Lineage Tracing of Neuronal Progenitor Cells Expressing *dlx1a/2a* in the Zebrafish Brain

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ABSTRACT

The *Distal-less homeobox* (*Dlx)* genes encode homeodomain transcription factors that play important roles in the development of limbs, sensory organs, branchial arches and the forebrain. In the forebrain, *Dlx1* and *Dlx2* are expressed in neuronal progenitor cells and play essential roles in GABAergic neuron differentiation and migration. In order to understand the fate of neuronal progenitor cells that express *dlx1a/2a* genes in the brain, we produced lines of *Tg(dlx1a/2a:CreER^{T2})* transgenic fish expressing the CreER^{T2} recombinase driven by regulatory elements from the *dlx1a/2a* locus. *CreER^{T2}* expression in these fish faithfully recapitulates that of *dlx1a/2a* genes in the forebrain. These fish were mated with *Tg(ubi:Switch)* reporter fish that express a loxP-flanked GFP gene followed by mCherry, driven by the ubiquitin promoter. Upon tamoxifen treatment, the double transgenic fish express mCherry in *dlx1a/2a*-expressing cells. Live imaging data showed that mCherry-expressing cells were observed first in the telencephalon and prethalamus, regions from which they migrated and populated the telencephalon, prethalamus and hypothalamus by 10dpf. Fate mapping of mCherry-expressing cells in double transgenic fish demonstrated that a majority of *dlx1a/2a*-expressing cells give rise to GABAergic neurons. Furthermore, as zebrafish produce new neurons throughout life, the role of *dlx1a/2a* during adult neurogenesis was examined. Our preliminary data showed that *dlx1a/2a*-expressing progenitor cells populate various domains of the forebrain during adult neurogenesis. Our lineage tracing system provides a powerful tool to investigate the origin of GABAergic neuron progenitors and the mechanisms by which they populate or repopulate the adult brain.

RÉSUMÉ

Les gènes *Distal-less homeobox (Dlx)* codent pour des facteurs de transcription à homéodomaine qui jouent des rôles importants dans le développement des membres, des organes sensoriels, des arcs branchiaux, et du cerveau antérieur. Dans le cerveau anterieur, *Dlx1* et *Dlx2* sont exprimés dans les cellules progénitrices neuronales et jouent des rôles essentiels pour la différenciation et la migration. Afin de comprendre le sort des cellules progénitrices neuronales qui expriment les gènes *dlx1a/dlx2a* dans le cerveau, nous avons produit des lignées de poissons transgéniques *Tg(dlx1a/2a:CreER^{T2})* qui expriment la recombinase CreER^{T2} placée sous le contrôle des élements régulateurs du locus *dlx1a/2a*. L'expression de CreER^{T2} récapitule celle des gènes *dlx1a/2a* dans le cerveau antérieur. Ces poissons ont été croisés avec des poissons rapporteurs *Tg(ubi:Switch)* qui expriment la GFP flanquée de sites loxP, suivi par mCherry, et le tout sous le contrôle du promoteur Ubiquitine. Lorsque les poissons double-transgéniques sont traités avec du Tamoxifen, ils expriment mCherry dans les cellules exprimant *dlx1a/2a.* L'imagerie in vivo démontre que les cellules exprimant mCherry ont premièrement été observées dans le télencéphale et le préthalamus, des régions depuis lequelles les cellules ont migré afin de peupler le télencéphale, le préthalamus et l'hypothalamus à 10 jours après la fécondation. La cartographie de la destinéede cellules exprimant mCherry dans les poissons doubles transgéniques a démontré que la majorité de cellules exprimant *dlx1a/2a* donnent naissance à des neurones GABAergiques. Des plus, puisque les poissons-zèbres produisent de nouveauxneurones pendant toute leur vie, le rôle de *dlx1a/2a* durant la neurogénèse adulte a été examiné. Nos données préliminaires ont montré que les cellules progénitrices exprimant *dlx1a/2a* peuplent des domaines variés du cerveau antérieur durant la neurogénèse adulte. Notre système de cartographie de la destinée nous fournit

un important outil afin étudier l'origine de progéniteurs de neurones GABAergiques et les mécanismes par lesquels ils peuplent ou repeuplent le cerveau adulte.

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LIST OF ABBREVIATIONS AND ACRONYMS

Ascl – achaete-scute complex like

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

bHLH – basic helix-loop-helix

CB – calbindin

CCK – cholecystokinin

CNTNAP2– contactin-associated protein-like 2

CR – calretinin

CRE – *cis*-regulatory element

CGE – caudal ganglionic eminence

Cre – cyclization recombination

DIG – digoxygenin

Dll – Distal-less

Dlx – Distal-less homeobox

dpf – days post fertilization

dpi – days post induction

EGFP – enhanced green fluorescence protein

FMR1– fragile X mental retardation protein

GABA – gamma-aminobutyric acid

$GABA_AR$ – $GABA_A$ receptor

Gad – glutamic acid decarboxylase

GFAP – glial fibrillary acidic protein

hpf – hours post-fertilization

LGE – lateral ganglionic eminence

loxP – locus of crossover in P1

MECP2 – methyl-CpG-binding protein 2

MGE – medial ganglionic eminence

mopi – months post induction

NBT – nitroblue terazolium

nNOS – neuronal nitric oxide synthase

NPY – neuropeptide Y

PFA – paraformaldehyde

POA – preoptic area

polyA – polyadenylation signal

PV – parvalbumin

SOM – somatostatin

SVZ – subventricular zone

URE – upstream regulatory element

VIP – vaso-active intestinal peptide

VZ – ventricular zone

βG – beta Globin

4-OHT – 4-hydroxytamoxifen

STATEMENT OF CONTRIBUTIONS

Shengrui Feng performed most of the experiments and analyses presented in this thesis, including producing the transgene construct, generating *Tg(dlx1a/2a:CreER^{T2})* transgenic fish, live imaging and fate mapping analyses. Dr. Cynthia Solek helped design the transgene construct. The work on adult neurogenesis is a collaboration with Elyssa Mahoney and is mainly conducted by Elyssa Mahoney.

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INTRODUCTION

The main objective of the work presented in this thesis was to understand the role of *dlx1a/2a* in the zebrafish brain, especially to characterize their contribution to GABAergic neurons. In this introduction, the neural induction in vertebrates, the function of GABAergic neurons, and the mechanisms regulating GABAergic neuron development are discussed first. Then various aspects of the *Dlx* genes, including their homolog *Dll* gene in *Drosophila*, their genomic organization, their expression and function in the brain and their *cis*-regulatory elements, are reviewed. Furthermore, the concept of Cre/*loxP* based lineage tracing, a cell labeling technique used in this study, is described. Lastly, the objective and hypothesis of this study are explained.

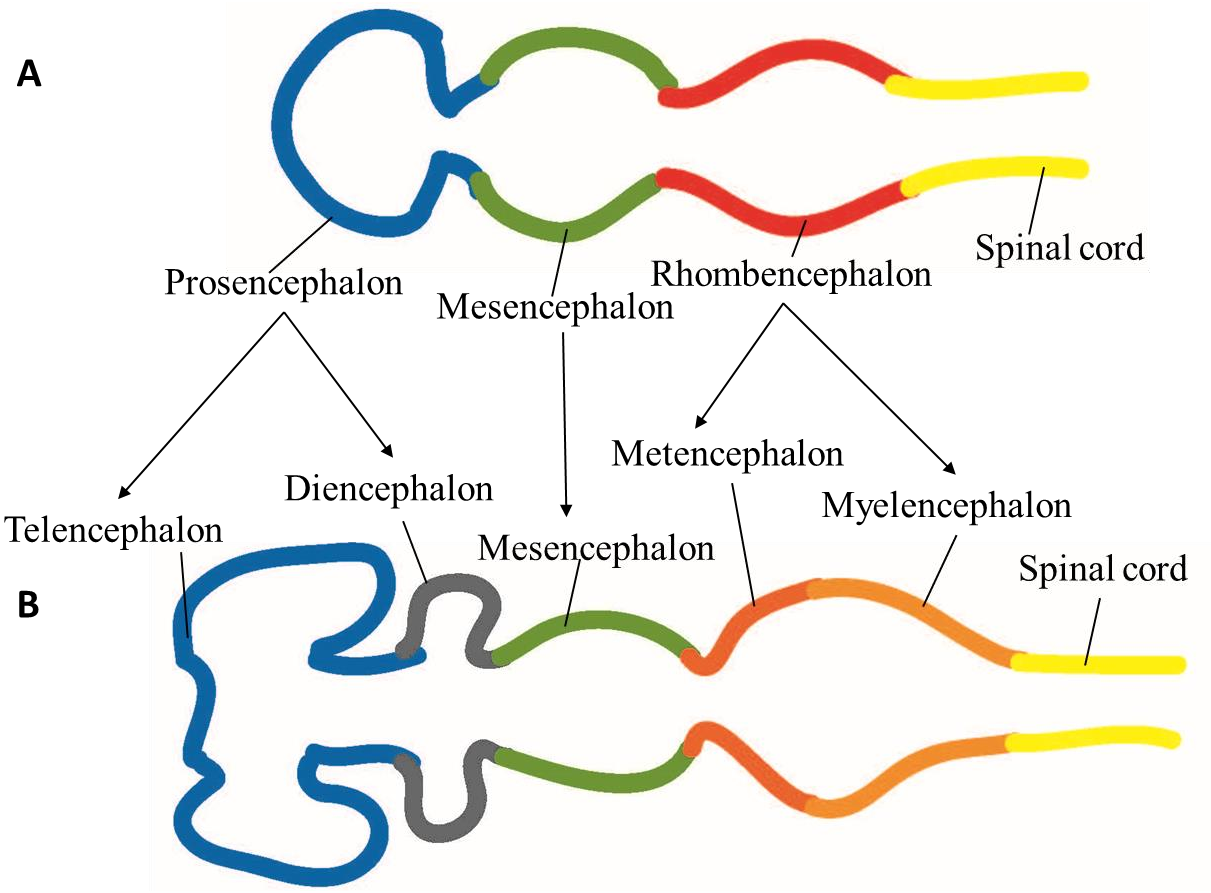
1.1 Neural Induction in Vertebrates

In vertebrates, gastrulation is followed by the induction of the nervous system. Three germ-layers, the ectoderm, mesoderm and endoderm are established from the blastula through gastrulation. The future nervous system originates from the neuroectoderm and segregates from the ventrolateral ectoderm which develops into epidermal skin. The dorsal-most ectoderm overlaying the notochord thickens and roll upwards to form the neural plate, a key-hole structure with the anterior broad end. In most vertebrates, the lateral edges of the neural plate fold and fuse at the dorsal midline, forming a hollow, cerebrospinal fluid-filled neural tube through neurulation. In comparison, the zebrafish neural plate initially forms a solid structure named neural keel by infolding of the neural plate. Neural keel then undergoes detachment of cells in the centre and give rise to a neurocoel (Papan and Campos-Ortega, 1994). The neural tube (or neural keel in zebrafish) subsequently develops into the central

nervous system. More specifically, the anterior neural tube gives rise to the brain, which is subsequently composed of a series of vesicles along the anterior-posterior axis, whereas the caudal neural tube maintains a tubular structure to form the spinal cord (Nicholls et al. 2012).

The developing brain first forms one combined forebrain (prosencephalon) and midbrain (mesencephalon) vesicle, and one hindbrain (rhombencephalon) vesicle. Then the brain further develops into three more defined vesicles including prosencephalon, mesencephalon and rhombencephalon vesicles (figure 1.1A). Finally, the prosencephalon is divided into the telencephalon and diencephalon and the rhombencephalon is divided into the metencephalon and myelencephalon (figure 1.1B) (Wolpert and Tickle 2011).

Figure 1.1: Schematic representation of the developing vertebrate brain. (A) The early developing brain becomes subdivided into three primary vesicles: the prosencephalon, the mesencephalon, and the rhombencephalon. Below represents a longitudinal section of the neural tube at the same developmental stage. (B) As development progresses, the prosencephalon divides into the telencephalon and diencephalon, and the rhombencephalon divides into the metencephalon and myelencephalon, establishing the major functional subdivisions of the brain. Below indicates a longitudinal section of the brain at the same developmental stage (Adapted from Purves et al. 2001).


A
Prosencephalon
Mesencephalon
Rhombencephalon
Spinal cord
Telencephalon
Diencephalon
Mesencephalon
Metencephalon
Myelencephalon
Spinal cord
B

1.2.1 GABAergic Neurons Function

GABAergic neurons secrete gamma-aminobutyric acid (GABA) as the primary neurotransmitter (Stuhmer et al., 2002). GABAergic neurons make up 20% of all neurons in the cerebral cortex and hippocampus and 95% of neurons within the striatum (Wonders and Anderson, 2006). In the adult mammalian brain, GABAergic neurons are the principal source of inhibitory neurons and glutamatergic neurons (neurons use glutamate as the primary neurotransmitter) are the principal excitatory neurons. The neurotransmitter GABA plays its inhibitory role by activating chloride conducting receptors. The activation of chloride conducting receptors results in a fast increase in conductance, which initiates an inhibitory postsynaptic potential in postsynaptic neurons (Nicholls et al. 2012). The balance of the excitatory and inhibitory systems in the cerebral cortex must be maintained to avoid pathological consequences including epilepsy, autism and schizophrenia (Lewis, 2000). However, GABA plays excitatory roles in the immature brain, and glutamatergic synapses are established after GABAergic neurons in many species and structures (Ben-Ari, 2002). In the early developing brain, GABAergic excitatory synapses establish the cortical network and then become inhibitory after the excitation of the developing neurons.

GABAergic neurons are heterogeneous and can be classified into various groups based on connectivity, morphology, and electrophysiology (Markram et al., 2004; Flames and Marin, 2005; Gelman and Marin, 2010). Based on the morphology of axons and dendrites, they can be classified into basket cells, bipolar cells, bitufted cells, double bouquet cells, Chandelier cells and Martinotti cells. GABAergic neurons can be further classified into various subtypes based on the expression patterns of markers including calcium-binding proteins, such as calbindin (CB), calretinin (CR) and parvalbumin (PV), neuropetides, such as somatostatin

(SOM), neuropeptide Y (NPY), vaso-active intestinal peptide (VIP) and cholecystokinin (CCK), and neurotransmitters such as neuronal nitric oxide synthase (nNOS) (Markram et al., 2004; Wonders and Anderson, 2006; Gelman and Marin, 2010).

GABA is synthesized from glutamate by the enzyme named glutamic acid decarboxylase (*Gad*). In mice, *Gad* has two isoforms which are *Gad65* and *Gad67* (commonly referred to as *Gad1* and *Gad2* respectively). Approximately 95% of both *Gad* genes are co-localized in all GABA cells within the hippocampus. At least two *gad* genes which are *gad1b* and *gad2* are found in zebrafish (Martin et al. 1998). The *gad1b* and *gad2* genes are expressed in the telencephalon, diencephalon and spinal cord, all domains positive for GABA (Martin et al. 1998). Due to the teleost genome duplication, a third *gad* gene possibly exists in zebrafish, although little is known about its expression (Bosma et al. 1999).

1.2.2 Regulatory Mechanisms Controlling GABAergic Neuron Development

GABAergic neurons develop from various regions of the neuroepithelium and are produced in all of the major domains of the brain (Achim et al., 2014). Both shared and unique mechanisms regulate GABAergic neuron development in the different domains of the developing brain.

In the rodent telencephalon, GABAergic neurons first originate from the subpallial neuroepithelium that comprises the lateral, medial, and caudal ganglionic eminences (LGE, MGE, CGE) and the preoptic area (POA), where they tangentially migrate towards the olfactory bulb, hippocampus and neocortex (Panganiban and Rubenstein, 2002). Likewise, in zebrafish GABAergic neurons are generated near the medial subpallial ventricular wall at 24 hours post fertilization (hpf) and migrate dorsolaterally to the cortex (Mueller et al., 2006).

In the developing mouse diencephalon, GABAergic neurons are generated from the neuroepithelium of prethalamus, the rostral thalamus, the juxtacommissural pretectum and the commissural pretectum. The most dorsal regions of the midbrain produces mix populations of GABAergic and glutamatergic neurons (Achim et al., 2014). GABAergic progenitors also exist in the ventral and dorsal regions of hindbrain. In mice, GABAergic neurons are derived from *Nkx6-1*-expressing progenitors in the ventral hindbrain and from *Ptf1a*-expressing progenitors in the dorsal hindbrain (Fujiyama et al., 2009; Lahti et al., 2013; Achim et al., 2014).

In mouse, the proneural gene *Ascl1* (*Mash1*) encodes a basic helix-loop-helix (bHLH) transcription factor that plays key roles in GABAergic neurogenesis (Allan and Thor, 2003). *Ascl1* is expressed in almost all of the GABAergic progenitor domains in the developing brain (Lo et al., 1991). *Ascl1* not only regulates cell cycle exit, but also promotes development of GABAergic identity (Achim et al., 2014). Although proneural gene *Ascl1* promotes GABAergic neurogenesis, further specification of GABAergic neurons involves additional regulatory factors. Distinct molecular toolkits are used for GABAergic fate determination in different domains of the developing brain as discussed below.

In the rodent forebrain, *Dlx* gene family have been shown to play essential roles in GABAegic neuron specification. *Dlx1* and *Dlx2* expression can be both detected in the subventricular and ventricular zones (SVZ and VZ) of the medial and lateral ganglionic eminences. *Ascl1* mutants exhibit altered telencephalic expression of the *Dlx* genes and *Gad1* (Gad67) (Casarosa et al., 1999; Long, Cobos et al., 2009). The overlapping expression of *Ascl1* and *Dlx* genes in the developing mouse forebrain further suggests that *Ascl1* acts upstream of *Dlx1*/*Dlx2*. Indeed, *Ascl1* directly binds to a *Dlx1*/*Dlx2* regulatory

element I12b to initiate transcription of *Dlx1/Dlx2* (Poitras et al., 2007). It has been shown that *Dlx1/Dlx2* may act upstream of *Dlx5/Dlx6* by regulating via the I56i enhancer (Anderson, Qiu et al., 1997; Zerucha et al., 2000). An almost complete loss of all GABAergic neurons from striatum, cortex, olfactory bulbs, and hippocampus has been observed in *Dlx1/2* double mutant mice. (Anderson, Eisenstat et al., 1997; Anderson, Qiu et al., 1997; Long et al., 2007). It has been shown that *Dlx* genes regulate GABAergic neuron both directly and through activation of the expression of *Gad1* and *Gad2* which encode enzymes responsible for the synthesis of GABA (Stuhmer et al., 2002; Long, Cobos et al., 2009; Long, Swan et al., 2009).

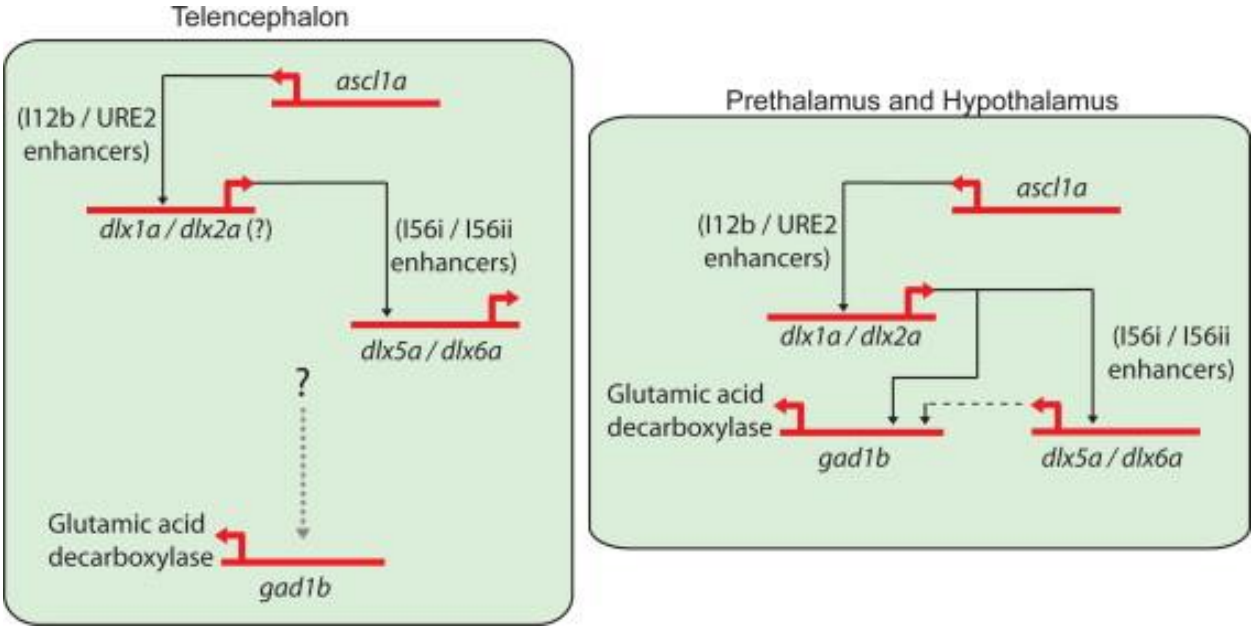
In the zebrafish forebrain, the regulatory relationships between the *ascl1a, dlx* and *gad1b* genes has been examined as well (MacDonald et al., 2013). *Ascl1* has two orthologs named *ascl1a* and *ascl1b* in zebrafish. The *ascl1a* and *ascl1b* genes are expressed in the subpallial telencephalon and prethalamus of the embryonic forebrain, similar to *Ascl1* expression pattern in the mouse forebrain, but each has unique and overlapping expression patterns (Allende and Weinberg, 1994; Wullimann and Mueller, 2002). The observation that little co-localization of *ascl1b* with *dlx1a* or *ascl1a* at 24 hpf indicates that *ascl1b* may not initiate *dlx* genes at the early developing forebrain (MacDonald et al., 2013). In the telencephalon, *ascl1a* controls *dlx1a*/*2a* expression via URE2 and I12b enhancers (Figure 1.2). The *dlx1a*/*2a* genes modulate *dlx5a/6a* expression via I56i and I56ii enhancers. Knockdown of *ascl1a*, *dlx1a*/*2a* or *dlx5a*/*6a* has almost no effect on *gad1b* expression which suggests an unknown gene regulatory pathway regulates the expression of *gad1b* in the telencephalon (MacDonald et al., 2013). In the ventral prethalamus and hypothalamus of diencephalon, *ascl1a* modulates *dlx1a*/*2a* expression (Figure 1.2). The *dlx1a*/*2a* genes control the expression of *dlx5a*/*6a* and *gad1b* in these domains. The

dlx5a/*6a* genes may play a minor role in *gad1b* expression in the diencephalon as knockdown of *dlx5*a/*6a* causes a mild reduction in *gad1b* signal compared to *dlx1a*/*2a* knockdown (MacDonald et al., 2013).

In the developing mouse midbrain, transcription factor genes *Gata2* and *Tal2* are required for GABAegic neuron specification. *Gata2* is expressed after *Ascl1* in GABAergic progenitors soon after their cell cycle exit in the midbrain (Kala et al., 2009). A similar expression pattern of *Tal2* in GABAergic neuron precursors is seen in the midbrain (Achim et al., 2013). *Gata2* and *Tal2* mutants have similar midbrain phenotype, exhibiting a transformation of all midbrain GABAergic progenitors into glutamatergic neurons (Kala et al., 2009; Achim et al., 2013). In addition, *Gata2* and *Tal2* are essential for the activation of transcription factor genes, including *Tal1, Gata3, Six3,* and *Sox14*. These downstream genes are involved in the GABAergic neuron development and the maintenance of the GABAergic neurons (Achim et al., 2014).

In the developing mouse ventral and dorsal hindbrain, *Tal1* and *Ptf1a* play essential roles in GABAegic neuron specification, respectively. Progenitors that express *Nkx6-1* give rise to GABAegic precursors in the ventral hindbrain. *Tal1* expression is required for this process and the loss of *Tal1* results in deficiency of GABAergic neurons in this domain (Achim et al., 2012). *Ptf1a* is crucial to the GABAergic neuron specification in the dorsal hindbrain. Lineage tracing experiment has shown that *Ptf1a*-expression cells give rise to GABAergic neurons in the cerebellum. *Ptf1a* mutants also exhibit an almost complete loss of GABAergic neurons of the cerebellum and cerebellar nuclei (Minaki et al., 2008).

Figure 1.2 Model of the regulatory relationships between the *dlx* and *gad1b* genes in the zebrafish forebrain. In the telencephalon, *ascl1a* controls *dlx1a*/*2a* expression via URE2 and I12b enhancers. The *dlx1a*/*2a* genes modulate *dlx5a/6a* expression via I56i and I56ii enhancers. An unknown gene regulatory pathway regulates the expression of *gad1b* in the telencephalon. In the diencephalon, *ascl1a* modulates *dlx1a*/*2a* expression. The *dlx1a*/*2a* genes controls the expression of *dlx5a*/*6a* and *gad1b* in these domains. The *dlx5a*/*6a* genes may play a minor role in *gad1b* expression in the diencephalon (MacDonald et al., 2013).


Telencephalon
ascl1a
(I12b / URE2
enhancers)
dlx1a / dlx2a (?)
(I56i / I56ii
enhancers)
dlx5a / dlx6a
?
Glutamic acid
decarboxylase
gad1b
Prethalamus and Hypothalamus
ascl1a
(I12b / URE2
enhancers)
dlx1a / dlx2a
(I56i / I56ii
enhancers)
Glutamic acid
decarboxylase
gad1b
dlx5a / dlx6a

1.3.1 The *Drosophila Distal-less* (*Dll*) Gene

Distal-less (*Dll*) gene was initially identified in *Drosophila* and is essential for proper proximo-distal patterning in *Drosophila* limb (Panganiban and Rubenstein, 2002). *Dll* encodes a homeodomain-containing transcription factor that plays key roles in the specification of several distal appendages, including the limbs, antennae, optic lobe of the brain and glial cells of the ventral nerve cord in Drosophila (Kaphingst and Kunes 1994; Panganiban and Rubenstein, 2002). *Dll* null flies lack rudimentary larval limbs and antennae and therefore die at the early embryonic stage, while ectopic *Dll* expression can lead to the formation of proximo-distal axis (Cohen et al., 1989; Cohen and Jurgens, 1989; Panganiban and Rubenstein, 2002). While *Dll* is expressed in both brain and ventral nerve cord, its functional implication in central nervous system is largely unknown.

1.3.2 The Organization of the *Dlx* Genes

The vertebrate *distal-less homeobox (Dlx)* genes, *dll* homologs, are found in all vertebrate species and they encode a group of homeodomain-containing proteins that function as transcription factors, which are important for the development of forebrain, branchial arches, sensory organs and limbs (Panganiban and Rubenstein, 2002). *Dlx* genes are generally organized as convergently transcribed bigene clusters with overlapping expression patterns. Six *Dlx* genes have been characterized in mouse and human (Price et al., 1991; Robinson et al., 1991; Simeone et al., 1994; Weiss et al., 1994; Nakamura et al., 1996). They are arranged as three convergently transcribed bigene pairs (*Dlx*1/*Dlx*2, *Dlx*3/*Dlx*4 and *Dlx*5/*Dlx*6) separated by relatively short intergenic (3.5-16 kb) region (Zerucha and Ekker, 2000). In zebrafish, there are eight known *dlx* genes. Six of them (*dlx*1a/*dlx*2a, *dlx3b/dlx4b* and

dlx5a/dlx6a) are organized in a way similar to their mammalian counterparts (Quint et al., 2000). The zebrafish *dlx2b* and *dlx4a* are two additional unlinked genes which are considered to be duplicates of ancestral *Dlx*2 and *Dlx*4, respectively. This duplication might occur through a genome duplication event that is specific to the teleost lineage (Amores et al., 1998; Ellies, Langille, et al., 1997; Stock et al., 1996). All *Dlx* genes share a similar exon-intron organization which contains three exons and two introns (Ellies, Stock, et al., 1997, Liu et al., 1997). *Dlx* genes encode 243 to 333-amino acid protein products and the highly conserved 61 amino acid homeobox is split between exons 2 and 3 (Liu et al., 1997).

1.3.3 The Expression and Function of *Dlx* Genes in the Brain

The *Dlx* genes are expressed in a number of organs and tissues including the forebrain, branchial arches, sensory organs, limbs/fin buds, bone, cartilage, hematopoeitic and immune systems, and are important for their development (Bendall and Abate-Shen, 2000; Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002). The expression patterns of *Dlx* genes from the same bigene pair are highly overlapping, while subtle differences in their expression patterns have been observed which indicates that *Dlx* genes have both redundant and distinct functions (Akimenko et al., 1994; Ellies, Langille, et al., 1997; Quint et al., 2000). In addition, expression patterns of *Dlx* genes are considered to be evolutionarily conserved among vertebrates including human, mouse and zebrafish (Akimenko et al. 1994; Ellies, Stock, et al., 1997; Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002). In the forebrain and branchial arches, the *Dlx* genes are expressed in nested pattern along the proximal-distal axis (Panganiban and Rubenstein, 2002). Here, the expression pattern and function of *Dlx* genes in the brain is the primary concern of this study.

In mammals, *Dlx2*, *Dlx1*, *Dlx5* and *Dlx6* are sequentially expressed in the developing forebrain (Bulfone, Puelles, et al., 1993; Liu et al., 1997; Eisenstat et al., 1999; Zerucha et al., 2000). Among the eight zebrafish *dlx* genes, *dlx1a*, *dlx2a*, *dlx5a*, *dlx6a*, and *dlx2b* are expressed in the developing forebrain (Akimenko et al. 1994; Ellies, Stock, et al., 1997). In the developing forebrain, *Dlx* genes are expressed in the ventral telencephalon and the diencephalon which includes the prethalamus and hypothalamus (Robinson et al., 1991; Bulfone, Kim, et al., 1993).

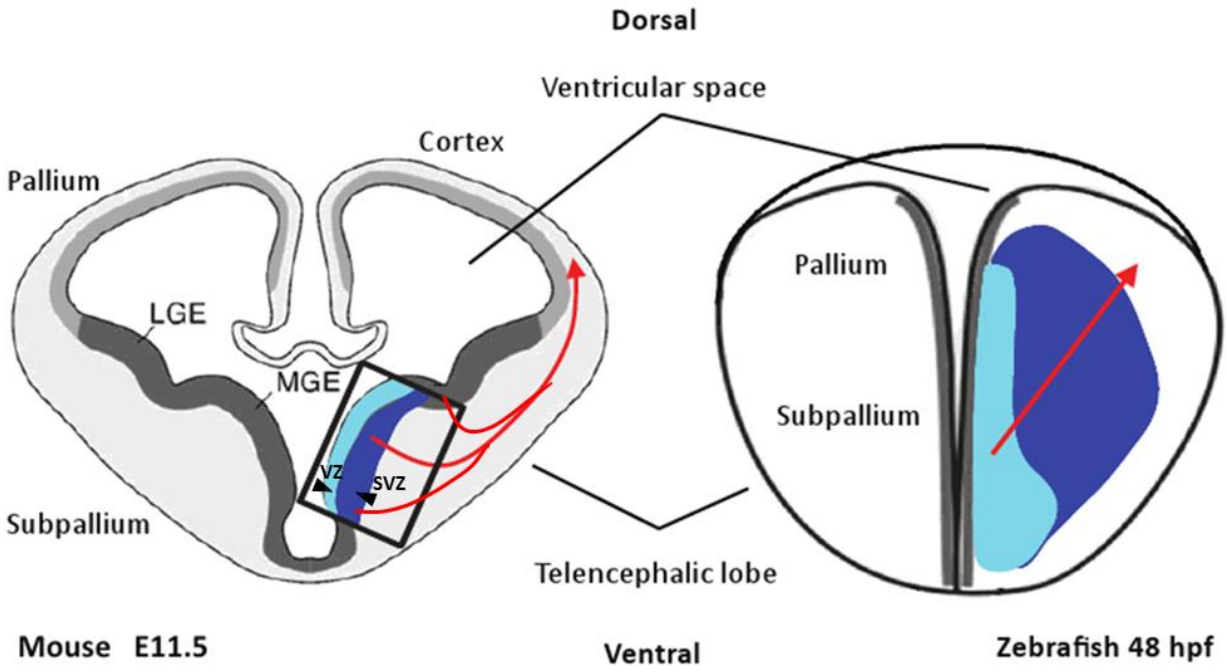
In mice, *Dlx* gene expression partially colocalizes with GABAergic neuron migration and differentiation during forebrain development. Likewise, partially overlapping expression patterns of *dlx* genes with *gad1b* have been observed in the developing zebrafish forebrain (Macdonald *et al*, 2010). As mentioned above, *Dlx1* and *Dlx2* are expressed earlier than *Dlx*5 and *Dlx*6, both in progenitor cells in the SVZ and VZ of the medial and lateral ganglionic eminences (Figure 1.3). *Dlx5* and *Dlx6* are expressed in the post-mitotic differentiating neurons migrating in the subventricular and mantle zones (Figure 1.3) (Panganiban and Rubenstein, 2002).

Dlx knockout mice have been generated to examine the roles of *Dlx* genes in the developing brain. Due to craniofacial defects or deficiency in the enteric nervous system, *Dlx1* mutants die within three weeks after birth, while *Dlx2* and *Dlx5* mutants die very quickly after birth (Qiu et al., 1995; Qiu et al., 1997; Acampora et al., 1999). Although brain development in *Dlx1* mutants appears largely intact, postnatal *Dlx1* mutants exhibit a loss of CR+, SOM+ and NPY+ interneurons in the cortex and hippocampus in a time-dependent fashion. In addition, possibly due to the deficiency of neuronal inhibition mediated by GABAergic interneurons, *Dlx1* mutants exhibit an epileptic and hyperexcitable phenotype

(Cobos et al., 2005). Although no morphological or histological abnormalities of the developing forebrain in *Dlx2* mutants have been found, *Dlx2* mutants exhibit reduced dopaminergic neurons in the olfactory bulb (Qiu et al., 1995). Similarly, *Dlx5* mutants have seemingly normal developing forebrain, but a reduced number of GABAergic neurons are found in the olfactory bulb (Acampora et al., 1999). The characterization of *Dlx6* mutants is hitherto missing.

In contrast, differentiation of late born neurons within the subcortical telencephalon in the *Dlx1*/*Dlx2* double mutants is largely aborted (Anderson, Eisenstat et al., 1997; Marin et al. 2000). This blocks the development of basal ganglia late-born GABAergic projection neurons and the production of several types of GABAergic, dopaminergic and cholinergic interneurons (Anderson, Eisenstat et al., 1997; Marin et al. 2000). Thus, *Dlx1*/*Dlx2* double mutants exhibit a massive decrease in the GABAergic interneurons of the cerebral cortex (Anderson, Eisenstat et al., 1997; Anderson, Qiu et al., 1997; Bulfone et al., 1998; Marin et al., 2000). Moreover, there is almost no *Dlx5* and *Dlx6* expression in the forebrain of *Dlx1*/*Dlx2* double mutants, which suggests that *Dlx1* and *Dlx2* act upstream of *Dlx5* and *Dlx6* in the forebrain development (Anderson, Eisenstat et al., 1997; Zerucha et al. 2000). Unfortunately, the forebrain phenotype of *Dlx5*/*Dlx6* mutants cannot be assessed further owing to exencephaly (Robledo et al. 2002).

Figure 1.3 Schematic representation of *Dlx* expression in the developing mouse and zebrafish telencephalon. The left and right diagrams represent the transverse sections of the developing mouse and zebrafish telencephalon, respectively. The proliferative ventricular zones are indicated in dark gray, and pallial ventricular zone in mouse is indicated in light gray. Light blue domains represent the expression of *Dlx1/Dlx2* (*dlx1a/dlx2a* in zebrafish*)*, dark blue domains represent *Dlx5/Dlx6* (*dlx5a/dlx6a* in zebrafish) expression, and the red arrow indicates the migration path of GABAergic neuron precursor cells born in the ventricular zone. LGE: lateral ganglionic eminence; MGE: medial ganglionic eminence (Adapted from Pollack, 2013).


Dorsal
Ventricular space
Cortex
Pallium
LGE
MGE
VZ
SVZ
Subpallium
Telencephalic lobe
Pallium
Subpallium
Mouse E11.5
Ventral
Zebrafish 48 hpf

1.3.4 *Cis*-regulatory Elements of *Dlx* Genes

The largely overlapping expression of *Dlx* genes in a given bigene cluster and the existence of an intergenic region suggests that this region may contain *cis*-regulatory elements (CREs) responsible for the overlapping expression patterns (Ellies, Stock, et al., 1997).

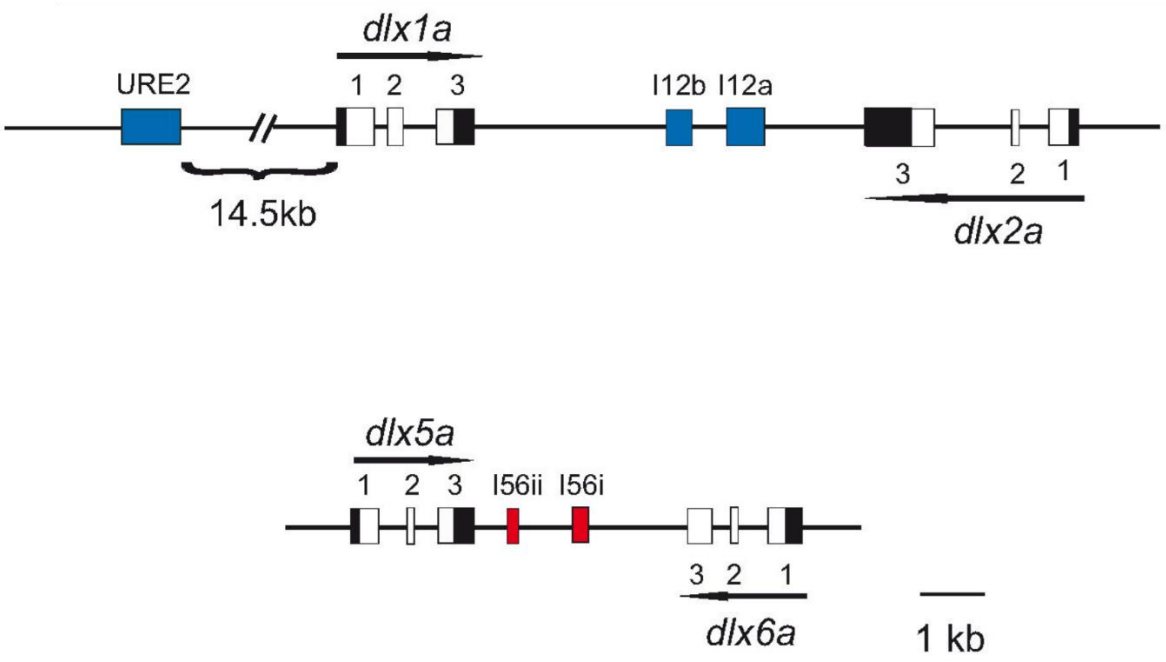
This is supported by the fact that the intergenic region between *Dlx1* and *Dlx2* contains highly conserved CREs named I12a (550bp) and I12b (450 bp) (Figure 1.4) (Ghanem et al. 2003). The intergenic region between *Dlx1* and *Dlx2* was compared among five vertebrate species (human, mouse, zebrafish, *Takifugu rubripes* and *Spheroides nephelus*) to identify the CREs. I12a and I12b were identified as their sequences were highly conserved in all five species (Ghanem et al. 2003). Further studies demonstrate that the I12b enhancer drives reporter gene expression in the forebrain, while the I12a enhancer drives reporter gene expression in the mesenchymal cells of the first two branchial arches (Ghanem et al. 2003, Park et al. 2004). Moreover, the activities of I12a and I12b mimic endogenous expression of *Dlx1/2* in the forebrain and in the branchial arches respectively (Ghanem et al. 2003).

Two additional regulatory elements are located upstream of the *Dlx1* transcriptional start named upstream regulatory element (URE) 1 and URE2 (Figure 1.4) (Ghanem et al. 2007; Jackman and Stock, 2008). URE1 enhancer is active specifically in the retina, a tissue in which *Dlx1* and *Dlx2* are expressed, whereas URE2 targets reporter expression to the forebrain (Dolle et al. 1992; de Melo et al. 2003).

The intergenic region of the *Dlx5* and *Dlx6* bigene cluster contains two enhancers, named I56i (400 bp) and I56ii (300bp) (Figure 1.4) (Zerucha et al. 2000). Between mice and

zebrafish, I56i and I56ii are conserved and share 83% and 85% sequence identity, respectively (Zerucha et al., 2000). I56i and I56ii are active in the forebrain and olfactory placodes (Zerucha et al. 2000). A 1.4 kb intergenic sequence of zebrafish *dlx5a/6a* cluster is sufficient to drive reporter gene expression faithfully mimicking endogenous expression of *dlx5a/dlx6a* (*Dlx5/Dlx6*) in the forebrain of mice and zebrafish (Zerucha et al., 2000). In mice, URE2, I12b and I56i enhancers are active in interneuron progenitors of the ganglionic eminence and mark both unique and overlapping neuronal populations (Ghanem et al. 2007; Potter *et al*., 2009), whereas I56ii is only active in post-mitotic neurons (Ghanem *et al.*, 2008).

Figure 1.4 Schematic representation of genomic organization of *dlx1a/2a and dlx5a/6a* bigene clusters in zebrafish. Within each bigene cluster, *dlx* genes are transcribed convergently. A number of highly conserved *cis*-regulatory elements (CREs) have been characterized regulating *dlx* expression in the developing embryos: URE2 is upstream of *dlx*1a, I12a and I12b within the *dlx*1a/*dlx*2a intergenic region, I56i and I56ii within the *dlx*5a/*dlx*6a intergenic region. Exons are indicated in white and UTRs in black. Blue (URE2, I12a and I12b) and red boxes (I56i and I56ii) represent regulatory elements (Adapted from MacDonald et al., 2010).


dlx1a
URE2
1 2 3
I12b I12a
3 2 1
14.5kb
dlx2a
dlx5a
1 2 3 I56ii I56i
3 2 1
dlx6a
1 kb

1.4 Cre/*loxP* Based Lineage Tracing

Understanding how cells are generated and organized and how they integrate into a complex functional organ such as the brain is both intriguing and challenging. Lineage tracing is an essential tool for studying cell proliferation, migration and differentiation. In lineage tracing, stem cells or progenitor/ precursor cells are labeled by lineage tracer and such label is transmitted to the progeny. Ideally the lineage tracer should be retained permanently and transmitted to all progeny of the founder cell, and should not be passed on to unrelated cells (Kretzschmar and Watt, 2012). Lineage tracing allows the characterization of the number of progeny of the founder cell, their location and their fates.

Cre/*loxP* recombination system is able to introduce site specific recombination event and has been successfully used in gene expression control and lineage tracing. This system, initially identified in bacteriophage P1, consists of a 38.5 kD recombinase named Cre (cyclization recombination) recombinase and a 34-bp asymmetric DNA sequence named *loxP* (locus of crossover in P1) (Sternberg and Hamilton, 1981). The *loxP* sequence contains an 8-bp asymmetrical recognition site flanked by two 13 bp inverted repeats for Cre binding.

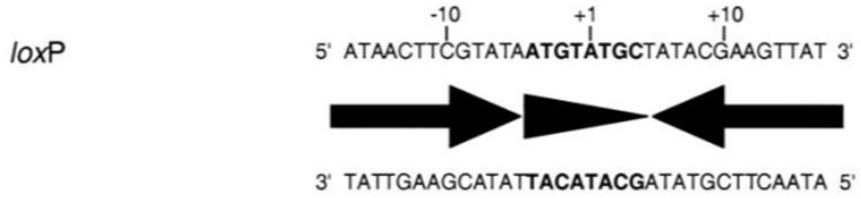
During recombination, two molecules of Cre monomers specifically recognize *loxP* and bind to the two inverted repeats in *loxP* sites to form a DNA synaptic complex for recombination. The outcome of the recombination depends on the relative orientation of the recognition sites with respect to one another (Hoess et al., 1982). Cre will delete the DNA sequence between two *loxP* sites if the two recognition sites in *loxP* sites are present in direct orientation, whereas Cre will invert the DNA between two *loxP* sites if

the two recognition sites are in opposite orientation. As *loxP* sites are absent from vertebrate genomes they can be exploited for Cre-mediated targeted recombination using transgenic approaches.

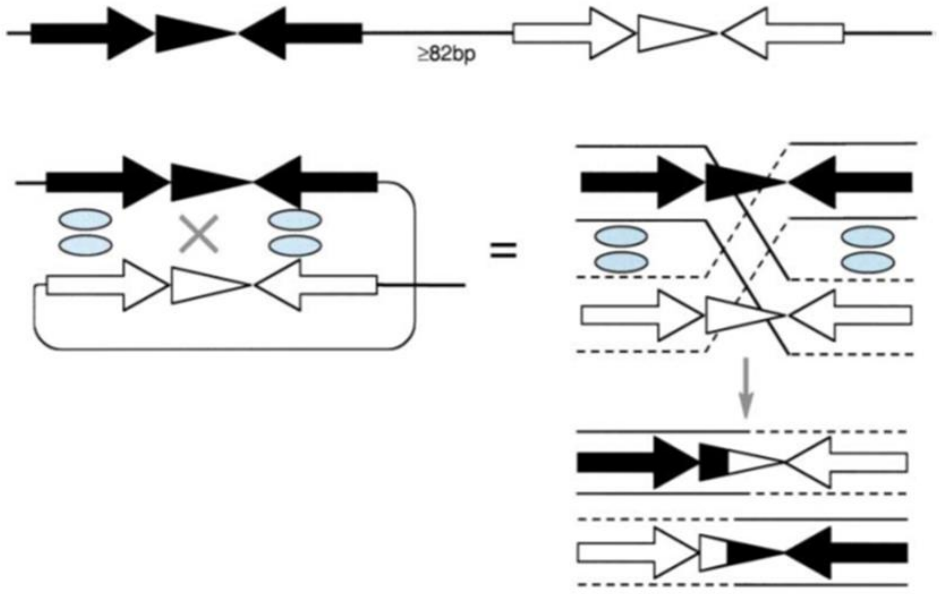
The Cre/*loxP* system has been widely applied for transgenesis studies in many models including human cells, mouse and zebrafish (Kowolik et al., 2004; Thummel et al., 2005; Hans et al., 2009; Ludes-Meyers et al., 2009; Seok et al., 2010). Inducible Cre/*loxP* recombination systems developed recently allows both spatial and temporal control of the Cre recombination. Temporal control of Cre/*loxP* recombination can also be obtained using a ligand-inducible chimeric Cre recombinase where Cre is fused to the mutant ligand-binding domain of the human estrogen receptor ($CreER^{T2}$). $CreER^{T2}$ is efficiently activated by exposure to tamoxifen or its active metabolite 4-hydroxytamoxifen (4-OHT) and provides improved temporal control over Cre-mediated recombination (Indra et al., 1999; Hans et al., 2009). Spatial control of Cre/*loxP* recombination can be achieved by directing Cre expression in a specific tissue or cell type using tissue- or cell type-specific promoters/regulatory elements.

Figure 1.5 Schematic representation of Cre/*loxP* mediated DNA recombination. (A) *lox*P, the Cre recombinase target site, contains one 8 bp asymmetrical spacer (indicated in bold) flanked by two 13 bp inverted repeats. (B) During recombination, binding of one Cre monomer to each inverted repeats forms a synaptic complex of two *loxP* sites and four Cre molecules followed by strand cleavage, exchange and ligation within the spacer regions. (C) If the two *loxP* sites are present in direct orientation, Cre/*loxP* mediated DNA recombination will excise a circular molecule from between two *loxP* sites, leading to deletion of the DNA between two *loxP* sites. (D) If the two *loxP* sites are present in opposite orientation, Cre will invert the DNA between two *loxP* sites. Blue oval represents Cre monomer (Adapted from Branda and Dymecki, 2004).

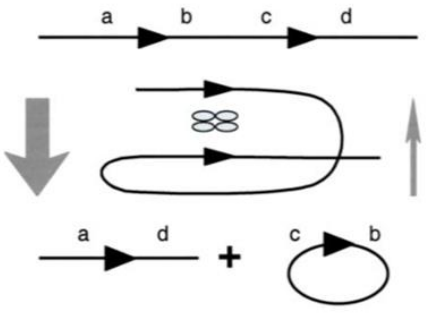
A Cre recombination target site



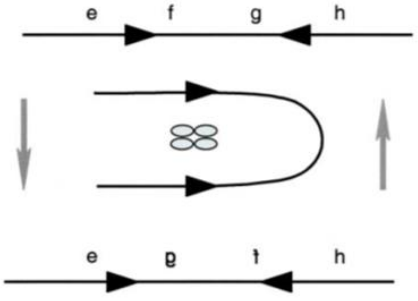
B Mechanism of Cre recombination



C Excision



D Inversion



= Cre Monomer = *loxP* site

1.5 Statement of Inquiry

GABAergic neurons play key inhibitory roles in the neuronal systems. Impaired GABAergic neuron function may result in an imbalance of neuronal inhibition and excitation, which has been associated with many neurodevelopmental disorders such as autism, Fragile X syndrome, Rett syndrome, schizophrenia and Tourette's syndrome. *Dlx1* and *Dlx2* are essential for GABAergic neuron differentiation and migration in the forebrain. *Dlx1/2* double mutants exhibit an almost complete loss of all GABAergic neurons from striatum, cortex, olfactory bulbs, and hippocampus (Anderson, Eisenstat et al., 1997; Anderson, Qiu et al., 1997; Long et al., 2007). In the mouse developing forebrain, *Dlx1* and *Dlx2* are expressed in progenitor cells in the SVZ and VZ of the medial and lateral ganglionic eminences, in a pattern which colocalizes with forebrain GABAergic neuron migration and differentiation. In agreement with this, partially overlapping expression patterns of *dlx1a/2a* genes with *gad1b* has been observed in the developing zebrafish forebrain (Macdonald *et al,* 2010).

Although *dlx1a/2a* are required for GABAergic neuron specification and migration, there is no direct evidence that in zebrafish *dlx1a/2a*-expressing cells give rise to GABAergic neurons. The primary objective of my research was to understand the fate of neuronal progenitor cells expressing *dlx1a/2a* in the zebrafish brain, especially to characterize their contribution to GABAergic neurons. We hypothesized that during zebrafish forebrain development, *dlx1a/dlx2a*-expressing cells give rise to GABAergic interneurons. Due to the transient nature of *dlx1a/2a* expression during development, a way to label *dlx1a/2a*-expressing cells specifically and permanently is needed for the research. To address this, inducible $CreER^{T2}$-mediated recombination system was introduced into this study. We generated lines of transgenic fish expressing the $CreER^{T2}$ recombinase driven by URE2 and I12b. We also showed that I12b and

URE2 drive CreER^{T2} expression in a pattern which closely resembles *dlx1a* and *dlx2a* expression in forebrain regions. These fish were mated with *Tg(ubi:Switch)* reporter fish that express a loxP-flanked GFP gene followed by mCherry, driven by the ubiquitin promoter (Mosimann et al., 2011). Upon tamoxifen treatment, *dlx1a/dlx2a*-expressing cells were labelled with mCherry in the double transgenic fish, which allow us to follow the development of *dlx1a/dlx2a*-expressing cells in the zebrafish brain. Using this system, we have examined questions including 1) Do *dlx1a/dlx2a*-expressing cells give rise to GABAergic neurons? If so, what would be the contribution of *dlx1a/dlx2a*-expressing cells to different subtypes of GABAergic neurons? 2) Would *dlx1a/dlx2a*-expressing cells present at different time points populate different brain regions or acquire different fates? 3) What is the role of *dlx1a/dlx2a*-expressing cells during adult neurogenesis? By examining these questions, we hope this research will provide insights into the fate of *dlx1a/dlx2a*-expressing progenitors and how they populate or repopulate the adult brain.

2 MATERIALS AND METHODS

2.1 Animal Maintenance

Zebrafish were raised at 28.5°C according to standard methods described in Nüsslein-Volhard and Dahm (2002). All experiments were performed according to the guidelines of the Canadian Council on Animal Care and were approved by the University of Ottawa animal care committee. Adult zebrafish were maintained and bred at 28.5°C with a controlled 14h light cycle. Newly born embryos were collected and sorted. Embryos were raised at similar densities in an incubator at 28.5°C. Embryos were euthanized by an overdose of tricainemesylate (ethyl 3-aminobenzoate methanesulfonate, Sigma).

2.2 Construct for Transgenesis

The *cmlc2-EGFP* sequence was PCR amplified from pTI156Cre available in our lab with designed primers 5' GGCGTCGACTAGCTTAAATCAGTTGTG 3' (forward) and 5' GCAAGCTTCTTGTTTATTGCAGCTTAT 3' (reverse). The amplified sequence was excised by *Sal*I and *Hin*dIII and then subcloned into the *Xho*I and *Hin*dIIII site of a pSP72 vector with Tol2 arms, a βGlobin (βG) minimal promoter and a polyadenylation signal (polyA), generating a vector named pSP72-c*mlc*2-*EGFP*.

The URE2 and I12b sequences were PCR amplified from zebrafish genomic DNA with primers 5' GCGGAGCTCGCAAAGCACAGAATTATTC 3' (URE2 forward with *Sac*I restriction endonuclease site), 5' GCGGAGCTCATCCCCTTTAGGGTTTTTG 3' (URE2 reverse with *Sac*I restriction endonuclease site), 5' GCGAAGCTTCGACATGCAGCTGTGGAG 3' (I12b forward with *Hin*dIIII restriction endonuclease site), and 5' GCGAAGCTTAGAAGAAAATAAAGCAGCC 3' (I12b reverse with *Hin*dIIII restriction

endonuclease site). Amplified DNA fragments were then ligated into pDrive vectors. The URE2 sequence was excised from pDrive vector by *Sac*I and subcloned into the *Sac*I site of pSP72-c*mlc*2-*EGFP* vector to produce pSP72-URE2-I12b-c*mlc*2-*EGFP* vector. The I12b sequence was then excised from pDrive vectors by *Hin*dIIII and subcloned into the *Hin*dIIII site of pSP72-URE2-I12b-c*mlc*2-*EGFP* vector. Following these steps, the *CreER^{T2}* sequence was then excised by *Eco*RI and *Not*I from pCAG-*CreER^{T2}* vector (available in our lab) and subcloned into the *Eco*RI and *Not*I site of pSP72-URE2-I12b-c*mlc*2-*EGFP* vector to produce the final construct named Tol2 (URE2&I12b*: CreER^{T2};cmlc2-EGFP).* The Tol2 (URE2&I12b*: CreER^{T2};cmlc2-EGFP)* construct was then sequenced to make sure that there is no mutation in any of the elements.

2.3 Zebrafish Transgenesis

Tol2-mediated transgenesis was used to generate transgenic zebrafish. The injection mixture was made fresh by mixing transgene plasmid (50ng/µl) and *Tol2* mRNA (100ng/µl) with 0.05% phenol red (a monitoring dye for injection) before the injection. The *Tol2* mRNA can be translated into a functional transposase capable of catalyzing transposition. The functional transposase excises the Tol2 construct from the plasmid and facilitates integration of the transgene into the genome of the zebrafish, increasing the efficiency of the transgenesis (Kawakami et al., 2000). The *Tol2* mRNA was synthesized by *in vitro* transcription using the mMessage mMachine SP6 kit (AM1340, Ambion). A Narishige IM300 microinjector was used for microinjection. Wild-type embryos were injected with the mixture at the one cell stage. Embryos that express EGFP in the heart were raised to adulthood (around four months). The offspring of EGFP+ injected fish and wild type fish were screened at 3dpf for EGFP expression in the heart to identify transgenic founder fish (F0) that transmit the transgene to the next generation. If F1 embryos showed EGFP expression

in the heart (*cmlc2* promoter targets EGFP expression in the heart), the F0 fish was considered as a founder fish. F1 embryos were then raised to establish stable lines.

2.4 4-OHT treatment for CreER^{T2} Induction

Hydroxy tamoxifen (4-OHT; H7904, Sigma) was dissolved in ethanol as a stock concentration of 10 mM. The stock solution was stored as single-use aliquots in the dark at –20°C. To induce CreER^{T2}-mediated recombination in embryos, 15-20 stage-matched embryos were placed in each well of a six-well culture plate. Embryos were treated with 5 µM 4-OHT (for embryos at 13hpf, 24hpf, 48hpf and 72hpf) or 10 µM 4-OHT (for embryos at 120hpf) in E3 medium (5.0 mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, 0.33mM $MgSO_4$, and diluted 50X in distilled water), freshly mixed. The treated embryos were incubated at 28.5°C in the dark for 24 hours. Embryos were then washed three times with 50 mL of E3 medium to remove 4-OHT completely.

To induce CreER^{T2}-mediated recombination in adult zebrafish, 5-8 month old fish were induced by soaking in 5µM 4-OHT for 6-8 hours in the dark. Induced fish were then placed in tanks with fresh system water for recovery.

2.5 Whole Mount *in situ* Hybridization

The antisense mRNA probes were labelled with digoxygenin (DIG)-UTP using DIG RNA Labeling Mix (11277073910, Roche) and synthesized from cDNA clones available in our lab: *dlxla* (Ellies, Stock, et al., 1997), *dlx2a* (Akimenko et al. 1994), *CreER^{T2}* (generated by Cynthia Solek). The vector that contained the cDNA clone was linearized with the appropriate enzyme and the antisense mRNA probes were synthesized using the T7 polymerase (Table 2.1).

Embryos were fixed at 48 hours post fertilization (hpf) in 4% paraformaldehyde (PFA), washed in PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.1% Tween 20, pH=7.4) and dehydrated in methanol for storage at -20°C. Embryos were then rehydrated sequentially in MeOH 75% / PBST 25%, MeOH 50% / PBST 50%, MeOH 25% / PBST 75% for 5 min each followed by four 5 min washes in PBST. To permeabilize embryos, 48hpf embryos were treated by 10 µg/ml of proteinase K for 5 min. Embryos were washed for 5 min in PBST, re-fixed in 4% PFA for 20 min and washed 5 times for 5 min in PBST. Embryos were then pre-hybridized in 1ml of hybridization mix (50% formamide, 5X SSC , 92 µM citric acid pH 6, 0.1% tween and sterile water), at 70°C for 2 hrs. For probe hybridization, embryos were incubated in 200 µL of hybridization mix 'plus' (hybridization mix with 50 µg/ml heparin and 500 µg/ml tRNA) with 100-200 ng of probe overnight at 70°C.

Embryos were gradually washed at 70°C in 75% hybridization mix / 25% 2X SSC, 50% hybridization mix / 50% 2X SSC, 25% hybridization mix / 75% 2X SSC and 100% 2X SSC for 15 min per wash. Embryos were then washed twice for 30 min in 0.2X SSC at 70°C. 0.2X SSC was progressively replaced with PBST through a series of 10-min washes at room temperature in 75% 0.2X SSC / 25% PBST, 50% 0.2X SSC / 50% PBST, 25% 0.2X SSC / 75% PBST and PBST. embryos were incubated for 2 hours at room temperature in blocking buffer (2% calf serum and 2 mg/ml bovine serum albumin in PBST) to saturate nonspecific binding sites for the antibody. Embryos were then incubated in anti-DIG antibody solution diluted at 1/1000 with blocking buffer overnight at 4°C. The antibody solution was discarded and the embryos were washed briefly in PBST, followed by six 15 min washes in PBST. Embryos were washed 3 times in phosphatase alkaline buffer, 5 min per wash. Embryos were treated in a staining solution of PAB, 226 µg/ml nitroblue terazolium (NBT), and 175 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP)

prepared fresh and kept in the dark. When the desired staining intensity was reached, the reaction was stopped by washing the embryos 3 times for 5 min per wash in 1 mM EDTA in PBST. They were then post-fixed in 4% PFA overnight at 4°C. Embryos were equilibrated in 100% glycerol before imaging.

2.6 Tissue Collection and Sectioning

Zebrafish were euthanized in 200-500mg/L tricainemesylate. The heads of adult fish were detached by cervical severing and dissected in a plate with 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH=7.4) buffer. Brains were removed and fixed in 4% PFA overnight at 4 °C. 10dpf and 45dpf larvae were fixed in 4% PFA overnight at 4 °C directly without dissection. Fixed samples were washed three times in 1X PBS solution then equilibrated in 30% sucrose/1X PBS at 4 °C overnight. The fixed samples were embedded in OCT compound (Tissue Tek) and immediately frozen in liquid nitrogen. Frozen cryostat blocks were sectioned using a Leica CM1850 sectioning machine. Transverse sections of 12µm thickness were collected on Superfrost Plus slides (Fisher). The slides were stored at -80 °C until further use.

Table 1: cDNA clones used to synthesize mRNA antisense probes for *in situ* hybridization

cDNA clone	**Plasmid**	**Linearization Enzyme**	**RNA Polymerase**	**Probe Length (Kb)**
dlxla	pBluescript-SK	BamHI	T7	0.78
dlx2a	pBluescript-SK	BamHI	T7	1.67
CreER^{T2}	pBluescript-SK	EcoRI	T7	1.8

2.7 *In situ* Hybridization on Sections

Frozen slides were thawed at room temperature for 2 hours before *in situ* hybridization. Probes were diluted in 1 mL hybridization buffer composed of 1X salt solution (0.2 M NaCl, 10 mM Tris-HCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1 mM Tris-base, 5 mM EDTA, pH=7.5), 50% deionized/ultrapure formamide, 10% dextran sulfate, yeast tRNA 1mg/mL, 1X Denhardts solution, and ddH_20. The probe mixture was denatured for 10 min at 70°C before hybridization. Slides were placed in a humidified chamber lined with paper towel soaked with solution A (1X SSC, 50% formamide, 0.1% Tween 20). Two hundred microliters of the probe mix were added to each slide. The slides were then covered with coverslips and incubated overnight at 70°C. Slides were transferred to a Coplin jar and washed twice for 30 min at 70°C with solution A after the incubation. Slides were washed twice in 1X TBST (140 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl pH=7.5, 0.1% Tween 20) for 30 min at room temperature. Slides were then transferred to a humidified chamber lined with paper towel soaked in distilled water. Slides were then blocked in blocking solution (10% FBS in 1x TBST) for 2 hours at room temperature. A 200 µL of 1:1000 dilution of anti-DIG AP Fab fragment antibody (Roche) in blocking solution was applied to each slide. Slides were then incubated overnight at 4°C. Slides were transferred to Coplin jars and washed five times for 20 min in 1X TBST at room temperature. Slides were then washed twice for 10 min in 1X NTMT staining buffer (100 mM NaCl, 100 mM Tris HCl pH=9.5, 50 mM $MgCl_2$, and 0.1% Tween 20). Slides were then stained with 250 µL of 1X NTMT containing 1.7 µL of 50 mg/mL BCIP and 2.3 µL of 50 mg/mL NBT and incubated in the dark for 2 hours. Following the staining, slides were washed twice in ddH_20 for 5 min to stop the staining reaction. Slides were fixed by adding 300 µL of 4% PFA for 20 min at room temperature, and then rinsed with ddH_20. Slides were

mounted with a Vectashield mounting medium (Vector Labs) and visualized with a Zeiss Axiophot fluorescence microscope.

2.8 Immunohistochemistry on Sections

Before immunostaining assays, frozen slides were thawed at room temperature for 2 hours. Slides were soaked in methanol overnight at -20^{o}C. The following day after three 10 min washes in 1X PBST, sections were incubated with a blocking solution (10% calf serum in 1X PBST) for 2 hours at room temperature. Slides were then incubated in a humid chamber overnight at 4^{o}C with primary antibodies diluted in a solution of 1% calf serum in PBST.

Samples were then washed three times for 15 min in PBST, followed by one 5 min wash in PBS. Sections were incubated in the dark at room temperature with secondary fluorescence-coupled antibody diluted in a solution of 1% calf serum in PBST for 2 hours. Slides were then washed three times in PBST for 15 min in the dark, followed by one 10 min wash in PBS. Slides were mounted with a Vectashield mounting medium (Vector Labs). Sections were visualized with a Zeiss Axiophot fluorescence microscope.

The following primary antibodies were used in this study: polyclonal rabbit anti-mCherry (1:500, Abcam), mouse anti-calretinin (1:500, Swant), polyclonal rabbit anti-GABA (1:1000, Sigma). The following secondary antibodies were used: Alexa Fluor 488® goat anti-rabbit IgG (H + L) (1:1000 Invitrogen), Alexa Fluor 488® goat anti-mouse IgG (H + L) (1:1000, Invitrogen), Alexa Fluor 594® goat anti-rabbit IgG (H + L) (1:500, Invitrogen).

2.9 Live Confocal Imaging

Live embryos and larvae were embedded dorsal side up in 1% low melting point agarose (A9414, Sigma) and imaged using a Nikon A1 MP Multiphoton Confocal Microscope equipped with a 25X water dipping objective. Excitation lasers were at 594 (Fluorescein). Z-stacks were taken at approximately 120-200 μm thickness.

3 RESULTS

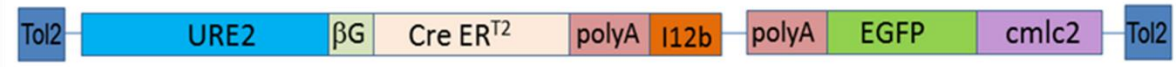
3.1 Generation of *Tg(dlx1a/2a:CreER^{T2})* and *Tg(dlx1a/2a:CreER^{T2}); Tg(ubi:Switch)* Transgenic Lines

In order to produce the *Tg(dlx1a/2a:CreER^{T2})* transgenic line, a Tol2(URE2&I12b*:CreER^{T2};cmlc2-EGFP)* construct was designed and generated (figure 3.1A). In the transgene construct, c*mlc*2 is a heart specific promoter which drives EGFP expression in the heart. URE2 and I12b, two enhancers of *dlx1a/2a,* are used to drive *CreER^{T2}* expression in *dlx1a/2a*- expressing cells. To make the construct, the *cmlc2-EGFP* sequence was subcloned into a pSP72 vector with Tol2 arms, a βGlobin (βG) minimal promoter and a polyadenylation signal (polyA) (made by Cynthia Solek and Gary Hatch). The URE2 and I12b sequences were PCR amplified from zebrafish genomic DNA and ligated into pDrive vectors. URE2, I12b, *CreER^{T2}* were subsequently subcloned into the pSP72 vector to produce the final construct. The Tol2(URE2&I12b*:CreER^{T2};cmlc2-EGFP)* construct was then sequenced to make sure that there is no mutation in any of the elements.

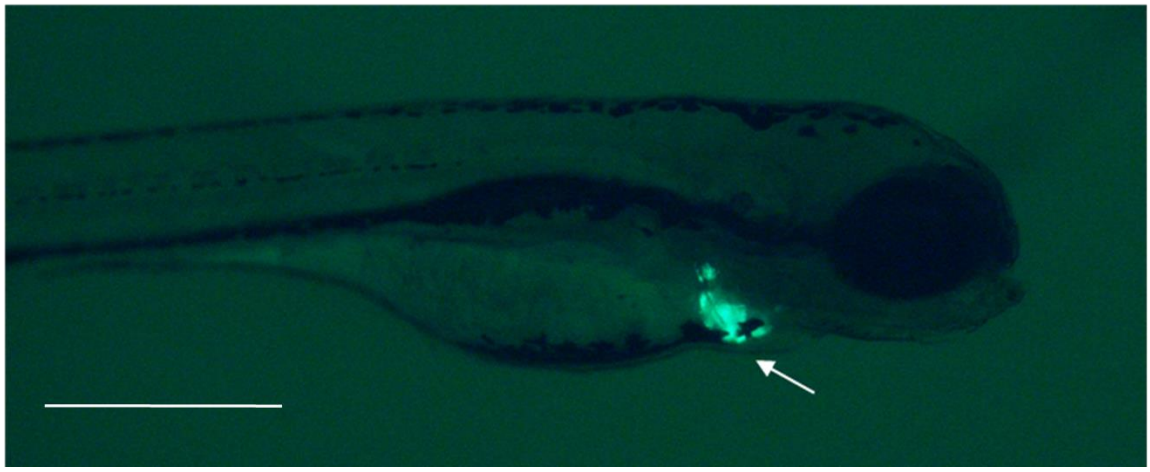
About 3000 embryos were injected with the DNA construct (50ng/µl) and *Tol2* transposase mRNA (100ng/µl) at the one cell stage. Embryos that harbor the transgene and thus express EGFP in the heart were identified using fluorescence microscopy (figure 3.1B). Approximately 250 EGFP+ injected fish survived and were raised to adulthood (around four months). To identify transgenic founder fish (F0) that transmit the transgene to the next generation, the EGFP+ injected fish were crossed with wild type fish. F1 embryos were screened for EGFP expression in the heart to determine transmission of the transgene. After screening 150 EGFP+ fish, 10 transgenic founder fish were identified. F1 embryos from the founder fish were raised to produce stable *Tg(dlx1a/2a:CreER^{T2})* transgenic lines.

Figure 3.1 Generation of *Tg(dlx1a/2a:CreER^{T2})* transgenic zebrafish (A) Schematic representation of the Tol2(URE2&I12b*: CreER^{T2};cmlc2-EGFP)* construct. To produce the construct, the *cmlc2-EGFP,* URE2, I12b, *CreER^{T2}* sequences were subsequently subcloned into a pSP72 vector with Tol2 arms, a βG minimal promoter and a polyA. In the transgene construct, c*mlc*2 is a heart specific promoter directing EGFP expression in the heart. URE2 and I12b are used to drive *CreER^{T2}* expression in *dlx1a/2a*- expressing cells. (B) EGFP expression in the heart of zebrafish injected with Tol2(URE2&I12b*: CreER^{T2};cmlc2-EGFP)* vector at 4dpf (marked by the arrow). Scale bar 1000 μm.

A



B



To generate *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) double transgenic lines, F1 *(dlx1a/2a:CreER^{T2})* individuals were crossed with *Tg*(*ubi:Switch*). The *Tg*(*ubi:Switch*) reporter fish express a loxP-flanked GFP gene followed by mCherry, driven by the ubiquitin promoter. The *ubi* promoter directs transgene expression in all examined adult organs and at all developmental stages (Mosimann et al., 2011). The offspring of *Tg(dlx1a/2a:CreER^{T2})* and *Tg*(*ubi:Switch*) consist of wild-type embryos, *Tg(dlx1a/2a:CreER^{T2})* embryos that exhibit strong EGFP expression in the heart (figure 3.2A), *Tg*(*ubi:Switch*) embryos that exhibit moderate ubiquitous EGFP expression (figure 3.2B), and *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) embryos that show both moderate ubiquitous EGFP expression and strong cardiac EGFP expression (figure 3.2C). The latter were selected for further analysis.

3.2 Characterization of *CreER^{T2}* Expression in Tg(*dlx1a/2a*: *CreER^{T2}*) Transgenic Lines

To determine if *CreER^{T2}* expression in *Tg(dlx1a/2a:CreER^{T2})* lines mimics the endogenous *dlx1a/2a* expression in the brain, *in situ* hybridization was performed using probes against *dlx1a*, *dlx2a* and *CreER^{T2}* in *Tg(dlx1a/2a:CreER^{T2})* transgenic animals (figure 3.3). At 48hpf, *CreER^{T2}* expression was found in the telencephalon, prethalamus, and hypothalamus in *Tg(dlx1a/2a:CreER^{T2})* (figure 3.3E, F), with patterns closely resembling the endogenous forebrain expression of *dlx1a* (figure 3.3A, B) and *dlx2a* (figure 3.3C, D). To examine if *CreER^{T2}* is also correctly expressed in the brain at later developmental stages, *in situ* hybridization was performed on the head sections of *Tg(dlx1a/2a:CreER^{T2})* larvae to examine the expression pattern of *dlx1a*, *dlx2a* and *CreER^{T2}*. At 45dpf, the expression of *dlx1a*, *dlx2a* and of *CreER^{T2}* was found in similar regions of the ventral telencephalon (figure 3.4). Together, these data suggest that *CreER^{T2}* is correctly expressed in *dlx1a/2a*- expressing cells in *Tg(dlx1a/2a:CreER^{T2})* at various developmental stages.

Figure 3.2 EGFP expression in *Tg(dlx1a/2a:CreER^{T2})*, *Tg*(*ubi:Switch*) and *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) at 3dpf. *Tg(dlx1a/2a:CreER^{T2})* embryos exhibit strong EGFP expression in the heart (A, arrow), *Tg*(*ubi:Switch*) embryos show moderate ubiquitous EGFP expression (B), and *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) embryos exhibit both moderate ubiquitous EGFP expression and strong cardiac EGFP expression (C, arrow). Scale bar 1000 µm.

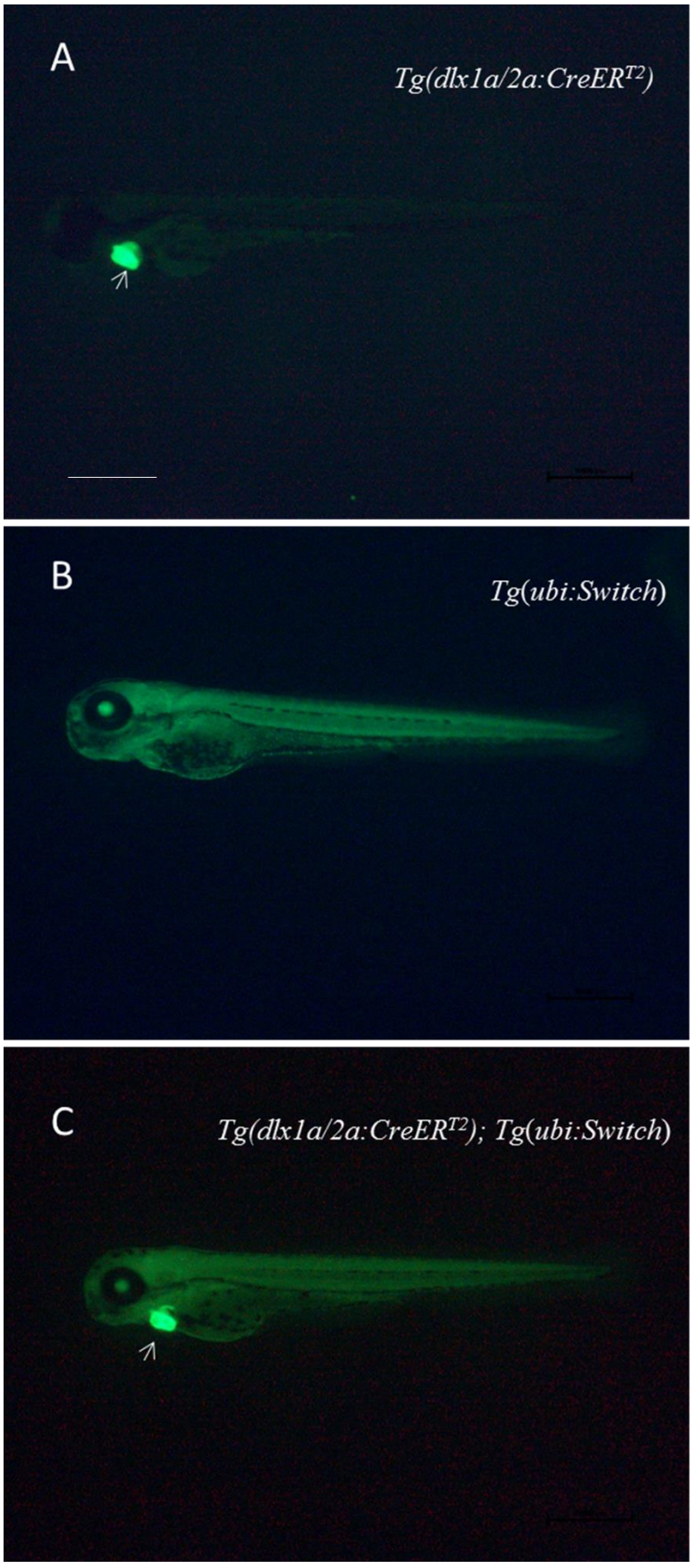

A
Tg(dlx1a/2a:CreER^T2)
B
Tg(ubi:Switch)
C
Tg(dlx1a/2a:CreER^T2); Tg(ubi:Switch)

Figure 3.3 Whole-mount *in situ* hybridization showing that *CreER^{T2}* expression in *Tg(dlx1a/2a:CreER^{T2})* zebrafish recapitulates endogenous *dlx1a/2a* expression in the forebrain at 48hpf. (A, B) Expression of *dlx1a* is seen in the telencephalon, prethalamus and hypothalamus. (C, D) The *dlx2a* gene is expressed in similar domains of the telencephalon, prethalamus and hypothalamus. (E, F) *CreER^{T2}* is expressed in the same domains of the telencephalon, prethalamus, and hypothalamus in *Tg(dlx1a/2a:CreER^{T2})* embryos. A, C, E are lateral views, dorsal is up; B, D, F are dorsal views. Anterior is to the left. Tel, telencephalon; Pth, prethalamus; Hyp, hypothalamus. Scale bar 25 μm.

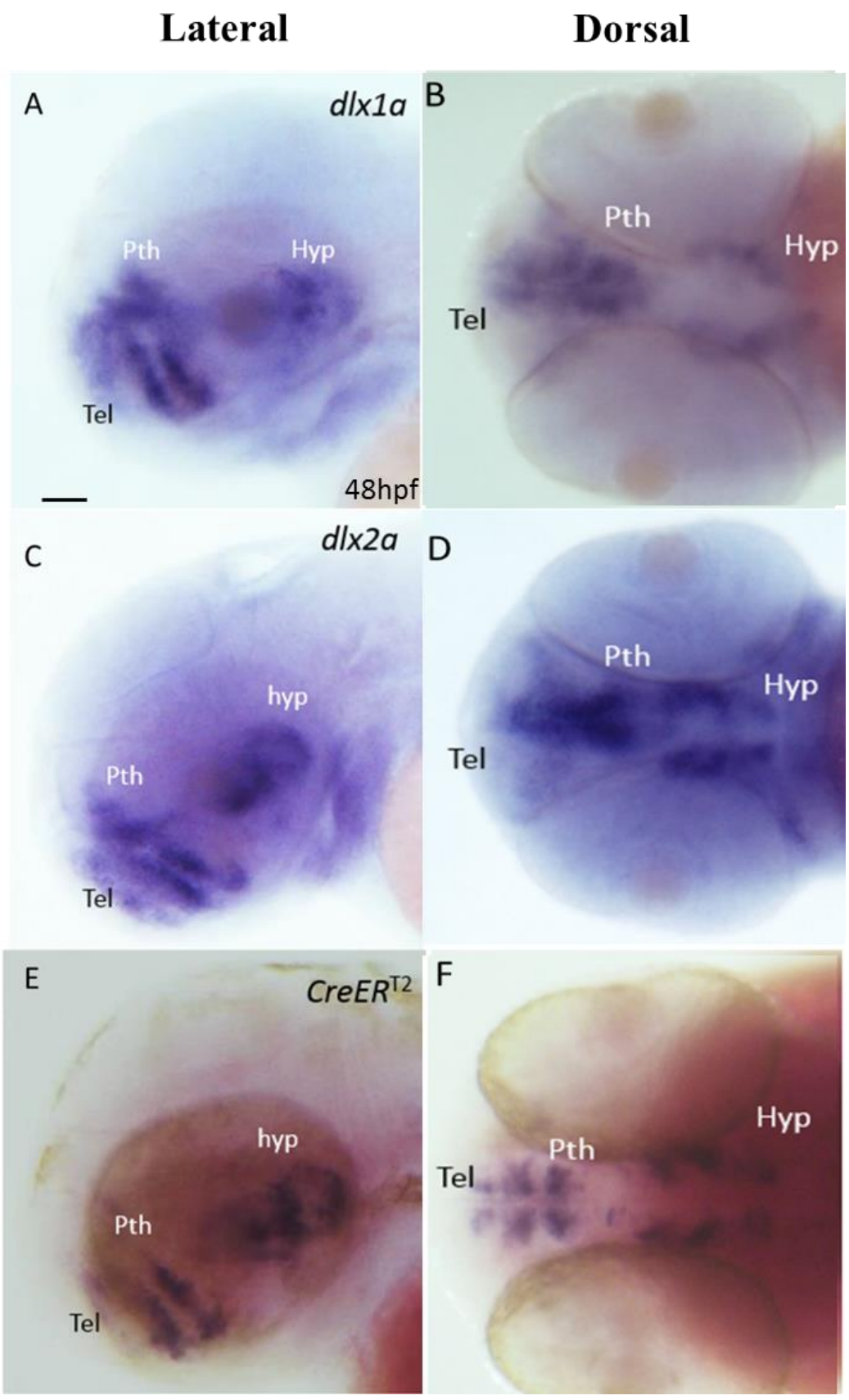
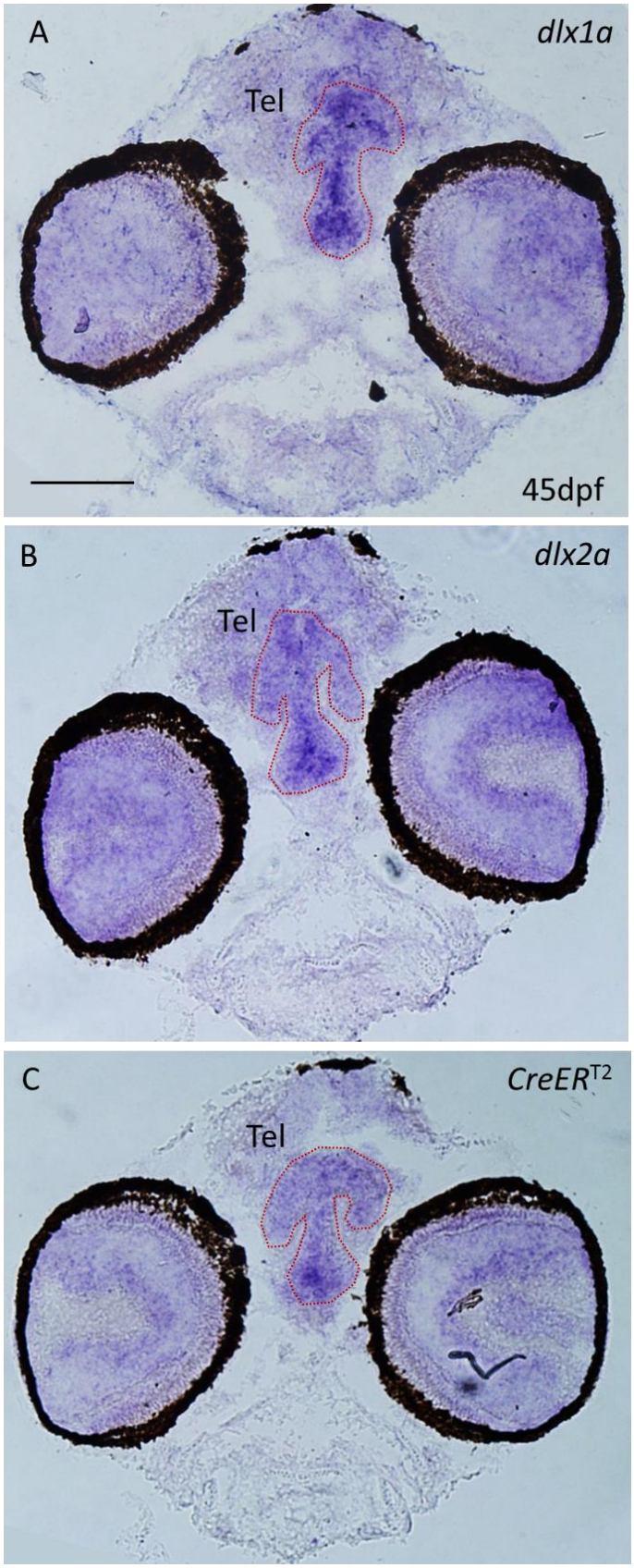

Lateral
Dorsal
A
dlx1a
Pth
Hyp
Tel
48hpf
B
Pth
Hyp
Tel
C
dlx2a
hyp
Pth
Tel
D
Pth
Hyp
Tel
E
CreER^T2
hyp
Pth
Tel
F
Hyp
Pth
Tel

Figure 3.4 *In situ* hybridization on transverse sections showing that *CreER^{T2}* expression recapitulates endogenous *dlx1a/2a* expression in the telencephalon of *Tg(dlx1a/2a:CreER^{T2})* zebrafish at 45dpf. (A) Expression of *dlx1a* is seen in the ventral telencephalon (dashed region). (B) The *dlx2a* gene is expressed in similar domains of the telencephalon (dashed region). (C) *CreER^{T2}* is expressed in the same domains of the telencephalon in *Tg(dlx1a/2a:CreER^{T2})* (dashed region) as the endogenous expression of the *dlx1a* and *dlx2a*. Tel, telencephalon. Scale bar 50 µm.


A
dlx1a
Tel
45dpf
B
dlx2a
Tel
C
CreER^{T2}
Tel

3.3 Live Imaging of *dlx1a/2a*-expressing Progenitors in Early Neurogenesis

To investigate the developing pattern of *dlx1a/2a*-expressing progenitors in the early developing brain, *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) were induced with tamoxifen at early developmental stages. Upon tamoxifen treatment, Cre/*loxP* recombination specifically occurs in the *dlx1a/dlx2a*-expressing cells of the double transgenic fish and, therefore, *dlx1a/dlx2a*-expressing cells will express mCherry permanently. The development of mCherry-expressing cells was followed by live imaging during early development. Since *dlx1a/2a* expression begins at 13hpf in the prospective forebrain (Akimenko et al. 1994; Ellies et al. 1997), *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) embryos were induced with 5μM 4-OHT from 13hpf to 24hpf. The *dlx1a/dlx2a*-expressing cells present from 13hpf to 24hpf were labeled with mCherry. The induced embryos were imaged from 2dpf to 10dpf using confocal microscopy (figure 3.5). At 2dpf, mCherry-expressing cells were detected in the telencephalon and prethalamus of double transgenic fish (figure 3.5B). This mCherry-expressing cell population remarkably increased and populated the telencephalon, prethalamus and hypothalamus by 10dpf (figure 3.5C-F).

To examine if *dlx1a/2a*-expressing progenitors present at later time points populate different brain regions, *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) embryos were induced with 4-OHT at 24hpf, 48hpf, 72hpf and 120hpf, and imaged, beginning on the day following 4-OHT induction and until 10dpf using confocal microscopy (figure 3.6-3.9). In embryos induced from 24hpf to 48hpf, mCherry-expressing cells originated from the telencephalon and prethalamus at 2dpf (figure 3.6B) and developed into large cell populations located in the telencephalon, prethalamus and hypothalamus by 10dpf (figure 3.6C-F), a pattern that is roughly similar to that obtained for *dlx1a/2a*-expressing progenitors present from 13hpf to 24hpf. In embryos induced at 48hpf and 72hpf, mCherry-expressing cells were detected in the telencephalon and prethalamus at 3dpf

(figure 3.7B) and 4dpf (figure 3.8B), respectively, and then developed into cell clusters located in the telencephalon, prethalamus and hypothalamus by 10dpf (figure 3.7C-E, figure 3.8C-E). Notably, only a few *dlx1a/2a*-expressing cells were found in the telencephalon and prethalamus at 6dpf (figure 3.9B) when the fish were induced from 120hpf to 144hpf, and those progenitors gave rise to smaller cell clusters located in the telencephalon, prethalamus and hypothalamus by 10dpf (figure 3.9D-E). Collectively, these data indicate that *dlx1a/2a-expressing* neuronal progenitors labelled at later time points seemingly gave rise to fewer progeny than the ones labelled at 13hpf or 24hpf.

Figure 3.5 Live confocal imaging of mCherry-expressing cells in double transgenic fish induced from 13hpf to 24hpf. *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) embryos were induced with 4-OHT from 13hpf to 24hpf and imaged from 2dpf to 10dpf (B-F) as illustrated in the diagram (A). The calibration of each dimension of the z-stack snapshot is indicated on the lower left corner of each image. The arrowheads point to mCherry-expressing cells. Tel, telencephalon; Pth, prethalamus; Hyp, hypothalamus.

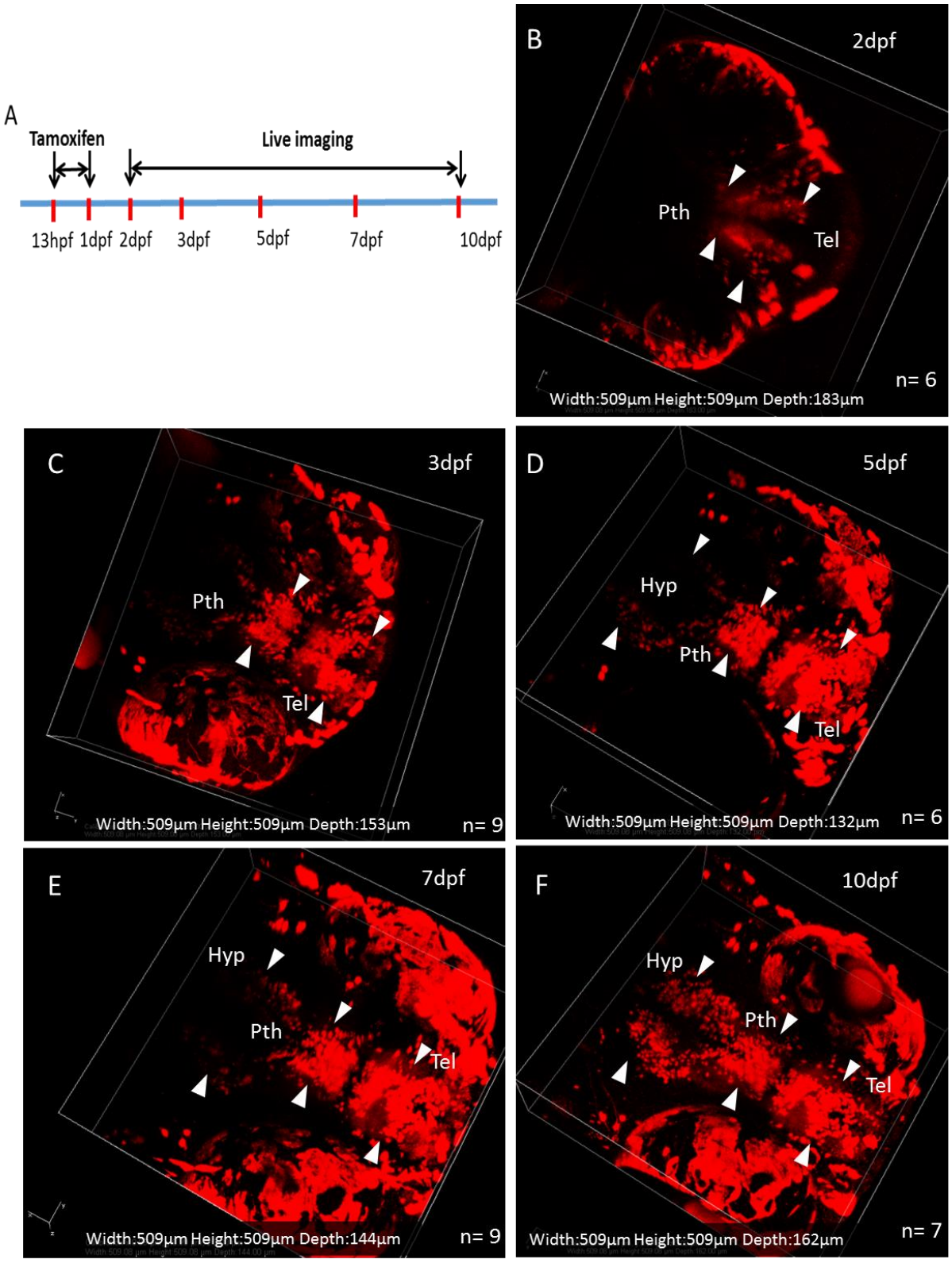

A
Tamoxifen
Live imaging
13hpf 1dpf 2dpf 3dpf 5dpf 7dpf 10dpf
B
2dpf
Pth
Tel
n= 6
Width:509µm Height:509µm Depth:183µm
C
3dpf
Pth
Tel
Width:509µm Height:509µm Depth:153µm
n= 9
D
5dpf
Hyp
Pth
Tel
Width:509µm Height:509µm Depth:132µm
n= 6
E
7dpf
Hyp
Pth
Tel
Width:509µm Height:509µm Depth:144µm
n= 9
F
10dpf
Hyp
Pth
Tel
Width:509µm Height:509µm Depth:162µm
n= 7

Figure 3.6 Live confocal imaging of mCherry-expressing cells in double transgenic fish induced from 24hpf to 48hpf. *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) embryos were induced with 4-OHT from 24hpf to 48hpf and imaged from 2dpf to 10dpf (B-F) as illustrated in the diagram (A). The calibration of each dimension of the z-stack snapshot is indicated on the lower left corner of each image. The arrowheads point to mCherry-expressing cells. Tel, telencephalon; Pth, prethalamus; Hyp, hypothalamus.

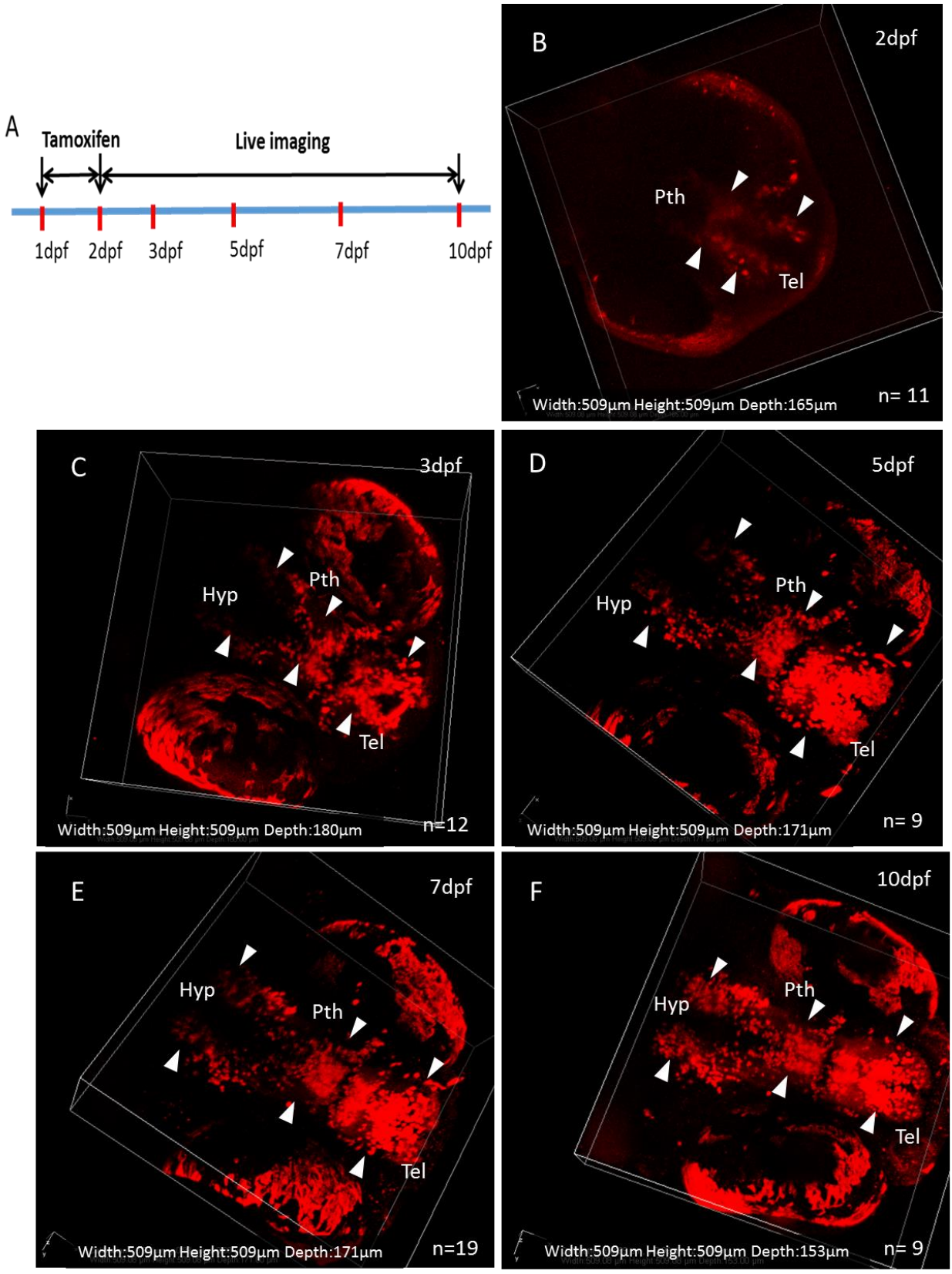

A
Tamoxifen
Live imaging
1dpf
2dpf
3dpf
5dpf
7dpf
10dpf
B
2dpf
Pth
Tel
Width:509µm Height:509µm Depth:165µm
n= 11
C
3dpf
Hyp
Pth
Tel
Width:509µm Height:509µm Depth:180µm
n=12
D
5dpf
Hyp
Pth
Tel
Width:509µm Height:509µm Depth:171µm
n= 9
E
7dpf
Hyp
Pth
Tel
Width:509µm Height:509µm Depth:171µm
n=19
F
10dpf
Hyp
Pth
Tel
Width:509µm Height:509µm Depth:153µm
n= 9

Figure 3.7 Live confocal imaging of mCherry-expressing cells in double transgenic fish induced from 48hpf to 72hpf. *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) embryos were induced with 4-OHT from 48hpf to 72hpf and imaged from 3dpf to 10dpf (B-E) as illustrated in the diagram (A). The calibration of each dimension of the z-stack snapshot is indicated on the lower left corner of each image. The arrowheads point to mCherry-expressing cells. Tel, telencephalon; Pth, prethalamus; Hyp, hypothalamus.

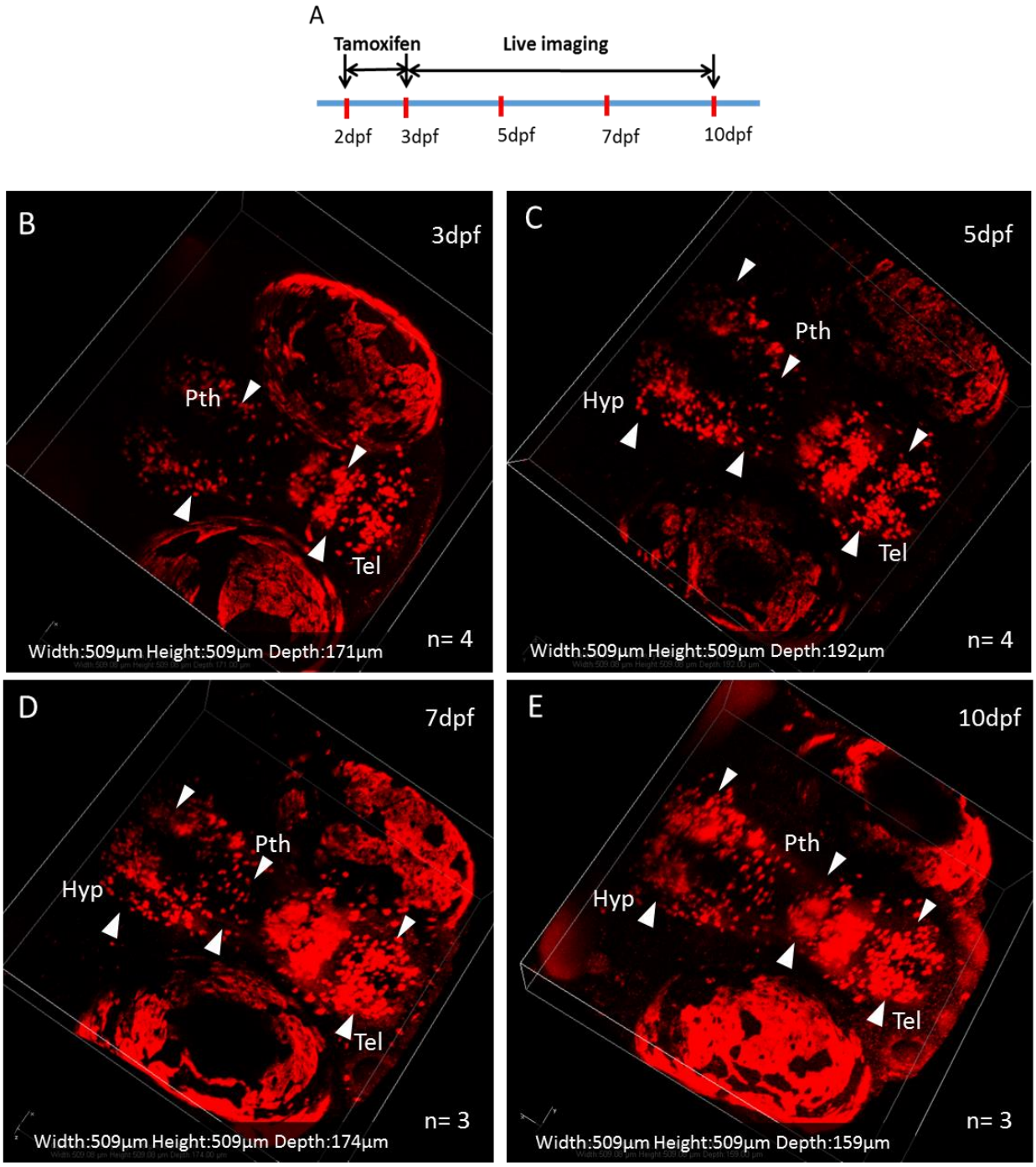

A
Tamoxifen
Live imaging
2dpf
3dpf
5dpf
7dpf
10dpf
B
3dpf
Pth
Tel
Width:509µm Height:509µm Depth:171µm
n= 4
C
5dpf
Pth
Hyp
Tel
Width:509µm Height:509µm Depth:192µm
n= 4
D
7dpf
Pth
Hyp
Tel
Width:509µm Height:509µm Depth:174µm
n= 3
E
10dpf
Pth
Hyp
Tel
Width:509µm Height:509µm Depth:159µm
n= 3

Figure 3.8 Live confocal imaging of mCherry-expressing cells in double transgenic fish induced from 72hpf to 96hpf. *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) embryos were induced with 4-OHT from 72hpf to 96hpf and imaged from 4dpf to 10dpf (B-E) as illustrated in the diagram (A). The calibration of each dimension of the z-stack snapshot is indicated on the lower left corner of each image. The arrowheads point to mCherry-expressing cells. Tel, telencephalon; Pth, prethalamus; Hyp, hypothalamus.

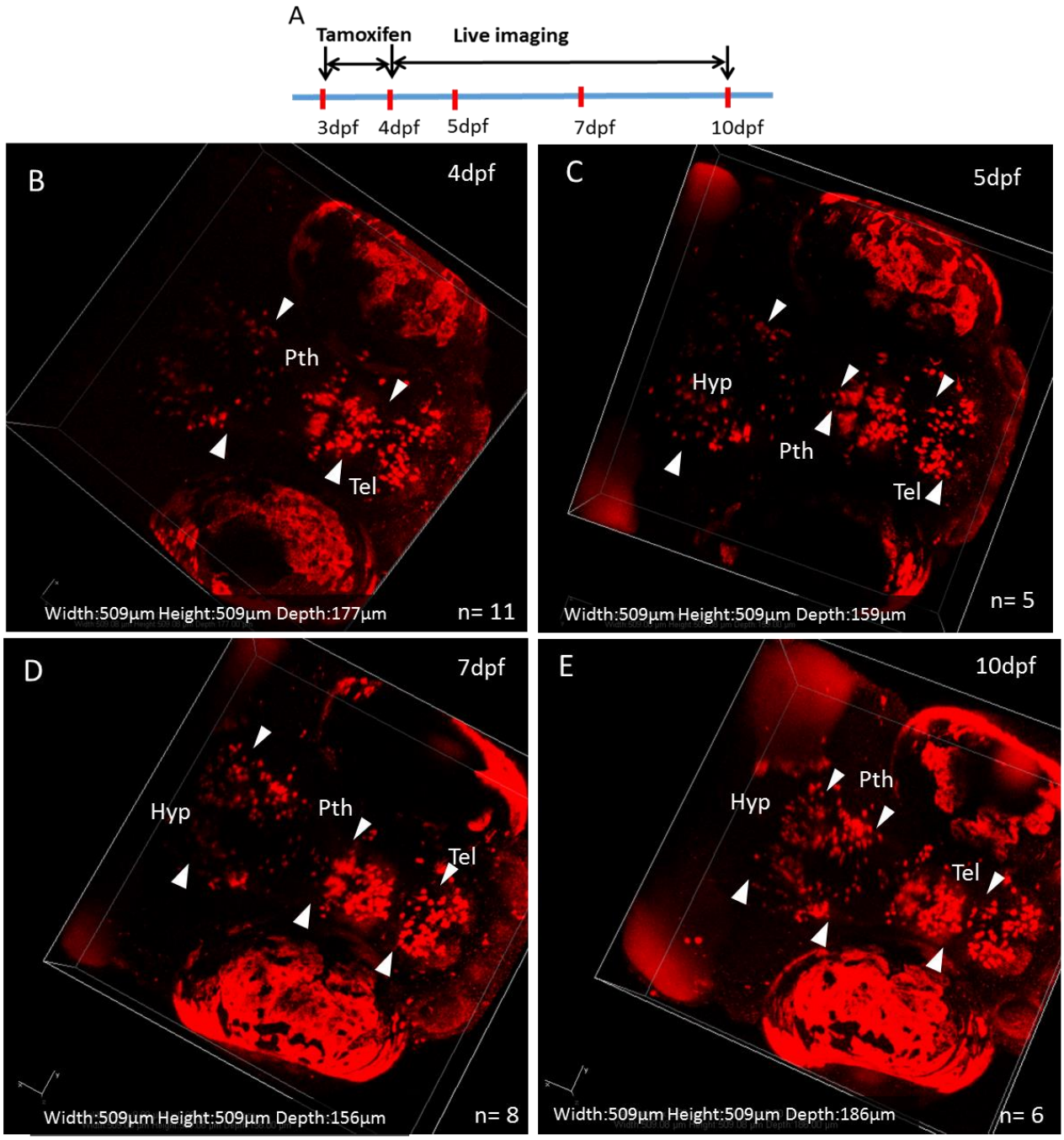
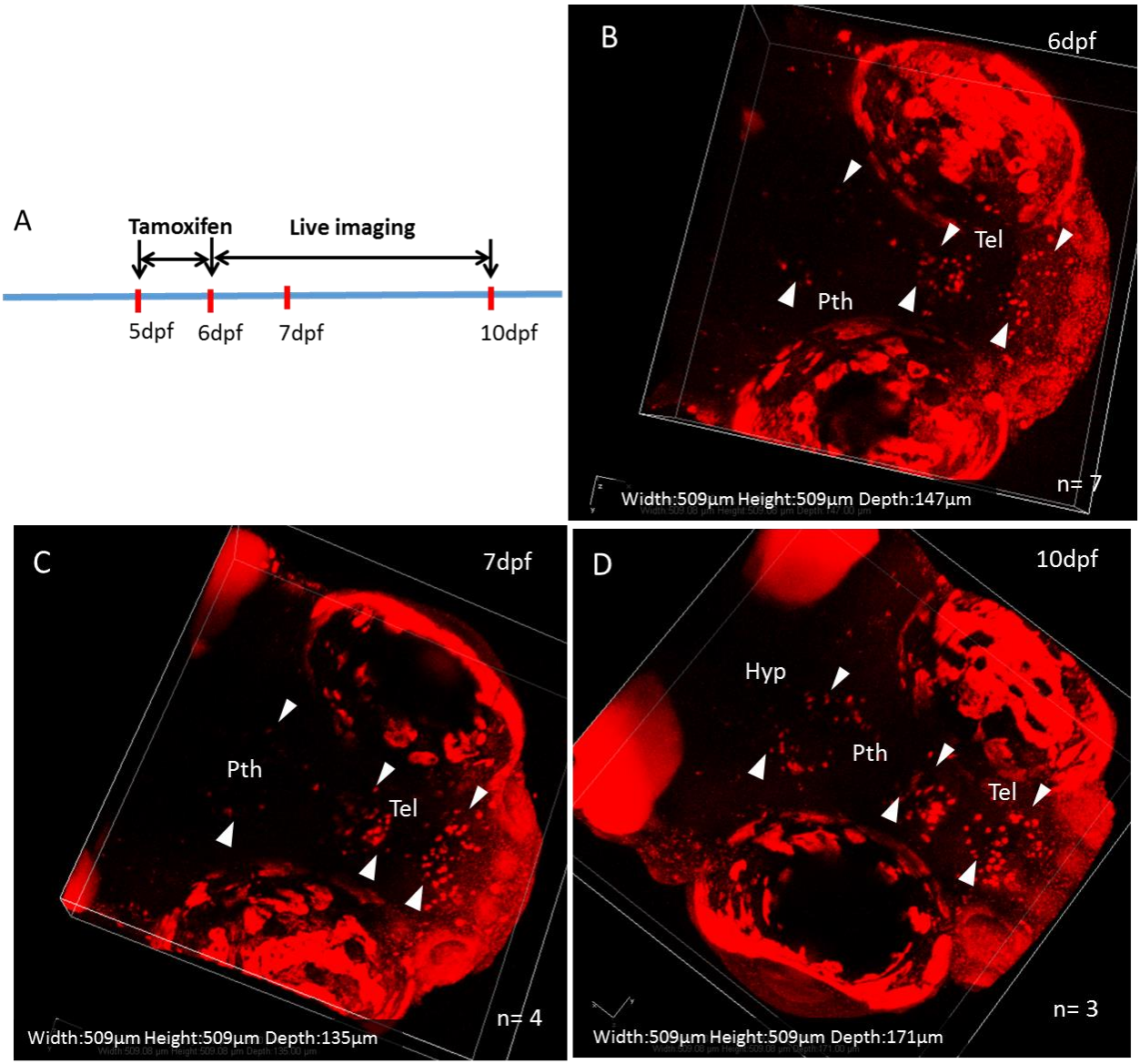

A
Tamoxifen
Live imaging
3dpf
4dpf
5dpf
7dpf
10dpf
B
4dpf
Pth
Tel
Width:509µm Height:509µm Depth:177µm
n= 11
C
5dpf
Hyp
Pth
Tel
Width:509µm Height:509µm Depth:159µm
n= 5
D
7dpf
Hyp
Pth
Tel
Width:509µm Height:509µm Depth:156µm
n= 8
E
10dpf
Hyp
Pth
Tel
Width:509µm Height:509µm Depth:186µm
n= 6

Figure 3.9 Live confocal imaging of mCherry-expressing cells in double transgenic fish induced from 120hpf to 144hpf. *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) embryos were induced with 4-OHT from 120hpf to 144hpf and imaged from 6dpf to 10dpf (B-D) as illustrated in the diagram (A). The calibration of each dimension of the z-stack snapshot is indicated on the lower left corner of each image. The arrowheads point to mCherry-expressing cells. Tel, telencephalon; Pth, prethalamus; Hyp, hypothalamus.


A
Tamoxifen
Live imaging
5dpf
6dpf
7dpf
10dpf
B
6dpf
Tel
Pth
n= 7
Width:509µm Height:509µm Depth:147µm
C
7dpf
Pth
Tel
n= 4
Width:509µm Height:509µm Depth:135µm
D
10dpf
Hyp
Pth
Tel
n= 3
Width:509µm Height:509µm Depth:171µm

3.4 Fate Mapping of *dlx1a/2a*-expressing Progenitors in the Brain

To understand the fate of neuronal progenitor cells expressing *dlx1a/2a* in the zebrafish brain, embryos from the *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) line were induced with 4-OHT from 24hpf to 48hpf and raised until 10dpf. To examine if *dlx1a/2a*-expressing progenitors give rise to GABAergic neurons, immunohistochemistry using antibodies against mCherry and GABA was performed on the transverse sections of the head of 10dpf *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* fish. We observed that a majority of mCherry-expressing cells in the ventral telencephalon, hypothalamus and inferior lobe also express GABA (figure 3.10), indicating that a large proportion of the progeny of *dlx1a/2a*-expressing progenitors become GABAergic neurons. It should be noted that a small number of mCherry-expressing cells in these domains remain free of GABA staining (figure 3.10C, F, I, arrowheads), which suggests that *dlx1a/2a-expressing* progenitors may also contribute to other cell types.

GABAergic neurons can be classified into multiple subtypes based upon the expression patterns of CB, CR, PV, SOM, NPY, VIP, nNOS and CCK. (Markram et al., 2004; Wonders and Anderson, 2006; Gelman and Marin, 2010). In mice, Cre transgenic lines that express Cre-recombinase under the control of I12b and URE2 have been generated (Potter et al., 2009). Fate-mapping data from this study shows that most Neuropeptide Y+, nNOS+, parvalbumin+, and somatostatin+ cells are marked by I12b-Cre in the cortex and hippocampus, and 25-40% of these interneuron subtypes are labelled by URE2-Cre (Potter et al., 2009). To further examine the contribution of *dlx1a/2a*-expressing progenitors to different subtypes of GABAergic neurons in zebrafish, co-localization of mCherry with some of the aforementioned markers was performed on brain sections of *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* fish. To date, data have been obtained for Calretinin on transverse sections of the brain of 10dpf *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* fish

induced with 4-OHT from 24hpf to 48hpf. Comparison of mCherry expression with Calretinin revealed that a vast majority of mCherry-expressing cells within the telencephalon and diencephalon of 10dpf embryos are not co-labelled by Calretinin (figure 3.11 and data not shown). Nonetheless, we did observe some sparse co-localization of mCherry and Calretinin in the ventral telencephalon and diencephalon (figure 3.11 arrowheads).

In order to characterize the distribution of mCherry-expressing cells in the adult zebrafish brain, embryos from the *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) line were induced with 4-OHT from 24hpf to 48hpf and raised into adults. Immunohistochemistry using antibodies against mCherry was performed on brain sections of these adult fish. Based on our observations, the distribution of mCherry-expressing cells was restricted in the olfactory bulb and to the forebrain. More specifically, we observed sparse mCherry-expressing cells located in the internal cellular layer and glomerular layer of olfactory bulb (figure 3.12A arrows). A few mCherry-expressing cells were also detected in the central zone of dorsal telencephalic area and in the dorsal nucleus of ventral telencephalic area (figure 3.12B arrows). The majority of mCherry-expressing cells were found in the ventral nucleus of the ventral telencephalic area (figure 3.12C arrows), in the posterior tuberal nucleus and in the periventricular nucleus of the posterior tuberculum (figure 3.12D arrows). In addition, mCherry-expressing cells were found lining up in the region adjacent to the caudal zone of periventricular hypothalamus (figure 3.12E arrows). Lastly, some mCherry-expressing cells were located within the dorsal zone of the periventricular hypothalamus and in the caudal zone of the periventricular hypothalamus (figure 3.12F arrows).

Figure 3.10 Double immunostaining on transverse sections showing colocalization of mCherry and GABA in the brain of *Tg(dlx1a/2a:CreER^{T2}); Tg(ubi:Switch)* larvae at 10dpf. A majority of mCherry-expressing cells in the ventral telencephalon (A), hypothalamus (D) and in the inferior lobe (G) coexpress GABA (B, E, and H). Note that a small number of mCherry-expressing cells are free of GABA labeling (C, F, I, arrowheads). A–C, D–F and G–I are representative transverse sections at the telencephalon, hypothalamus and inferior lobe of the diencephalon, respectively. Dorsal is upwards in all panels. C, F, and I are merged images of (A and B), (D and E) and (G and H), respectively. Tel, telencephalon; OT, optic tectum; Hyp, hypothalamus; IL, inferior lobe. Scale bar 10 μm.

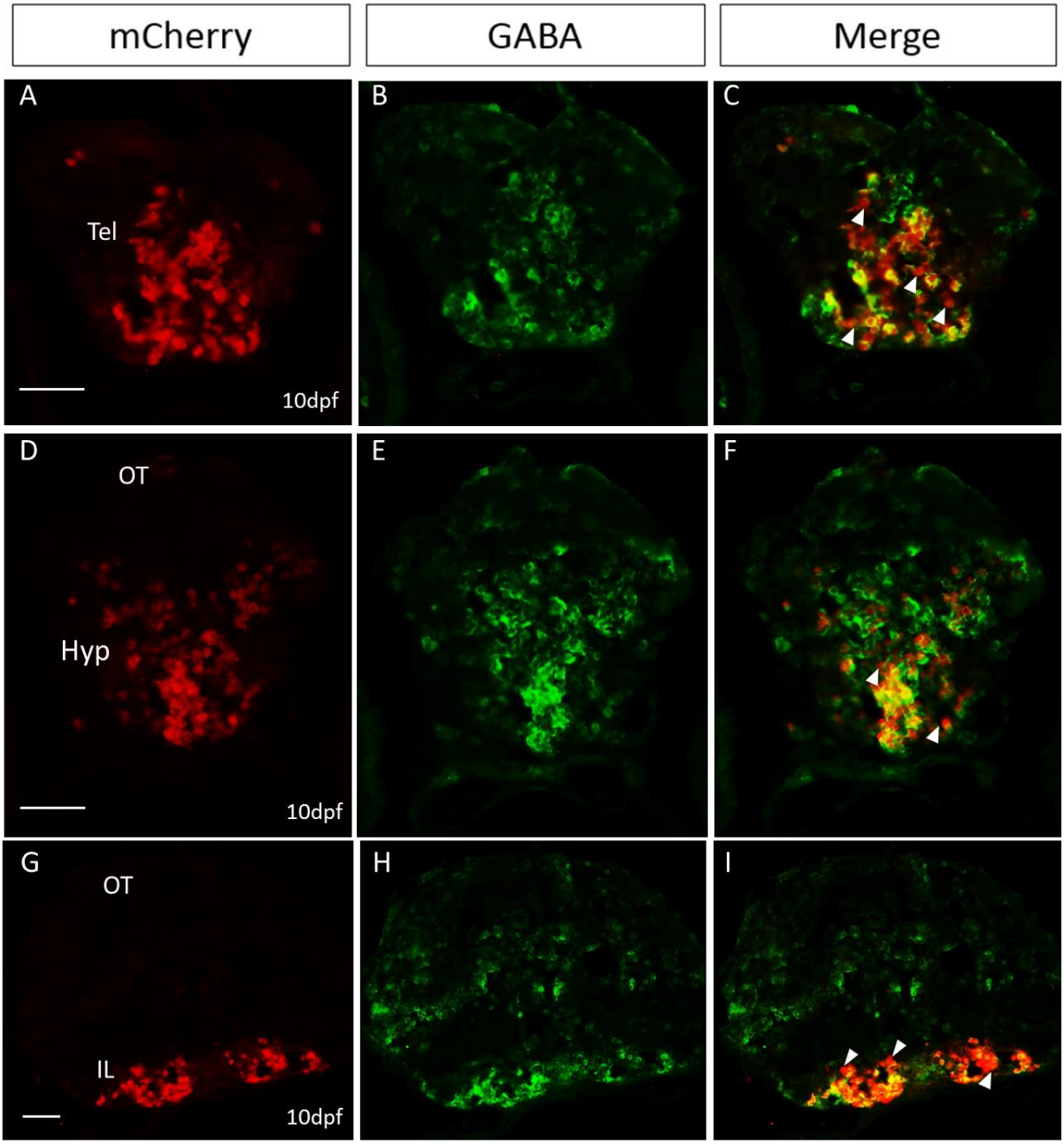
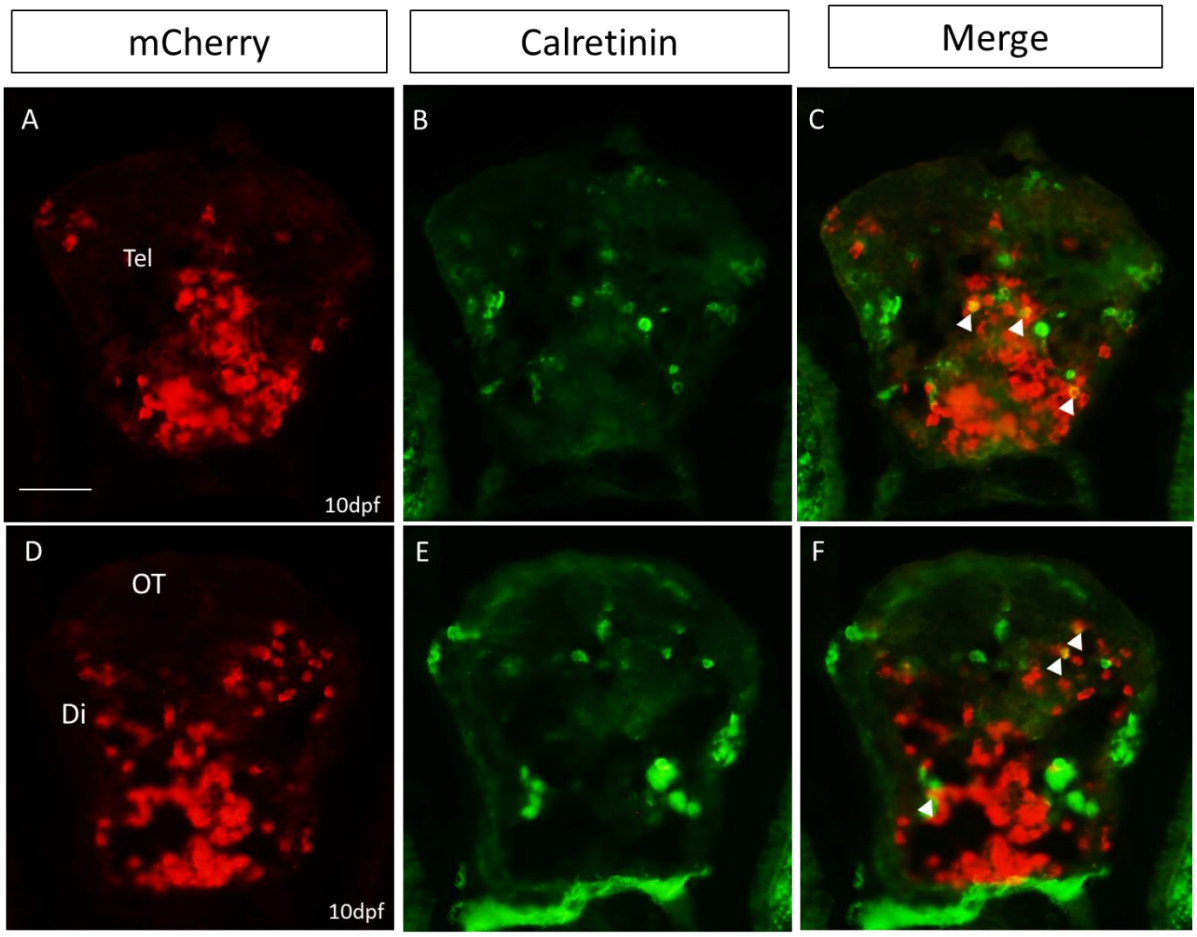

mCherry
GABA
Merge
A
B
C
Tel
10dpf
D
E
F
OT
Hyp
10dpf
G
H
I
OT
IL
10dpf

Figure 3.11 Double immunostaining on transverse sections showing sparse colocalization of mCherry and Calretinin in the brain of *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) larvae at 10dpf. A vast majority of mCherry-expressing cells (A, D) within the telencephalon and diencephalon of 10dpf embryos are not co-labelled with Calretinin (B, E). However, sparse co-localization of mCherry and Calretinin can be identified in in the ventral telencephalon and diencephalon (C, F, arrowheads). A–C and D–F are representative transverse sections of the telencephalon and diencephalon, respectively. Dorsal is upwards in all panels. C and F are merged images of (A and B) and (D and E), respectively. Tel, telencephalon; OT, optic tectum; Di, diencephalon; IL, inferior lobe. Scale bar 10 μm.


mCherry
Calretinin
Merge
A
Tel
10dpf
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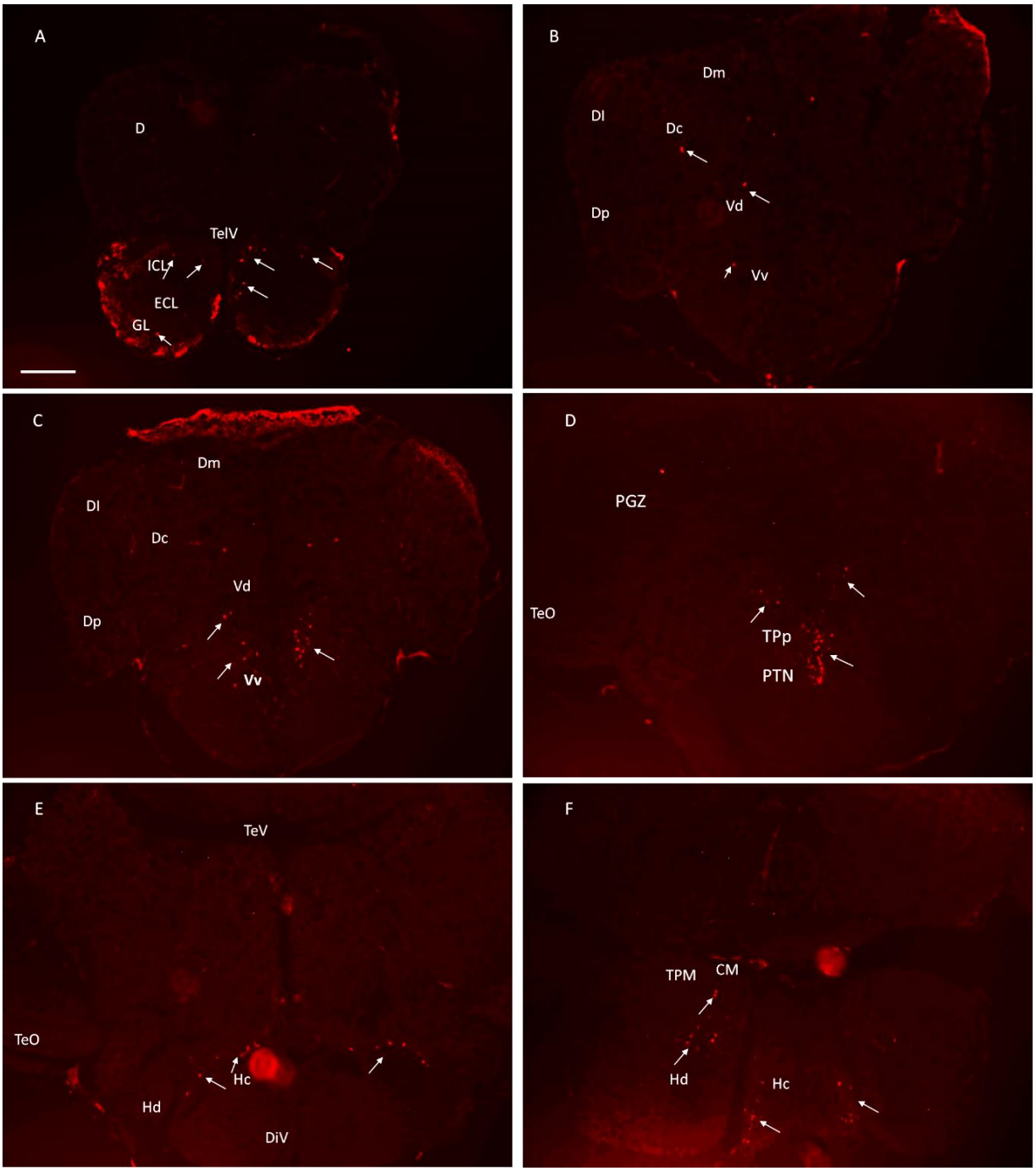
C
D
OT
Di
10dpf
E
F

Figure 3.12. Distribution of mCherry-expressing cells in the adult brain. Immunohistochemistry using antibodies against mCherry was performed on brain sections of adult *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* zebrafish induced with 4-OHT between 24hpf and 48hpf. (A) mCherry-expressing cells are found in the internal cellular layer and in the glomerular layer of the olfactory bulb. (B) A few mCherry-expressing cells can be detected in the central zone of the dorsal telencephalic area and in the dorsal nucleus of the ventral telencephalic area. (C) Clusters of mCherry-expressing cells are found in the ventral nucleus of the ventral telencephalic area. (D) The mCherry-expressing cells are located in the posterior tuberal nucleus and in the periventricular nucleus of the posterior tuberculum. (E) Some mCherry-expressing cells are found lining up in the region adjacent to the caudal zone of periventricular hypothalamus. (F) The mCherry-expressing cells are located within the dorsal zone of periventricular hypothalamus and in the caudal zone of the periventricular hypothalamus. A–C and D–F are representative transverse sections at the level of the telencephalon and diencephalon, respectively. The arrows point to mCherry-expressing cells. D, Dorsal telencephalic area; Dc, central zone of dorsal telencephalic area; DiV, diencephalic ventricle; Dm, medial zone of dorsal telencephalic area; Dp, posterior zone of dorsal telencephalic area; ECL, external cellular layer of olfactory bulb; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; ICL, internal cellular layer of olfactory bulb; GL, glomerular layer of olfactiry bulb; V, ventral telencephalic area; Vv, ventral nucleus of ventral telencephalic area; Vd, dorsal nucleus of ventral telencephalic area; TeO, optic tectum; TelV, telencephalic ventricle; TeV, tectal ventricle; TPp, periventricular nucleus of posterior tuberculum; PGZ, periventricular grey zone; PTN, posterior tuberal nucleus. Scale bar 50 µm.


A
D
TelV
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GL
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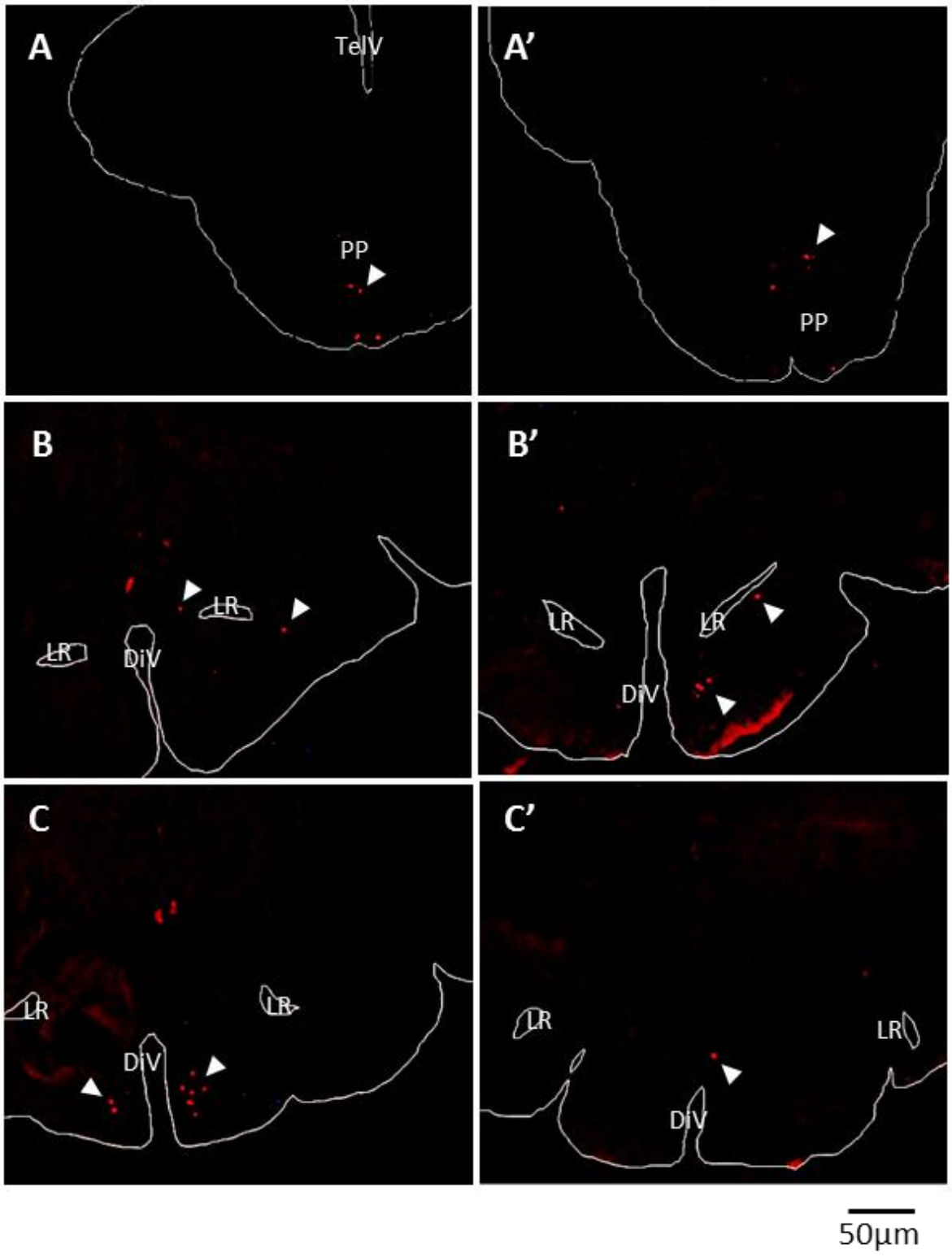
Dm
Dl
Dc
Vd
Dp
Vv
C
Dm
Dl
Dc
Vd
Dp
Vv
D
PGZ
TeO
TPp
PTN
E
TeV
TeO
Hc
Hd
DiV
F
TPM
CM
Hd
Hc

3.5 Lineage Tracing of *dlx1a/2a*-expressing Cells during Adult Neurogenesis

To characterize the contribution of *dlx1a/2a*-expressing neuronal progenitor cells during adult neurogenesis, adult fish from *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* were induced with 5µM 4-OHT for 6-8 hours. Two fish were sacrificed at each of the following time points: 1day post induction (dpi), 7dpi, 14dpi, 21dpi, 1month post induction (mopi) and 2mopi. To date, we have examined mCherry-expressing cells in the brain of *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* fish at 1mopi and 2mopi (figure 3.13). At 1mopi, mCherry-expressing cells were found in the telencephalon and diencephalon. In the telencephalon, mCherry-expressing cells were detected in the external cellular layer, in the internal cellular layer and in the glomerular layer of the olfactory bulb, as well as in the medial and central zones of the dorsal telencephalic area, in the dorsal and ventral nuclei of the ventral telencephalic area, and in the parvocellular preoptic nucleus (figure 3.13 and data not shown). In the diencephalon, mCherry-expressing cells were present in the dorsal, caudal and ventral zones of the periventricular hypothalamus, and in the anterior tuberal nucleus. At 2mopi, mCherry-expressing cells were seen in the same domains of the telencephalon and diencephalon as 1mopi. However, the distribution of mCherry-expressing cells in the olfactory bulbs is unknown since the olfactory bulbs of the 2mopi fish were damaged during dissection. Future work will focus on the characterization of mCherry-expressing cells at 1dpi, 7dpi, 14dpi and 21dpi to understand the contribution of *dlx1a/2a* during adult neurogenesis. These preliminary data suggest that *dlx1a/2a* are active in the adult brain and *dlx1a/2a*-expressing progenitors populate various regions of the telencephalon and diencephalon.

Figure 3.13 Lineage tracing of mCherry-expressing cells during adult neurogenesis. Adult fish from the *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* line were induced with 5μM 4-OHT for 6-8 hours. Cross sections of the telencephalon (A, A') and diencephalon (B, B', C, C') of induced fish at 1mopi (A, B, C), and 2mopi (A', B', C') showing the location of mCherry-expressing cells (marked by white arrowheads). TelV, telencephalic ventricle; PP, parvocellular preoptic nucleus; DiV, diencephalic ventricle; LR, lateral recess of diencephalic ventricle.


A
TelV
PP
A'
PP
B
LR
LR
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B'
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LR
DiV
C
LR
LR
DiV
C'
LR
LR
DiV
50µm

4 DISCUSSION

4.1 *Tg(dlx1a/2a:CreER^{T2})* as a Tool to Follow the Development of *dlx1a/2a*-expressing Cells in the Brain

Dlx1 and *Dlx2* encode homeodomain-containing transcription factors, which play important roles in multiple organs during development (Panganiban and Rubenstein, 2002). In the developing forebrain of the mouse, the expression of *Dlx1* and *Dlx2* is observed in progenitor cells in the subventricular and ventricular zones of the medial and lateral ganglionic eminences, which colocalizes with forebrain GABAergic neuron migration and differentiation. Similarly, partially overlapping expression patterns of *dlx* genes with *gad1b* has been seen in the developing zebrafish forebrain (Macdonald *et al,* 2010). To understand the fates of neuronal progenitor cells that express *dlx1a/2a* in the zebrafish brain, we have developed a *Tg(dlx1a/2a:CreER^{T2})* transgenic line in which *dlx1a/2a*-expressing cells are marked by *CreER^{T2}*. URE2 and I12b, two enhancers of *dlx1a/2a,* are used to drive *CreER^{T2}* expression in the transgene construct, as the activity of URE2 and I12b enhancers is sufficient to recapitulate expression of *dlx1a/2a* in the forebrain (Yu et al., 2011). Comparison of *CreER^{T2}* expression with *dlx1a* and *dlx2a* expression by *in situ* hybridization suggested that URE2 and I12b drive *CreER^{T2}* expression in a pattern closely resembling the endogenous *dlx1a/2a* expression in forebrain regions at various developmental stages.

By crossing *Tg(dlx1a/2a:CreER^{T2})* with *Tg*(*ubi:Switch*), we generated *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) double transgenic animals. By treating double transgenic animals with 4-OHT at early developmental stages, *dlx1a/2a*-expressing cells were labeled with mCherry due to Cre/*loxP* recombination. For example, in embryos induced from 24hpf to 48hpf, mCherry-expressing cells originated from the telencephalon and prethalamus at 2dpf,

and developed into large cell populations in the telencephalon, prethalamus and hypothalamus by 10dpf. The mCherry-expressing cells were distributed in various regions of the adult forebrain. In addition, mCherry-expressing cells were observed in various regions of the forebrain at both 1 month and 2 months after the adult double transgenic fish were induced with 4-OHT. Therefore, the development of *dlx1a/2a*-expressing cells can be traced throughout development in both early neurogenesis and adult neurogenesis using this transgenic zebrafish system.

4.2 Activity of *dlx1a/2a*-expressing Cells in Early Neurogenesis

We followed the development of *dlx1a/2a*-expressing cells present at different time points in early neurogenesis by inducing the double transgenic embryos with 4-OHT at different time points. In the embryos induced at different time points, mCherry-expressing cells were always observed first in the telencephalon and prethalamus, regions from which they migrated and populated the telencephalon, prethalamus and hypothalamus by 10dpf. However, *dlx1a/2a*-expressing neuronal progenitors present at later time points gave rise to fewer progeny than the ones present at 13hpf or 24hpf. For instance, *dlx1a/2a*-expressing progenitors present from 24hpf to 48hpf underwent robust cell proliferation and gave rise to a large population of cells by 10dpf, whereas *dlx1a/2a*-expressing progenitors present from 120hpf to 144hpf underwent very limited proliferation and developed into seemingly much smaller cell clusters. This suggests that *dlx1a/2a* are mainly active in the proliferating progenitors at very early developmental stages, while at later developmental stages *dlx1a/2a*-expressing cells are mainly postmitotic precursors or they are in a quiescent state.

4.3 Implication of *dlx1a/2a* in GABAergic Neuron Development

GABAergic neurons are generated from all of the major domains of the brain. Different molecular toolkits are used for GABAergic neuron differentiation in different domains of the developing brain: *Dlx* genes in the forebrain, *Gata2* in the midbrain, *Tal1* and *Ptf1a* in the hindbrain (Achim et al., 2014). In mice, forebrain GABAergic neurons first originate from the subpallial neuroepithelium that comprises LGE MGE, CGE and POA, where they tangentially migrate towards the olfactory bulb, hippocampus and neocortex (Potter et al., 2009). Likewise, in the zebrafish forebrain GABAergic neurons are derived from a region near the medial subpallial ventricular wall at 24hpf and migrate dorsolaterally to the cortex (Mueller et al., 2006). Mouse *Dlx1/2* double mutants exhibit an almost complete loss of all GABAergic neurons in the striatum, cortex, olfactory bulbs, and hippocampus, suggesting *Dlx1* and *Dlx2* are essential for GABAergic neuron differentiation and migration (Anderson, Eisenstat et al., 1997; Anderson, Qiu et al., 1997; Long et al., 2007). In mice, the I12b-Cre and URE2-Cre lineage cells has been analysed in the adult brain (Potter et al., 2009). Both I12b-Cre and URE2-Cre labeled cells populate various forebrain domains including the globus pallidus, caudate–putamen, accumbens, preoptic area, amygdala, hippocampus, cortex, thalamic reticular nucleus, hypothalamus and axonal projections from the basal ganglia to the substantia nigra. In addition, I12b-Cre and URE2-Cre lineage cells are found in the granule cells, in the external plexiform layer, and in the periglomerular cells of the olfactory bulb cells (Potter et al., 2009). In the cortex and hippocampus, about 85% of I12b-Cre labeled cells and 75% of URE2-Cre labeled cells expressed GABA. A well-known limitation in GABA-detection probably results in the lack of 100% overlap (Pow, 1997; Potter et al., 2009). Noteworthy, a small portion of basal forebrain cholinergic neurons in the diagonal band, magnocellular preoptic area, substantia innominata, and hypothalamus are co-labeled by I12b-Cre

but not URE2-Cre. These data together suggest that, in mice, *Dlx1/ Dlx2*-expressing cells give rise to GABAergic neurons in the cortex and hippocampus and a subset of basal forebrain cholinergic neurons (Potter et al., 2009).

However, there is no direct evidence that *dlx1a/2a*-expressing cells give rise to GABAergic neurons in zebrafish. Our fate mapping analysis suggests that a large proportion of the progeny of *dlx1a/2a*-expressing progenitors in the ventral telencephalon, hypothalamus and inferior lobes became GABAergic neurons at 10dpf. Nevertheless, a small number of mCherry-expressing cells in the forebrain were not labeled with GABA, suggesting that *dlx1a/2a*-expressing progenitors may also contribute to other cell types, although it is possible that the aforementioned limitation in GABA-detection accounted for it or that some mCherry-expressing cells were still immature neurons by 10dpf. Future studies will examine if *dlx1a/2a*-expressing cells exclusively give rise to GABAergic neurons by co-labeling mCherry and GABA in the adult brain. If not, co-labeling of mCherry with neuronal marker HuC/D will be performed to examine if *dlx1a/2a*-expressing cells all become neurons. If some mCherry-expressing cells are not labeled with HuC/D, this might suggest that not all *dlx1a/2a*-expressing cells become neurons in the zebrafish brain. Co-labeling of mCherry with glia cell markers such as Olig2 and GFAP will be further conducted to investigate if *dlx1a/2a*-expressing cells contribute to different types of glia cells. We will also co-label mCherry with markers for other neuron types such as cholinergic neurons to examine if *dlx1a/2a*-expressing cells become other types of neurons.

GABAergic neurons are heterogeneous and can be classified into various subtypes based upon the expression patterns of CB, CR, PV, SOM, NPY, VIP, nNOS and CCK (Markram et al., 2004; Wonders and Anderson, 2006; Gelman and Marin, 2010). It has been shown that different expression patterns of these markers are associated with distinct physiological characteristics (Butt

et al., 2005; Miyoshi et al., 2007). In the mouse cortex and hippocampus, most NPY+, nNOS+, PV+, SOM+ cells, and over 60% of CR+ cells are marked by I12b-Cre, while 25–40% of these GABAergic neuron subtypes are labeled by URE2-Cre. In addition, about 18% and 4% of CB+ cells in these domains are labeled by I12b-Cre and URE2-Cre, respectively (Potter et al., 2009). Our comparison of mCherry expression with Calretinin revealed that a few mCherry-expressing cells within the telencephalon and diencephalon of 10dpf embryos were co-labeled by Calretinin. Future studies will examine the co-expression of mCherry and other interneuron subtype markers to characterize the contribution of *dlx1a/2a*-expressing cells to different GABAergic neuron populations. Furthermore, to test if *dlx1a/2a-expressing* cells cells present at different time points populate different subpopulations of GABAergic neurons, co-labeling of mCherry with GABAergic neuron subtype markers will be performed on brain sections of *Tg(dlx1a/2a:CreER^{T2}); Tg*(*ubi:Switch*) animals induced with 4-OHT from 13hpf, 24hpf, 48hpf, 72hpf and 120hpf.

4.4 Activity of *dlx1a/2a*-expressing Cells in Adult Neurogenesis

The ability to produce new neurons in the adult brain depends on the constitutive activity of progenitor/stem cells (Lindsey and Tropepe, 2007). In adult mammals, adult neurogenesis is limited and the neurogenic progenitor/stem cell niches are restricted to the telencephalon (VelleMa et al., 2010). Adult neural stem cells are present in two neurogenic niches, the subventricular zone of the forebrain lateral ventricle and the subgranular zone of the hippocampus in mammals (Luskin, 1993; Eriksson et al., 1998). In contrast, zebrafish can constitutively produce new neurons along the whole rostro-caudal brain axis throughout life and 16 different progenitor niches have been found along the entire rostro-caudal brain axis (Grandel et al., 2006). The progenitor niches in the telencephalon is best characterized in adult zebrafish. Two distinct progenitor zones located at the

ventral ventricular region and the dorsal ventricular region are found in the zebrafish telencephalon. In zebrafish, *dlx2a* is expressed in ventricular progenitors of the adult telencephalon (Adolf et al., 2006). In agreement with this, *Dlx1* and *Dlx2* are expressed in the progenitors in the subventricular zone of the adult mouse forebrain (Saino-Saito et al., 2003). However, the role of *dlx1a/2a* during adult neurogenesis still remains to be thoroughly investigated.

To investigate the activity of *dlx1a/2a*-expressing cells during adult neurogenesis, we labelled the *dlx1a/2a*-expressing cells present in the adult brain by soaking the adult double transgenic animals in 4-OHT and sacrificed them at 1dpi, 7dpi, 14dpi, 21dpi, 1mopi and 2mopi. At 1mopi and 2mopi, mCherry-expressing cells were found in various domains in the telencephalon and diencephalon. The distribution of mCherry-expressing cells appeared very similar between the two time points. Interestingly, some of these domains are shared by the domains of mCherry-expressing cells in the adult brain induced from 24hpf to 48hpf, such as the internal cellular layer and glomerular layer of olfactory bulb, the central zone of dorsal telencephalic area, the ventral nucleus of the ventral telencephalic area, and the caudal and dorsal zone of periventricular hypothalamus. This might suggest that at least some *dlx1a/2a*-expressing cells populate the same brain regions during adult neurogenesis as they do during development. Future work will focus on the characterization of mCherry-expressing cells at 1dpi, 7dpi, 14dpi and 21dpi to better understand the differentiation and migration of *dlx1a/2a*-expressing cells during adult neurogenesis. Furthermore, co-labeling of mCherry with markers for GABAergic neurons will be performed to examine the potential of the *dlx1a/2a*-expressing cells to become GABAergic neurons as well as the contribution of the *dlx1a/2a* to different subpopulations of GABAergic neurons during adult neurogenesis.

4.5 GABAergic Interneurons in Neurodevelopmental Disorders

The balance of the excitatory and inhibitory systems in the cerebral cortex is crucial to the proper function of the brain. About 20% of all the neurons in the cerebral cortex are inhibitory GABAergic neurons, while 80% of them are excitatory glutamatergic neurons. The balance of inhibition and excitation depends on the relative numbers and activities of GABAergic and glutamatergic neurons (Rubenstein and Merzenich, 2003). Either decreased GABAergic signaling or increased glutamatergic signaling, could result in the imbalance of inhibition and excitation. As will be discussed below, alterations in the GABAergic neurons have been associated with in a number of neurodevelopmental disorders.

Autism spectrum disorders describe a range of a group of syndromes with a common set of core symptoms characterized by compromised social interaction, communication difficulties, and stereotyped or repetitive behaviors (Pizzarelli and Cherubini, 2011). Impaired GABA signalling via $GABA_A$ receptors ($GABA_ARs$) is present in all these syndromes. Mutations in $GABA_AR$ subunits have been identified as risk factors and expression of autism-related genes have been found in GABAergic interneurons (Coghlan et al., 2012; Xu et al., 2014). In mice, the absence of *contactin-associated protein-like 2* (*CNTNAP2*), an autism-related gene, leads to decreased number of parvalbumin, calretinin and neuropeptid Y interneurons and core autism-related deficits (Penagarikano et al., 2011).

Fragile X syndrome, the most common cause of inherited mental retardation, is a monogenic disorder caused by mutations in the *Fragile X mental retardation protein* (*FMR1)* gene on the X chromosome (Bagni and Greenough, 2005). The imbalance of neuronal excitation and inhibition has been hypothesized in these patients (Coghlan et al., 2012). Accordingly, the *Fmr1* null mice exhibit decreased number of parvalbumin interneurons, lower expression level of different

$GABA_AR$ subunits and reduced expression of *Gad1* (Selby et al., 2007; Olmos-Serrano et al., 2010).

Rett syndrome, one of the most common causes of mental retardation in females, is a progressive neurodevelopmental disorder. Rett syndrome patients show no obvious developmental anomalies until the age of 6–18 months, then they gradually lose purposeful hand use and speech, and develop symptoms including autism, microcephaly, ataxia, seizures, stereotypic hand movements and intermittent hyperventilation (Amir et al., 1999). It has been reported that Rett syndrome is caused by mutations in X-linked *MECP2*, which encodes methyl-CpG-binding protein 2 (Amir et al., 1999). Impaired GABAergic signaling has been found in Rett syndrome patients (Blue et al., 1999). Accordingly, the absence of *MeCP2* in GABAergic neurons in mice results in impaired GABAergic function, which mediates Rett syndrome phenotypes including increased stereotypical behaviors, progressive motor and respiratory dysfunctions, deficits in social behavior and premature lethal (Chao et al., 2010).

Schizophrenia is a neurodevelopmental disorder often characterized by dysfunction in cognition and psychosis (Lewis et al., 2012). Many studies have shown that GABAergic signaling are involved in the development of schizophrenia. For instance, disturbances in the axon terminals of the chandelier class of GABA neurons may lead to anomalies in GABA neurotransmission in the dorsolateral prefrontal cortex, which is associated with the pathophysiology of schizophrenia (Lewis, 2000). Furthermore, schizophrenia patients exhibit decreased number of calbindin interneurons of the anterior cingulate cortex and a reduction in somatostatin, parvalbumin and cholecystokinin interneuron populations in different brain regions (Cotter et al., 2002; Hashimoto et al., 2008; Fung et al., 2010).

Tourette syndrome is a neurologic disorder characterized by childhood-onset tics, accompanied by uncontrollable noises and utterances, obsessive compulsive behavior, anxiety, attention deficit hyperactivity disorder and other coexisting behavioral problems (Jankovic, 2001). Impaired GABAergic signaling and imbalanced neuronal excitatory/inhibitory ratio has been implicated in Tourette syndrome. Accordingly, decreased number of parvalbumin interneurons has been observed in the caudate and putamen of Tourette syndrome patients, while increased parvalbumin interneuron population has been seen in the globus pallidus pars interna (Kalanithi et al., 2005; Kataoka et al., 2010).

As discussed above, altered GABAergic signaling has been associated with a wide range of neurodevelopmental disorders. Zebrafish has the potential to generate GABAergic neurons throughout life and, possibly, the capacity of regenerating GABAergic neuron after injury (Kroehne et al., 2011). Understanding how GABAergic neuron progenitors populate or repopulate the adult brain in zebrafish might provide new insights into neuro-regenerative therapy in humans. Our studies suggest that *dlx1a/2a*-expressing progenitor cells give rise to GABAergic neuron in the zebrafish brain, which may shed light on the origin of GABAergic neuron progenitors and how they populate or repopulate the adult brain. Provided that our lineage tracing system labels forebrain GABAergic neurons in zebrafish, the *Tg(dlx1a/2a:CreER^{T2})* transgenic line may provide a powerful tool for further lineage-analysis or conditional gene inactivation studies of forebrain GABAergic neurons.

5. CONCLUSION

In summary, we produced lines of *Tg(dlx1a/2a:CreER^{T2})* transgenic fish that allow us to follow the development of *dlx1a/2a-expressing* cells in the zebrafish brain. We have shown that *CreER^{T2}* expression in these fish faithfully recapitulates that of *dlx1a/2a* genes in the forebrain. In Tg(*dlx1a/2a*:*CreER^{T2}*); Tg(*ubi:Switch*) double transgenic fish, *dlx1a/2a*-expressing cells were labeled with mCherry upon 4-OHT treatment. During early neurogenesis, *dlx1a/2a*-expressing progenitors present at later time points seemingly gave rise to fewer progeny than the ones present at 13hpf or 24hpf, suggesting that *dlx1a/2a*-expressing cells are mainly proliferating progenitors at very early developmental stages, while at later developmental stages most of *dlx1a/2a*-expressing cells are postmitotic precursors or in a quiescent state. Fate mapping of mCherry-expressing cells in double transgenic fish have demonstrated that a majority of *dlx1a/2a*-expressing cells give rise to GABAergic neurons. Furthermore, *dlx1a/2a*-expressing progenitor cells populate various domains of the forebrain during adult neurogenesis. The *Tg(dlx1a/2a:CreER^{T2})* transgenic animal will be a powerful tool for further lineage-analysis or conditional gene inactivation studies of forebrain GABAergic neurons.

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