP+ and CR+ cells scattered from the base of the subpallium near the ac out to the M4 and not reaching the pallium, dorsally (Figure 3.12F). There is also a “U” shaped pattern of co-expressing GFP+ and CR+ cells spanning from the base of the ac, out to the M4 and dorsally to about halfway up the inner edges of the eyes. Unlike the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line, many of the CR+ cells are not GFP+ in the ventral region and since there are much less GFP+ cells, there are also much less GFP+ and CR+ cells (Figure 3.12C, F).

At the midbrain, there are virtually no cells co-expressing GFP+ and CR+ from the Po out to the lfb up to the VT and DT. The GFP+ cells that are CR- are concentrated from the base of the Po and line up the midline dorsally to the EmT. The CR+ cells that span two horizontal stripes from the centre of the dorsal part of the EmT out to the VT and line the lateral-most region of the dorsal part of the brain are all GFP-. This is unlike the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line in which there are many cells co-expressing GFP and CR in both the ventral and dorsal region.

Caudally, there is a significant increase in CR+ cells, as seen in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line, however, there is also a significant decrease in GFP+ cells (Figure 3.13G, H, J, K). There is also virtually no co-expression between GFP and CR in any of the cells in the caudal portion of the brain (Figure 3.13L). This is in contrast to the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line, in which there are many co-expressing GFP and CR cells in all the regions of the caudal portion, including the dorsal area (Figure 3.13I).

The line *Tg(6kbdlx1a/dlx2a:EGFP)* shows very little co-expression between GFP and CR in the rostral region of the brain and virtually no co-expression more caudally

(Figure 3.12F, Figure 3.13L). This is in contrast to the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line in which there is a large amount of co-expression of GFP and CR in the rostral portion of the brain as well as some co-expression more caudally, including the dorsal area of the brain (Figure 3.12C, Figure 3.13I).

3.8.3 Expression of CR and *Tg(6kbdlx1a/dlx2a:EGFP)* in the 4dpf zebrafish brain

At 4dpf, rostrally, most of the CR+ cells that are scattered from the base of the subpallium out to the OE and up to the pallium are GFP+ (Figure 3.13F). This continues to the point where there is co-expression of GFP and CR from the base of the ac out laterally to the M4 and up to the Tve. This is consistent with the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line since there is co-expression between CR and GFP in virtually all the cells ventrally and dorsally in the rostral portion of the brain (Figure 3.13C). There are, however, CR+ cells that are GFP- in the most dorsal region of the pallium in the *Tg(6kbdlx1a/dlx2a:EGFP)* line (Figure 3.13F). This is in contrast to the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line, in which there are dorsally located CR+ and GFP+ cells in the rostral regions of the brain (Figure 3.13C).

Further caudally, there is little co-expression between CR and GFP from the base of the Po and up the centre region through the Sd towards the pallium (Figure 3.15L). These cells are mostly GFP+ and CR-, whereas the lateral regions from the lateral edges of the Po out to the M3 and up to the dorsal region of the pallium are CR+ and GFP-. There is some co-expression (approximately >30 cells) of GFP and CR near the central region of the EmT that extends laterally towards the lfb (Figure 3.15L). Dorsally, near the

P and T_{Ve}, the CR⁺ cells are virtually all GFP⁻, it must be noted that there are almost no GFP⁺ cells in the dorsal region of the brain at this point in development (Figure 3.15J,L). This is in contrast to the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line in which there are many CR⁺ and GFP⁺ cells that appear to migrate in a ventral to dorsal manner to occupy the pallium (Figure 3.15I).

Dorsally, the number of CR⁺ cells increases significantly, similar to what is observed in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line, however, the number of GFP⁺ cells decreases significantly (Figure 3.15I, J, L). This leaves for almost no co-expression of GFP and CR in the dorsal region (Figure 3.14L). Ventrally, however, there is some overlapping expression patterns of cells in a “U” shaped pattern extending from the Po out to the lfb (Figure 3.15L).

The *Tg(6kbdlx1a/dlx2a:EGFP)* line is similar to the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line at the rostral region of the brain where there is similar patterns of co-expression of CR and GFP (Figure 3.14C, F). From the midbrain to the caudal portion of the brain, however, the line is different than the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line. The *Tg(6kbdlx1a/dlx2a:EGFP)* line shows almost no co-expression between CR and GFP in the dorsal region of the brain, whereas the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line shows a high incidence of co-expression between GFP and CR cells dorsally (Figure 3.15I, L).

4. DISCUSSION

4.1 The CREs between *dlx1a* and *dlx2a* and the CREs between *dlx5a* and *dlx6a* target the reporter transgene (GFP) differentially in the developing zebrafish brain

In this study, the activity of the CREs between *dlx5a* and *dlx6a* and the CREs between *dlx1a* and *dlx2a* were compared using a reporter transgene in the developing brains of the transgenic zebrafish lines *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kb-dlx1a/dlx2a:EGFP)*, respectively.

At 2dpf, there are many GFP positive cells occupying the subpallium that appear to travel via the marginal regions, dorsally towards the pallium. There are overlapping patterns of reporter transgene expression in the ventral regions of the forebrain at the level of the subpallium between both lines. There appears to be many more GFP positive cells in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line compared to the *Tg(6kb-dlx1a/dlx2a:EGFP)* line, in which there are virtually no GFP positive cells in the pallium. It cannot be distinguished as to which specific enhancer is active in these cells since the transgenic lines were generated to report both the I56i and I56ii in the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line and I12a and I12b in the *Tg(6kb-dlx1a/dlx2a:EGFP)* line. Previous studies, however, show that the mouse I56i enhancer and the zebrafish I56i enhancer targets expression of *lacZ* in transgenic mice and zebrafish to the forebrain with higher efficiency than the I56ii enhancer alone (Zerucha, Stuhmer et al. 2000; Ghanem, Jarinova et al. 2003). Furthermore, in mice, *I12b-lacZ* and *I56i-lacZ* display nearly indistinguishable reporter expression patterns at E11.5 and E12.5 in the subpallium of transgenic mice (Ghanem, Yu et al. 2007). This could account for the overlapping patterns

of expression observed in the subpallium between both lines since the I56ii enhancer contributes to the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line transgene expression and the I12b enhancer contributes to the *Tg(6kb-dlx1a/dlx2a:EGFP)* line transgene expression. In addition, the mouse I12b enhancer targets expression to the forebrain of transgenic mice, whereas I12a targets *lacZ* expression to the mandibular component of the first branchial arch and hyoid arch, but not the brain. It is, therefore, possible to suggest that the enhancer activity seen in the *Tg(6kb-dlx1a/dlx2a:EGFP)* line is attributed to the I12b enhancer, whereas, I12a is not active in the brain but is, however, active in the branchial arch region. There is the possibility, however, that the I12a enhancer is dependent on the I12b enhancer for proper function in the brain, in which case the I12a enhancer may be active in the brain. It would be necessary to generate separate lines of transgenic zebrafish reporting containing individual enhancers to determine which enhancer, individually, is active in the cells.

At 3dpf and 4dpf, there is an increase in the number of GFP positive cells in both lines at the level of the subpallium, in which similar patterns of GFP expression is also observed. There is also an increase in GFP positive cells in the pallium of the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line but very few are seen in the *Tg(6kb-dlx1a/dlx2a:EGFP)* line. In this study, results suggest that cells in which the *dlx5a/6a* CREs play a role, are born in the subpallial region by at least 2dpf and migrate in a ventral to dorsal manner via the lateral edges of the brain into the pallium. This is in accordance with the Mione *et al.* study, in which the transgenic line, *Tg(1.4kb-dlx5a/dlx6a:EGFP)*, was analyzed via time lapse analysis and the migration tracked through the brain (Mione, Baldessari et al. 2008). As mentioned, between 2dpf and 4dpf, observations show that the *dlx1a/2a* CREs

are active in a very small subset of cells in the pallium. *In-situ* hybridization studies show at 2pf, that *dlx2a*-expressing cells are not observed in the pallium but by 3dpf, *dlx2a*-expressing cells begin to invade the pallium (Mueller, Wullimann et al. 2008). Since *dlx1a/2a* CRE activity is observed in the subpallium but there is virtually no activity in the pallium, the I12a and/or I12b enhancers are involved in *dlx1a* and *dlx2a* regulation while the cells are in the subpallium but may not play a role in regulation once the cells reach the pallium. Since there are *dlx2a*-positive cells located in the pallium of the Mueller *et al.* study but no *dlx1a/2a* CRE activity in our study, it could be suggested that either the *dlx1a* and *dlx2a* transcripts are extremely stable in order to maintain expression once the cells reach the pallium or else there are other regulatory elements involved in *dlx* expression (Mueller, Wullimann et al. 2008). It has been shown that the distinct CREs, I12b and URE2, display partially overlapping activities in the mouse forebrain (Ghanem, Yu et al. 2007). These studies also show that the *URE2-lacZ* and *I12b-lacZ*-positive cells express *Dlx* genes (using pan-Dll antibody) in the ganglionic eminences of transgenic mice at E13.5. It can, therefore, be suggested that the *dlx1a/2a* CRE, URE2 (located upstream of *dlx1a*), may be involved in maintaining *dlx1a* and *dlx2a* expression in the cells occupying the pallium. A transgenic line of zebrafish using GFP as a reporter gene driven by the URE2 enhancer has recently been generated (MacDonald et al, unpublished observations). Studies using *dlx1a/dlx2a in-situ hybridization* coupled with GFP immunohistochemistry using this line of transgenic zebrafish should be performed to determine whether the enhancer plays a regulatory role in *dlx1a/dlx2a* expression in the cells located in the pallium.

4.2 *dlx* gene enhancers I56i and/or I56ii are active in GAD65/67 positive cells

In this study, GAD65/67 was used to identify GABAergic interneurons in the developing zebrafish brain. It has previously been shown that where GAD is detected in the early embryonic zebrafish brain, GABA is also detected (Martin et al., 1998). A previous description of GABAergic cells has been completed and the results at 2dpf and 3dpf in this study are consistent with those results (Mueller, Vernier et al. 2006). At 2dpf, there is a similar expression pattern at the most rostral and lateral region of the subpallium. There is also a similarity at the rostral level, where the pallium is GAD65/67-free at this early stage in development. It has been reported that the earliest differentiating zebrafish brain neurons responsible for building the early axonal scaffold contain GABA at 24hpf (Mueller, Vernier et al. 2006). This could suggest that GAD65/67 positive cells are born and differentiate in the subpallium between 24hpf and 2dpf, as seen in our results at 2dpf, but have not yet migrated to the pallium.

At 3dpf and 4dpf, some GAD65/67 positive cells seem to have invaded the pallium and may have migrated from the subpallium. This could indicate that GABA positive cells differentiate around 2dpf in the subpallium and begin to migrate to the pallium around 3dpf. The developmental pattern of GABA positive cells has been studied in a number of vertebrates including the mouse. Mueller et al. have documented almost perfect correspondence between the cellular distribution of GAD (E 12.5) in the mouse and GABA in the zebrafish (3dpf) (Mueller, Vernier et al. 2006). There is also a temporal correspondence of GAD/GABA positivity between the two species.

At 2dpf, 3dpf, and 4dpf it can be seen, via immunohistochemistry using the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line of zebrafish, that either the enhancer I56i, I56ii or both I56i and I56ii are active in GAD65/67 positive cells in the developing zebrafish brain.

At 2dpf, ventrally in the subpallium, it can be determined that most of the cells in which these enhancers are active, are also GAD65/67 positive. At this point in time, however, the majority of GFP positive cells in the dorsal region at the level of the pallium are GAD65/67 negative. Since it is known that the GABA positive cells occupy the pallium at 3dpf in the developing zebrafish brain and this is what we observe in our results, it can be suggested that GAD65/67 positive cells that are also GFP positive, begin to migrate from the subpallium to the pallium around 3dpf.

At 4dpf, there are less GFP positive cells that are also GAD65/67 positive in the subpallium but many GFP positive cells that are GAD65/67 positive in the pallium. The migration continues via the marginal region. These results can be compared to the tangential migration seen in the developing mouse brain. At E13.5 (comparable to 4dpf in zebrafish according to Mueller et al. 2006), GAD65/67 and the reporter transgene *zfdlx4/6-lacZ* (zebrafish *dlx5a* and *dlx6a* intergenic region) are expressed in the basal telencephalon, ventral thalamus, and parts of the hypothalamus. These genes and transgene are also robustly expressed in cells migrating from the basal telencephalon to the cerebral cortex via the marginal zones (Liu, Ghattas et al. 1997; Zerucha, Stuhmer et al. 2000; Stuhmer, Puelles et al. 2002). In our study, a distinct route of migration cannot be defined, yet it is observed that there is a ventral to dorsal route of migration from the subpallium to the pallium, mainly via the marginal region, of GFP positive cells that are also GAD65/67 positive. In addition from the same study, double-immunolabeling for

GABA and X-Gal performed on the cerebral cortex in adult mice showed >90% of X-Gal positive cells also expressed GABA (Stuhmer, Puelles et al. 2002). Although our study was not performed on adult zebrafish, our results show that the number of GAD65/67 positive cells that are also GFP positive found in the pallium increases as the zebrafish forebrain develops.

4.3 *dlx* gene enhancers I12a and/or I12b are active in GAD65/67 positive cells

At 2dpf, 3dpf, and 4dpf it can be seen via immunohistochemistry using the *Tg(6kbdlx1a/dlx2a:EGFP)* line of zebrafish that either the enhancer I12a, I12b or both I12a and I12b (but most likely just I12b, see above) are active in GAD65/67 positive cells in the developing zebrafish brain.

At 2dpf in the ventral region at the level of the subpallium, it is observed that there is a low incidence of GFP positive cells that are GAD65/67 positive. There appears to be no GFP positive cells in the dorsal region of the brain at the pallial level. At 3dpf and 4dpf, however, there is an increased incidence of cells positive for both GFP and GAD65/67 located at the level of the subpallium. There are also GFP positive and GAD65/67 positive cells at the marginal zone of the forebrain leading from the subpallium towards the pallium. Similar results were seen in mice, since previous studies show that co-labelling with *I12b-lacZ* and GABA results in >93% co-expression in adult cortical interneurons (Ghanem, Yu et al. 2007). These interneurons migrate from the basal telencephalon to the cortex via migration in a manner similar to the ventral to dorsal migration from the subpallium towards pallium as seen in the zebrafish in this study.

4.4 *dlx* gene enhancers I56i and/or I56ii are active in calretinin expressing cells

At 2dpf, there is a scattered pattern of calretinin expressing cells, with many occupying the subpallium. In addition, there are many calretinin positive cells located along the marginal zones of the forebrain that seem to be migrating from the ventral region at the level of the subpallium towards the dorsal region at the level of the pallium. At 2dpf, it may be premature for the calretinin positive cells to reach the pallium or else the migratory route may not be complete this early on in development. The number of calretinin positive cells increases between 3dpf and 4dpf. Although there does not appear to be a defined route in which these calretinin positive cells are migrating, they do, however, appear to migrate in a ventral to dorsal manner. It cannot be determined from these results as to whether the calretinin positive cells observed at 3dpf and 4dpf have divided to produce an increased number of cells or whether cells have differentiated to express calretinin. Studies using BrdU labelling could identify which cells have proliferated as opposed to which cells have differentiated into calretinin expressing cells. In this study, it was also inconclusive as to whether the calretinin expressing cells are definitively GABAergic. This work needs to be completed since calcium binding proteins, such as calretinin, are expressed in both GABAergic and glutamatergic neurons (Celio 1990). This can be accomplished by co-labeling cells with calretinin and GABA or GAD65/67. It would also be useful to label the cells with TBR1, a marker of cortical glutamatergic neurons, to distinguish glutamatergic neurons from GABAergic interneurons.

At 2dpf, 3dpf, and 4dpf it can be seen via immunohistochemistry using the *Tg(1.4kb-dlx5a/dlx6a:EGFP)* line of zebrafish that either the enhancer I56i, I56ii or both I56i and I56ii are active in calretinin expressing cells in the developing zebrafish brain.

At 2dpf, there is a high incidence of GFP positive and calretinin positive cells in the subpallium. There are also GFP positive and calretinin positive cells scattered along the marginal zones and along the midline of the forebrain and pallium, which like the GAD65/67 positive cells, appear to migrate in a ventral to dorsal manner. Many of the GFP positive cells, however, do not express calretinin at this stage of development, but since there are several other interneuron molecular markers, it is possible that these cells may express a different marker other than calretinin. Calbindin is another calcium-binding protein used as an interneuron molecular marker and striatal projection neuron marker. In mice, it was shown that *I56ii-lacZ* cells in the cortex co-express calbindin and GABA (Ghanem, Yu et al. 2008). These cells also expressed *Meis2* and *Islet1*, markers of striatal projection neurons, leading to the conclusion that I56ii is active in GABAergic projection neurons and I56i is active in GABAergic interneurons with a subtype expressing calretinin.

At 3dpf and 4dpf, the number of calretinin and GFP positive cells has increased in both the subpallium and the pallium. There appears to be no definite pattern of expression in the forebrain, however, it is possible that the cells have migrated in a ventral to dorsal manner, as seen at 2dpf and the cells begin to reach their destination at 3dpf. In the mouse brain, studies co-labeling *I56i-lacZ* and calretinin showed >88% co-expression in cortical interneurons at P35 and that these cells migrate tangentially to the cortex between E11.5 and E13.5 (Ghanem, Yu et al. 2007). Therefore, the I56i enhancer most likely plays a

role in the GFP positive cells that are also calretinin positive in the pallium and these cells migrate in a ventral to dorsal manner (much like the tangential migration seen in the mouse) in the developing zebrafish brain. In order to distinguish the two enhancers in the zebrafish brain, it would be beneficial to co-label cells with *Meis2* or *Islet1* and various other molecular markers. Until further work is done, results from this study suggest that the CREs between *dlx5a* and *dlx6a* are active in a calretinin expressing population of cells that migrate from the ventral region at the level of the subpallium to the dorsal region at the level of the pallium.

4.5 *dlx* gene enhancers I12a and/or I12b are active in calretinin expressing cells

At 2dpf, 3dpf, and 4dpf it can be seen via immunohistochemistry using the *Tg(6kbdlx1a/dlx2a:EGFP)* line of zebrafish that either the enhancer I12a, I12b or both I12a and I2b are active in calretinin expressing cells in the developing zebrafish brain.

At 2dpf and 3dpf, it appears that most of the calretinin expressing cells are migrating in a ventral to dorsal manner from the subpallium to the pallium, however, most of these cells are not GFP positive. At 4dpf, however, the number of GFP positive cells that are also calretinin expressing increases in the subpallial region, however, there are virtually no GFP positive cells at the level of the pallium, whereas, there are many calretinin positive cells present. A similar increase in labeling can be seen in mice whereby, *I12b-lacZ* was expressed in >79% of calretinin expressing cortical interneurons (Ghanem, Yu et al. 2007). The *I12b-lacZ* and calretinin positive cells have been shown to migrate from the ventral telencephalon to the cortex in the developing mouse brain. Similar to what is seen in mice, the GFP positive and calretinin positive cells, observed in

the transgenic zebrafish in this study, may migrate in a ventral to dorsal manner but further studies need to be completed. In addition, *Il2b-lacZ* was also expressed in >85% of parvalbumin expressing interneurons and >75% of neuronal peptide-Y (NPY) expressing interneurons in mice (Ghanem, Yu et al. 2007). It may be possible that the relatively low incidence of GFP positive and calretinin positive cells in the zebrafish is due to the fact that *Il2b* is active in other subsets of interneurons, namely parvalbumin and NPY.

4.6. Possible connection between *Dlx* genes and autism

It is thought that autism, a severe neurobehavioural syndrome discovered in the first few years of life, may be due to a disproportionately high level of excitation or disproportionately weak level of inhibition in neural circuits that mediate language and social behaviours (Hamilton, Woo et al. 2005). The *Dlx* genes *Dlx1*, *Dlx2*, *Dlx5*, and *Dlx6* control the development of the basal ganglia and cortical interneurons. Furthermore, it is known that the *Dlx* genes have a central role in controlling the development, migration, and function of the inhibitory GABAergic interneurons in the forebrain. As previously mentioned, interneurons are crucial for telencephalic function since they regulate principal neuron activity via inhibition and along with projection neurons, maintain the spatio-temporal balance of excitation and inhibition of brain function. *Dlx* gene expression is under the control of a dynamic and complex regulation that changes temporally and spatially during embryonic development. Furthermore, the human *DLX* genes are close to the loci that have been linked with autism and for this reason, among others, variations of the genes have been studied as a cause of autism. In a comparison

study of autism probands and non-autistic siblings, variants within the *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* coding and non-coding sequences were observed, one located within the I56i enhancer. Although this research did not uncover the causes of autism, studies suggest that variants in the intergenic regions of the *Dlx* genes may play a role in the underlying causes of autism. The more knowledge that is acquired pertaining to *Dlx* gene regulation and function in the developing forebrain, the closer one gets to deciphering the molecular mechanisms leading to autism.

Conclusion

By using the transgenic zebrafish lines *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and *Tg(6kbdlx1a/dlx2a:EGFP)*, I have shown that the CREs between *dlx5a* and *dlx6a*, and the CREs between *dlx1a* and *dlx2a* target both overlapping and differential expression of the reporter transgene, GFP, in the developing zebrafish forebrain. The *dlx5a/6a* CREs play a role in a set of cells that migrate from the ventral subpallium to the pallium. The *dlx1a/2a* CREs, however, only play a role in a set of cells located in the subpallium. Further studies need to be done in order to determine whether there are other regulatory elements maintaining the expression of the *dlx1a/2a* genes in cells of the pallium. The *dlx1a/2a* CRE, URE2, may play a role in *dlx* regulation in cells located in the pallium.

There is a high occurrence of GAD65/67 expression in cells expressing *Tg(1.4kb-dlx5a/dlx6a:EGFP)* and in cells expressing *Tg(6kbdlx1a/dlxa:EGFP)* indicating that the CREs investigated in this study, are active in interneurons. Furthermore, the CREs are active in at least one subtype of interneurons, those expressing calretinin. Identifying the

function of regulatory elements in early *dlx* gene expression will provide further insights into the developmental mechanisms in which progenitor specification and neuronal migration occur. This study demonstrates that specific *dlx* enhancer elements contribute to defining molecularly distinct populations of cells, including GABAergic interneurons of the calretinin subtype, in the developing zebrafish brain. Results also reflect a dynamic regulation of *dlx* gene expression through several regulatory elements with distinct and overlapping functions.

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