

***dlx* genes, neurogenesis and regeneration in the adult zebrafish brain**

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ABSTRACT

The *Dlx* homeobox genes encode homeodomain transcription factors that are involved in multiple developmental aspects. In the brain, these genes take part in neuronal migration and differentiation, more precisely in the migration and differentiation of GABAergic neurons. Dysfunctions in the GABAergic system can lead to various pathological conditions, where impaired inhibitory function is one of the main causes of several neuropathies characterized by neuronal hyperexcitability. The *Dlx* genes are organized as bi-gene clusters and highly conserved *cis*-regulatory elements have been previously characterized to be fundamental for the regulation of *Dlx* expression in developing embryos of different vertebrates. The activity of these regulatory elements and the *Dlx* genes has been well studied in developmental stages of mice and zebrafish, but little is known about their activity in the adult brain. The extensive neurogenesis that takes place in the adult zebrafish brain provides an ideal platform for the visualization of mechanisms involving *dlx* genes during adulthood and their possible involvement in adult neurogenesis. Here we show novel information concerning the expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* in the adult zebrafish brain and provide insight into the identity of cells that express *dlx*. We also demonstrate the involvement of *dlx* genes in brain regeneration and through lineage tracing, their fate determination in the adult zebrafish brain. Analyses in the adult zebrafish has revealed that all four *dlx* paralogs are expressed in the forebrain and midbrain throughout adulthood and expression is found in almost all areas presenting continuous proliferation. Most *dlx*-expressing cells present GABAergic neuronal identity in the adult forebrain where, in some areas they were identified as the Calbindin subtype. In some areas of the midbrain, especially within the hypothalamus, many *dlx*-expressing cell co-localized with a marker for neural stem cells. However, cells expressing *dlx*

genes did not co-localize with markers for proliferating cells or for glia. Investigations during brain regeneration in response to injury in the adult zebrafish brain has revealed that *dlx5a* expression decreases shortly after lesion and that the *dlx5a/6a* bi-gene cluster, more specifically, *dlx5a*, is up regulated during the peak of regeneration response proposing a possible role for *dlx* during regeneration in adults. Studies of lineage tracing have shown the progeny of *dlx1a/2a*-expressing cells in adults are located within small clusters in different areas of the adult brain where they seem to become mature neurons. Our observations provide a better understanding about the role of *dlx* genes during adulthood, further contributing to the general knowledge of the molecular pathways involved in adult neurogenesis and regeneration in the zebrafish adult brain.

Resumé

Les gènes à homéoboîte de la famille *Dlx* codent pour des facteurs de transcription à homéodomaine qui sont impliqués dans de multiples aspects du développement embryonnaire.. Dans le cerveau, ces gènes participent à la migration et à la différenciation des neurones, plus particulièrement des neurones GABAergiques. Le mal-fonctionnement du système GABAergique mène à diverses conditions pathologiques. La perte de fonction inhibitrice des neurones GABAergiques est une des causes principales de neuropathies caractérisées par de l'hyperexcitabilité. Les gènes *Dlx* sont organisés en bigènes et des éléments de régulation agissant en *cis*, hautement conservés, ont été montré comme jouant un rôle fondamental dans le contrôle de l'expression des *Dlx* de nombreux vertébrés. L'activité de ces éléments de régulation et des gènes *Dlx* a été intensivement étudiée au cours des stades embryonnaires de la souris et du poisson-zèbre mais on sait peu de choses quant à leur activité dans le cerveau adulte. Une neurogénèse importante a lieu dans le cerveau du poisson-zèbre adulte et présente une plateforme idéale pour l'identification des mécanismes qui impliquent les gènes *dlx* chez l'adulte et leur rôle potentiel dans la neurogénèse adulte. Nous présentons ici des résultats nouveaux quant à l'expression des gènes *dlx1a*, *dlx2a*, *dlx5a*, et *dlx6a* dans le cerveau du poisson-zèbre adulte, quant à l'identité des cellules qui expriment ces gènes, leur rôle dans les processus régénératifs et quant à leur destinée cellulaire, telle que suggérée par des expériences de traçage de cellules. L'analyse du cerveau adulte du poisson-zèbre a montré que les quatre paralogues sont exprimés dans le cerveau antérieur et le cerveau médian tout au cours de la vie adulte et que leur expression se retrouve dans presque toutes les régions montrant une prolifération constitutive. La plupart des cellules exprimant les *dlx* correspondent à une identité neuronale GABAergique dans le cerveau adulte et, dans certaines régions, correspondent au sous-type de neurones GABAergiques exprimant la calbindine. Dans

certaines régions du cerveau médian, plus particulièrement dans l'hypothalamus, plusieurs cellules exprimant les *dlx* sont co-localisées avec un marqueur de cellules souches. Les cellules exprimant les *dlx* ne co-localisent pas avec des marqueurs de cellules prolifératives ou des cellules de la glie. Des expériences de régénération du cerveau du poisson-zèbre adulte en réponse à une blessure ont montré que l'expression de *dlx5a* diminue peu après la lésion et le bigène *dlx5a/dlx6a*, plus particulièrement, *dlx5a*, est induit au cours du pic de la réponse régénérative ce qui suggère un rôle pour les *dlx* dans le processus de régénération. Les expériences de traçage de cellules ont montré que la descendance des cellules exprimant *dlx1a/dlx2a* se retrouve dans de petits groupes de cellules dans diverses zones du cerveau où elles semblent devenir des neurones. Nos observations fournissent une meilleure compréhension du rôle des gènes *dlx* dans le cerveau adulte et contribue à une meilleure connaissance des voies moléculaires impliquées dans la neurogénèse ainsi que dans la régénération du cerveau adulte chez le poisson-zèbre.

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innate teaching skills Mom, that your daughter became a scientist in the first place! I am also grateful to my closest friends, my two older brothers and their families for being with me in this journey even from far away, you were my strength many times!

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"In the end, it doesn't matter how well we have performed or what we have accomplished, a life without heart, is not worth living".

John Eldredge

List of abbreviations

4OHT	- 4-hydroxytamoxifen
Ascl1	- Achaete-scute homolog 1
ASD	- Autism Spectrum Disorder
BCIP	- 5-bromo-4-chloro-3-indolyl phosphate
BMP	- bone-morphogenic proteins
bp	- base pairs
<i>cntpnap2</i>	- Contactin Associated Protein Like 2
CCe	- corpus cerebelli
cDNA	- complementary DNA
CNS	- central nervous system
Cre	- Causes recombination
CRE	- <i>cis</i> -regulatory element
D	- dorsal telencephalic area
<i>Dll</i>	- Distal-Less
Dlx	- <i>Drosophila</i> distal less homeobox
Dm	- medial zone of dorsal telencephalic area
dpf	- days post fertilization
dpl	- days post lesion
dpt	- days post treatment
<i>ef1a</i>	- elongation factor 1-alpha
EGFP	- enhanced green fluorescent protein
FISH	- fluorescent in situ hybridization
GABA	- gamma-aminobutyric acid

Gad	- glutamic acid decarboxylase
Gfap	- glial fibrillary acidic protein
GFP	- Green fluorescent protein
Ha	- habenula
Hc	- caudal hypothalamus
Hd	- dorsal zone of periventricular hypothalamus
HE	- Hematoxylin and Eosin
Hv	- ventral zone of periventricular hypothalamus
IHC	- immunohistochemistry
ISH	- in situ hybridization
LGE	- lateral ganglionic eminence
LH	- lateral hypothalamic nucleus
<i>loxP</i>	- locus of crossover in P1
mpf	- months post fertilization
mpt	- months post treatment
mRNA	- Messenger RNA
NBT	- Nitroblue tetrazolium
OB	- olfactory bulb
OCT	- Optimal cutting temperature
P	- pallium
PBS	- Phosphate-buffered saline
PCNA	- proliferation nuclear antigen
PFA	- paraformaldehyde
PGZ	- periventricular gray zone
Po	- preoptic region
PPa	- parvocellular preoptic nucleus, anterior part

PPp	- parvocellular preoptic nucleus, posterior part
qPCR	- quantitative polymerase chain reaction
qRT-PCR	- Real-time quantitative reverse transcription PCR
Sox2	- sex determining region Y-box 2
TBS	- Tris-buffered saline
TeO	- optic tectum
Tg	- transgene
TH	- Tyrosine Hydroxylase
TLE	- Temporal lobe epilepsy
TS	- Tourette Syndrome
URE	- upstream regulatory element
V	- ventral telencephalic area
Vc	- central nucleus of V
Vd	- dorsal nucleus of V
Vs	- supracommissural nucleus of V
Vv	- ventral nucleus of V
Vz	- ventricular zone
WT	- wild type
ypf	- years post fertilization
ypt	- years post treatment

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Statement of Contributions

The experiments presented in this thesis were executed by myself. However some contributions should be noted. Mariam Taktek, an undergraduate student who has worked several terms under my supervision has contributed for the results presented on Chapters 3.2 and 3.3 where she has done several experiments. Omid Lavasany, a fourth year honors student who carried his project under my supervision has contributed with results presented on Chapter 3.2, more specifically section 3.2.2 where he performed different experiments. Yuchen Luo, a fourth year honors student who also carried his project under my supervision, has contributed to results presented on Chapter 3.1 and 3.2. Finally, Thomas Duret a visiting Master student from University of Poitiers, has also worked under my supervision and contributed to results presented on Chapter 3.1.

CHAPTER 1.

General introduction

1.1 Homeodomain proteins

Homeodomain proteins are present in the entire eukaryote kingdom from unicellular organisms to plants, fungi and animals. These evolutionary conserved proteins execute several essential functions throughout development and adulthood. Members of this class of proteins are classified as transcription factors, which contain a homeodomain that can bind to DNA sequences of specific targets, thus controlling the activity of these genes they are often referred as Homeodomain-Containing Transcription Factors. This homeodomain is characterized by a 60-amino acid long domain composed of a helix-turn-helix (HTH) motif and can perform many variable binding assemblies and context-dependent functions (Bobola and Merabet 2017).

Homeodomain-Containing Transcription Factors represent about 15–30% of all transcription factors in plants and animals, and act in all tissues of the embryonic and the adult organism to regulate several diverse processes ranging from axis patterning, to stem cell maintenance and/or metabolic responses. A variety of research strategies have enabled the analyses of Homeodomain-Containing Transcription Factors binding (Kaplan and Biggin 2012). However the molecular mechanisms of these regulation activities still lays ground for extensive research and a pivotal question that still remains is how these transcription factors select their target genes in the genome using a DNA binding domain with very limited sequence specificity, primarily recognizing sequences of four to six nucleotides in length.

Developmental abnormalities, metabolic disorders, congenital syndromes and several other human diseases, have been linked to mutations in genes encoding these Homeodomain-containing proteins. The numbers of the mutations related to these conditions are expected to grow (Chi 2005). Analysis of the complete human genome has revealed that homeodomain-containing transcription factors represent one of the four major functional groups of proteins

whose germline mutations are the causes of known human diseases (Jimenez-Sanchez et al.2001).

Mutations of the *Pax4* gene illustrate well an example of such associations between homeodomain transcription factors and metabolic diseases. It is known that genetic variations may influence the ethnicity-specific clinical characteristics of Type 2 Diabetes in several ways. In a study conducted with approximately 4,000 Korean individuals and coding variants that are associated with Type 2 Diabetes, among the nonsynonymous variants, the *PAX4* Arg192His variant was most significantly associated with increased risk of Type 2 Diabetes in these patients (Kwak et al. 2018). Another study conducted using zebrafish knockdowns and screening of a consanguineous family, has showed that the Homeobox gene *NKX5-3* is associated with Oculo-Auricular Syndrome (Schorderet et al. 2008). Individuals with this Syndrome present a range of malformations including retinal dysplasia, deformation of the ear lobule and in certain stages advanced cataract development. Findings such as these depict the importance of extensive and detailed investigations of different aspects and roles of Homeodomain proteins.

1.2 *Dlx* genes

The *Dlx* homeobox gene family encode homeodomain-containing transcription factors in vertebrates and are homologues of the *Drosophila dilatal-less* (*Dll*), identified in the *Drosophila* Fruit Fly. Of all homeodomain-containing proteins, the *Dll* family is the earliest known gene to be specifically expressed in developing distal limbs and were initially identified showing an essential role in proper proximal-distal patterning of limbs in *Drosophila* (Cohen and Jürgens 1989). In addition to functioning during adult appendage development, the *Drosophila Dll* gene is also required for the formation of peripheral nervous system parts. *Dll* is required for the

expression of molecules required for differential affinities between proximal and distal cells in *Drosophila*. Several genes are present downstream of *Dll* in developing limbs and represent candidate targets for direct regulation by *Dll*, the loss of expression in *Dll* mutants has provided information that genes such as *Splinless* (*ss*), *Aristaless* (*al*) and *Disconnected* (*disco*) may be targets of Dll transcriptional activity in *Drosophila* (Panganiban and R Rubenstein 2002).

Homeobox genes of the *Dll* family have been identified in all metazoan groups where they play roles different roles in ontogeny. In the genome of vertebrates, *Dlx* genes are present as multiple paralogues generated by tandem duplication followed by whole genome duplications. They are organized into six orthologous groups based on sequence similarities of the homeodomains that they encode (Zerucha and Ekker 2000) (Appendix -3 Table1) . Like most homeobox genes encoding for transcription factors, *Dlx* homeobox genes seem to have multiple functions and targets at different stages of development in different tissues and cell types. Analyses of embryonic expression of *Dlx* genes in different species has supported the hypothesis that the nested *Dlx* expression in the pharyngeal arch is a shared feature among all jawed vertebrates (Takechi et al. 2013). Investigations on *Dlx2* and *Dlx5* by sustained misexpression in ovo, have also suggested that these genes are involved in defining where skeletogenic condensations will arise (Gordon et al. 2010). The *Dlx* genes are mainly involved in the development of the forebrain, branchial arches, sensory organs, and limbs

1.3 Expression and function of Dlx genes during development

The expression of *Dlx* gene family members has been demonstrated in the developing nervous system, neural crest, inner ear, brachial arches and developing appendages of vertebrates [(Ekker et al. 1992)(Acampora et al. 1999) (Evans 2007)]. Expression has been characterized mainly throughout embryonic development [for reviews see (Bendall and Abate-

Shen 2000)]. In the mouse forebrain, from embryonic day 10.5 to 12.5, *Dlx2* is expressed before *Dlx1*, which is expressed before *Dlx5* and these expressions are localized primarily to the nucleus of cells, although *Dlx5* transcripts can also be found in the cytoplasm (Eisenstat et al. 1999). In addition to indications given by expression patterns, the function of *Dlx* genes during development was studied in the mouse, mainly through functional studies and the production of targeted null mutations by homologous recombination.

Functional analyses provided the first clues that *Dlx* was involved in craniofacial structuring and neuronal differentiation in the forebrain of vertebrates (Qiu, Rubenstein 1995). In the mouse forebrain, four *Dlx* genes are expressed, *Dlx1*, *Dlx2*, *Dlx5*, and *Dlx6*, and the expression follows a temporal sequence (Fig. 1) (S. a. Anderson et al. 1997), *Dlx2* and *Dlx5* single mutants have reduced numbers of neurons in the olfactory bulb. *Dlx1* single mutants show very subtle phenotypes in embryos but adults have a propensity to develop epileptic seizures and reduced fear conditioning and *Dlx6* single mutants have not yet been reported (Cobos et al. 2005). These mild phenotypes may be explained by the fact that during development, the four *Dlx* paralogs are expressed in the same cells.

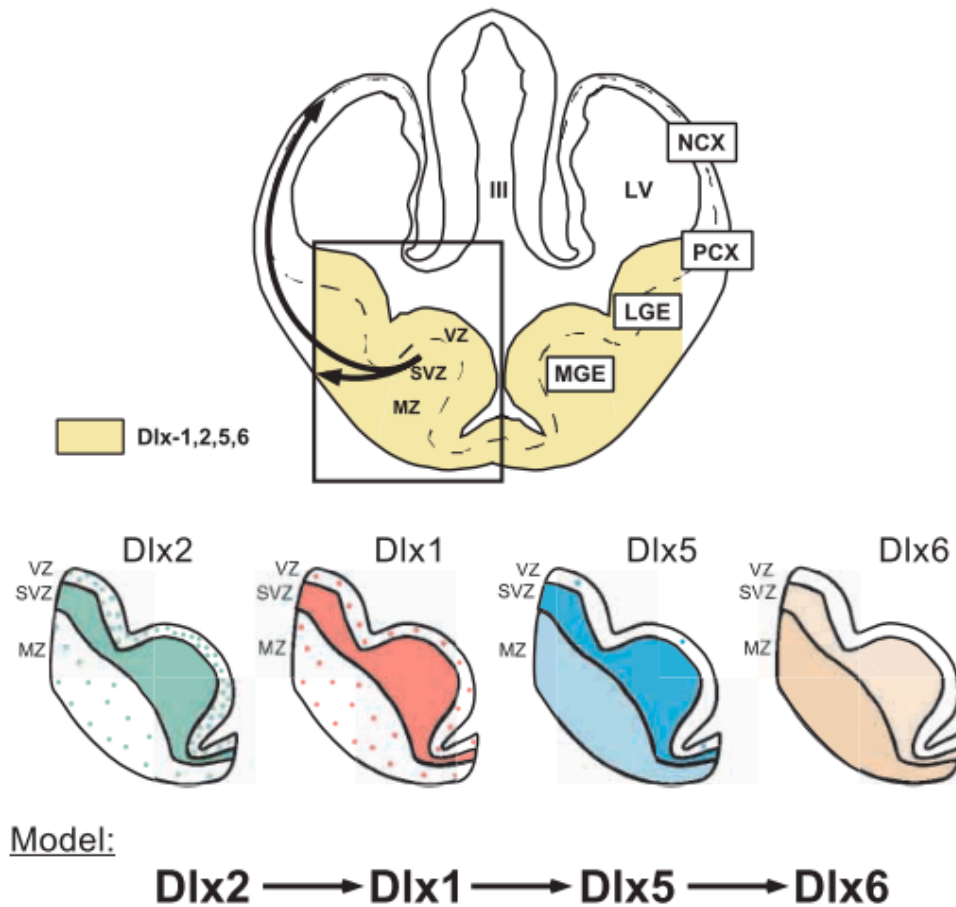


Figure 1 Expression of *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* during mouse brain development.

Top drawing represents the transverse section of an E12.5 mouse telencephalon showing the combined expression of four *Dlx* transcripts. The migration from the subpallium to the pallium (cortex) is indicated by arrows. Drawing depicted in the middle show uniform patches that represent abundant *Dlx* expression in differentiated cells and colour dots represent scattered expression. The model shown in the bottom is a hypothesized genetic and biochemical pathway that proposes *Dlx2* is the first to be expressed, followed by the expression of *Dlx1*, *Dlx5* and *Dlx6*. Adapted from (Panganiban and Rubenstein 2002)

Subsequent studies showed that homozygous *Dlx-1/2* mutant mice presented abnormal migration out of the LGE, resulting in an accumulation of partially differentiated neurons in this region of the brain, as well as reduced expression of glutamic acid decarboxylase (GAD) – an enzyme that synthesizes GABA - in the neocortex of these mutants (S. Anderson et al. 1999) (Fig. 3). This study provided important evidence that neuronal migration and establishment is highly *Dlx*-dependent in the forebrain and particularly for GABAergic interneurons. Furthermore, craniofacial malformations observed in mice knockout for both *Dlx5* and *Dlx5/6* as well as *Dlx5* loss-of-function embryos, suggested multiple and independent roles for these genes in the patterning of the branchial arches, in the morphogenesis of the vestibular organ and in osteoblast differentiation (Acampora et al. 1999).

Inactivation of *dlx* gene function was also carried out in the zebrafish using morpholino oligonucleotides (Solomon and Fritz 2002; MacDonald et al. 2013; Heude, Shaikho, and Ekker 2014). *Dlx* function in zebrafish was found to be essential for optic and olfactory placode development similar to what was found in mice. Importantly, loss of *dlx1a* and *dlx2a* function impairs *gad1b* expression in the prethalamus and hypothalamus, confirming that in teleost fish, *dlx* genes are essential for GABAergic neuron specification. A few mechanisms have been proposed to relate *Dlx* function and migration of interneuron precursors in zebrafish, which are going to be discussed further in this Thesis.

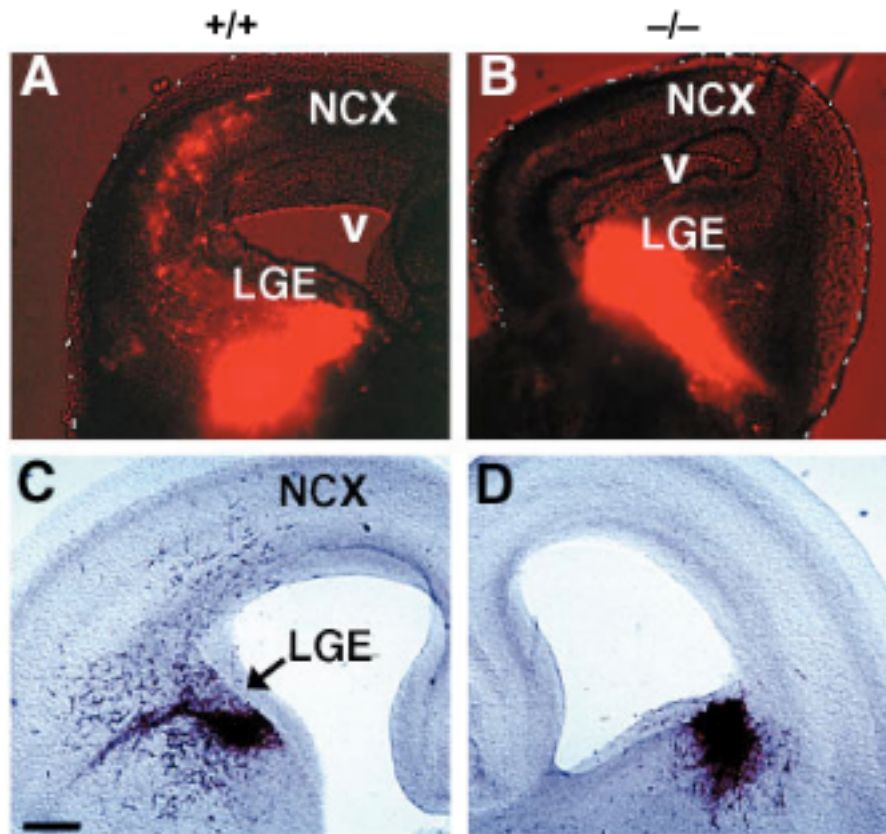


Figure 2. Cell migration out of the lateral ganglionic eminence (LGE) in sections from wild type (A and C) and *Dlx1/2* mutant mice (B and D).

In wild type embryos (A) cells have migrated from the LGE into the Neocortex (NCX), this migration is absent in the mutant sample (B). Slices from E15.5 animals showing, little or no migration into the neocortex occurred in the *Dlx1/2* mutant sample (C and D). Adapted from (S. A. Anderson et al. 1997).

The establishment and maintenance of *Dlx* expression requires upstream activity of other genes and pathways. For instance, in *Drosophila*, both *Decapentaplegic* (*Dpp*) and *Wingless* (*Wg*) have been identified as key factors for proper *Dll* expression. In mice, gain-of-function assays showed that Sonic Hedgehog (Shh) can induce *Dlx* expression in the forebrain, and mice lacking Shh activity show reduced levels of *Dlx2* expression in the forebrain (Gaiano et al. 1999). Finally in zebrafish, a forebrain nuclear zinc finger protein (Fez), has been demonstrated to regulate *dlx2* expression during neurodevelopment (Yang, Liu, and Lin 2001). Finding upstream key players acting on *dlx* as well as *dlx* targets, is a constant work in progress and important for elucidating molecular mechanisms involved in the proper function of *Dlx*.

1.4 Organization and structure of *Dlx* genes

The majority of vertebrates have six *Dlx* genes and in some fish species up to eight *dlx* genes have been characterized. These genes are generally organized as bigene clusters on distinct chromosomes on a tail-to-tail arrangement commonly arranged as: *Dlx1/Dlx2*, *Dlx3/Dlx4* and *Dlx5/Dlx6*. Within each bigene cluster, *Dlx* genes are transcribed convergently (Fig. 3) (Zerucha et al. 2000). For both *dlx1a/2a* and *dlx5a/6a* bi-gene clusters, the relative position and orientation of the conserved intergenic sequences are identical in five different vertebrate species including humans, mice and zebrafish (Ghanem et al. 2003).

Highly conserved cis-regulatory elements (CREs) have been characterized regulating *Dlx* expression in the developing mouse embryo. The upstream regulatory element 2 (URE2) was identified upstream of *dlx1a* while I12a and I12b reside within the *dlx1a/dlx2a* intergenic region (Ghanem et al. 2007). In the mouse, the activity of the I12b element begins at E.10 in the telencephalon, diencephalon and apical ectodermal ridge, whereas the I12a element is not

active in the forebrain but rather in the mesenchyme of the first brachial beginning at E9.5. (Ghanem et al. 2003) (Fig. 3).

To test if the orthologous sequences from the zebrafish genome have regulatory functions in the zebrafish, MacDonald and collaborators (2010) have designed two reporter constructs containing these genomic sequences selecting 6kb of the *dlx1a/2a* intergenic region, including I12a and I12b, and 900 bp encompassing URE2. The activity of the Zebrafish *dlx* enhancers was shown to be similar to their mouse counterparts (MacDonald et al. 2010).

The intergenic region of the *dlx5/dlx6* bigene cluster contains two enhancers, named I56i (approximately 400bp) and I56ii (approximately 300bp) (Zerucha et al. 2000). A 1.4 kb intergenic sequence of the zebrafish *dlx5a/6a* cluster is sufficient to drive reporter gene expression effectively mimicking endogenous expression of *dlx5a/6a* (*Dlx5/Dlx6*) in the forebrain of mice and zebrafish (Fig 3). The deletion of I56ii in mice has been shown to impair the expression of *Dlx* genes and of potential targets including *Gad2* as well as striatal markers *Islet1*, *Meis2*, and *Ebf1*.

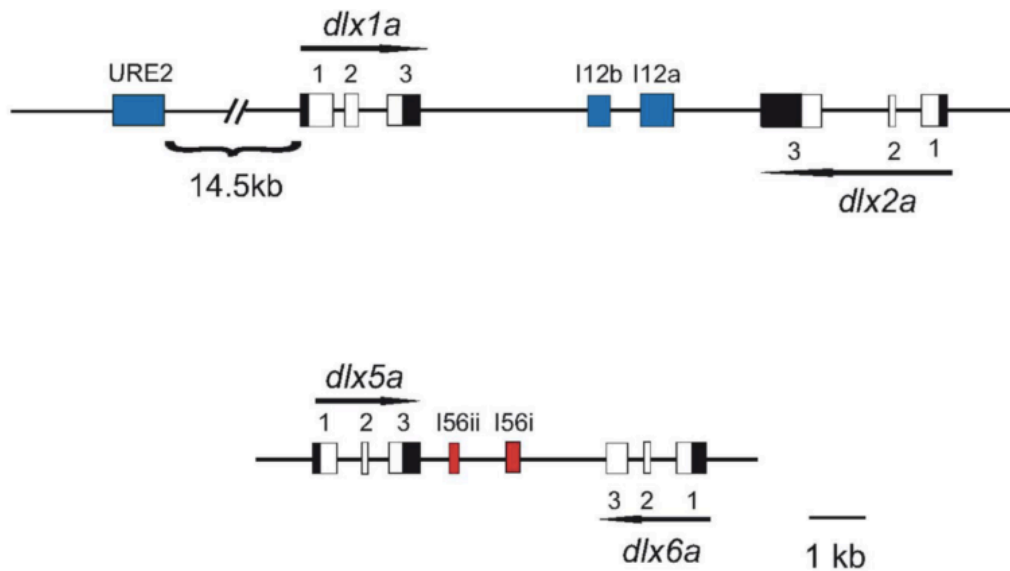


Figure 3. Schematic representation of genomic organization of *dlx1a/2a* and *dlx5a/6a* bigene clusters in zebrafish.

For each gene Untranslated regions (UTR) are indicated as black boxes and the three exons in white. In blue (URE2, I12a and I12b) and red boxes (I56i and I56ii) the regulatory elements are represented. Adapted from (MacDonald et al. 2010).

The identification of such regulatory elements was a starting point for the generation of a transgenic line used in this project, the Tg(*dlx6a*-1.4kb*dlx5a/dlx6a*:GFP). Zerucha and collaborators (2000) created a construct where GFP was placed immediately downstream of a 3.5-kb fragment of the *dlx6a* 5' flanking region including the promoter and part of the 5'UTR. This construct also contained 1.4-kb *dlx5a/6a* intergenic fragment containing I56i and I56ii and therefore named Tg(*dlx6a*-1.4kb*dlx5a/6a*:GFP). Zebrafish expressing this construct present expression of the GFP reporter transgene to the domains of *dlx* expression in the telencephalon and diencephalon (Zerucha et al. 2000; Ghanem et al. 2003).

1.5 *Dlx* genes in adulthood

The first indications of *Dlx* expression in adult vertebrates were obtained in rat using cDNA isolated from Chondrosarcoma - a type of bone cancer that develops in cartilage cells - encoding for a rat DLX. These analyses showed expression of rat DLX transcripts in multiple tissues during embryonic stages of rat development and in the adult heart (Zhao and de Crombrughe, 1994). Saino-Saito and collaborators (2003) have investigated whether *Dlx1* and *Dlx2* genes also are expressed in the adult brain, especially in pallial derivatives, such as the olfactory bulb, hippocampus, and possibly cortex. Widespread expression of both genes was found in adult mouse forebrain but not in the mid- or hindbrain.

The widespread expression of *Dlx* mRNA and protein found in the adult forebrain suggests that these genes may have additional roles in mature animals (Saino-Saito, Berlin, and Baker 2003). Still little is known about the role of *Dlx* genes in adulthood, particularly in species that present more pronounced rates of adult neurogenesis.

1.6 GABAergic neurons and their inhibitory roles

Neurons secreting Gamma-aminobutyric acid (GABA) neurotransmitters are the main inhibitory neurons in the mammalian adult brain and consist 20% of all neurons in the cerebral cortex and hippocampus, and 95% of neurons within the striatum (Maroof and Anderson 2010). In the early developing brain however, GABAergic excitatory synapses establish the cortical network and then become inhibitory after the excitation of the developing neurons. Thus in the context of the immature brain, GABA plays excitatory roles (Ben-Ari 2002).

Although the primary function of GABA is as a neurotransmitter, it presents the structure of an amino acid and therefore is referred to as amino acid neurotransmitter. GABA is synthesized by glutamate in a reaction catalyzed glutamic acid decarboxylase (Gad). The inhibitory role of GABA neurotransmitters takes place through the activation of chloride conducting receptors, which result in an increase in conductance and trigger an inhibitory postsynaptic potential. The activation of the chloride conducting receptors is caused by the binding of GABA to specific transmembrane receptors, which causes the opening of ion channels allowing the flow of negatively charged chloride ions into the cell or positively charged potassium ions out of the cell (Kuffler and Edwards 1957).

Neurons that produce GABA as their output are therefore called, GABAergic neurons. These 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) and neuropeptides, such as somatostatin (SOM), neuropeptide Y (NPY), vaso-active intestinal peptide (VIP) and cholecystokinin (CCK).

During vertebrate neurodevelopment, the migration of interneurons plays an essential role for the proper establishment and functioning of the CNS. In zebrafish, GABAergic neurons are generated near the medial subpallial ventricular wall at 24 hours post fertilization and during

the larval stage they migrate from the ventral telencephalon to the dorsal telencephalon where these neurons do not divide and may become post-mitotic, indicating that the presence of interneurons in that area is strictly due to migration (Mueller, Vernier, and Wullimann 2006). In fact, the precursors of most GABA-containing interneurons in the cerebral cortex migrate all the way from the subcortical progenitor zones (Pleasure et al. 2000).

1.7 Dysfunctions in the GABAergic system

As the major inhibitory neurotransmitter in the CNS, dysfunctions in the GABAergic system lead to various pathological conditions and impaired inhibitory function is one of the causes behind neuropathies characterized by neuronal hyper excitability. A proper balance of the excitatory and inhibitory systems in the cerebral cortex must be maintained to avoid disorders like epilepsy, schizophrenia, Tourette syndrome, bipolar depression and autism (Kalanithi et al. 2005) (Jankovic and Kurlan 2011) (Volk, Edelson, and Lewis 2016).

In experimental mice as well as in human temporal lobe Epilepsy (TLE), loss of GABAergic neurons has been reported (Bernard et al. 2000). This loss of inhibitory interneurons should lead to a decreased number of inhibitory synapses on the postsynaptic cells. A much earlier report in 1972, postulated that GABA dysfunction might play a central role in the pathophysiology of schizophrenia (Roberts *et al.*, 1972). In the context of schizophrenia on a speculative level, it has been previously hypothesized that a decreased GABAergic transmission in specific cortical areas could result in possibly enlargement, of sensory, memory and cognitive fields and thereby lead to disorganized thought processes (Benes and Berretta 1987). The so-called “GABAergic origin hypothesis,” is that N-methyl-D-aspartic acid-type glutamate receptors (NMDAR) hypofunction at GABAergic interneurons particularly, and would be sufficient for schizophrenia-like effects (Jiang, Cowell, and Nakazawa 2013).

Although scarce, postmortem brain neuropathological studies have demonstrated an association of Tourette syndrome (TS) to possible developmental defects in the migration of GABAergic neurons. Kalanithi and collaborators (2005) have presented a quantitative cellular characterization of specific cell types within the basal ganglia of subjects with severe and persistent TS. Their data demonstrate a consistent and profound parvalbumin-positive neuronal imbalance in the basal ganglia of the three patients examined postmortem that could indicate a defect in the migration of GABAergic neurons (Kalanithi et al. 2005).

Autism Spectrum Disorders (ASD), are a group of neurological and developmental disorders that affect communication, social interaction and behaviour on a variable scale. It is predicted that ASD affects 1 in every 59 children in the United States (J. Baio et al 2014). Evidence suggests that abnormalities in glutamate and GABA signalling are underlying causes of ASD symptoms [reviewed by (Rubenstein and Merzenich 2003) (Coghlan et al. 2015)]. These abnormalities have been observed with *in vivo*, *in vitro* and post-mortem studies. For instance, reductions in GABA levels have been reported in several brain regions in children with ASD. Alterations in levels of glutamate and glutamine in the cortex and basal ganglia of children, and in the basal ganglia in adults with ASD has been revealed by *in vivo*, proton magnetic resonance spectroscopy (Horder et al. 2018). Moreover in mice, absence of contactin-associated protein-like 2 (CNTNAP2), an autism-related gene, leads to a decreased number of parvalbumin and calretinin interneurons and autism-related deficits (Abrahams et al. 2011). In fact, zebrafish *cntnap2* mutants display a deficit specifically in *dlx5a/6a* expression within the telencephalon, but these fish did not display any regional deficits in glutamatergic neurons (Hoffman et al. 2016).

The many conditions associated with GABAergic deficits and dysfunction demonstrate how elucidating molecular mechanisms involved in normal GABAergic neurons development are critical. Understanding changes in neurodevelopmental events, neuroplasticity in post-injury

regeneration and during the progression of neurological disorders should provide important insights into the underlying mechanisms and potential therapeutic avenues (Jankovic and Kurlan 2011).

1.8 *Dlx* and GABAergic neurons

Genes of the *Dlx* family have been shown to play essential roles in GABAergic neuron specification as mentioned previously. For instance, 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). The migration of GABAergic neurons and specification into neuronal subtypes has therefore been shown to be highly dependent on the *Dlx* homeobox genes (Anderson et al, 1997).

Glutamic acid decarboxylase, encoded by the gene *Gad1*, is responsible for catalyzing the production of GABA from L-glutamic acid. Studies using triple fluorescent hybridization in zebrafish have shown overlapping expression of *dlx1a* and *dlx5a* and *gad1b* (orthologous to mouse *Gad1*) in the zebrafish forebrain at 24 hours post fertilization, also indicating that the *dlx* genes are regulated by a conserved genetic pathway and may play a role in GABAergic interneuron differentiation, this time in the zebrafish forebrain (MacDonald et al. 2010).

In mice, *Gad1* is downstream of *Dlx1* and *Dlx2*, what led MacDonald and collaborators (2013) to test this relationship in zebrafish. To assay the possible role for *dlx* genes in *gad1b* expression in the zebrafish forebrain at 24 and 48 hpf, they have used translation and splice blocking morpholinos against *dlx1a*, *dlx2a*, *dlx5a*, and *dlx6a*. They demonstrated that *ascl1a*

and *dlx1a/2a* regulate *dlx5a/6a* expression, which then regulates *gad1b* expression (Fig. 4). The gene *asc1a* encodes a member of the basic helix-loop-helix family of transcription factors.

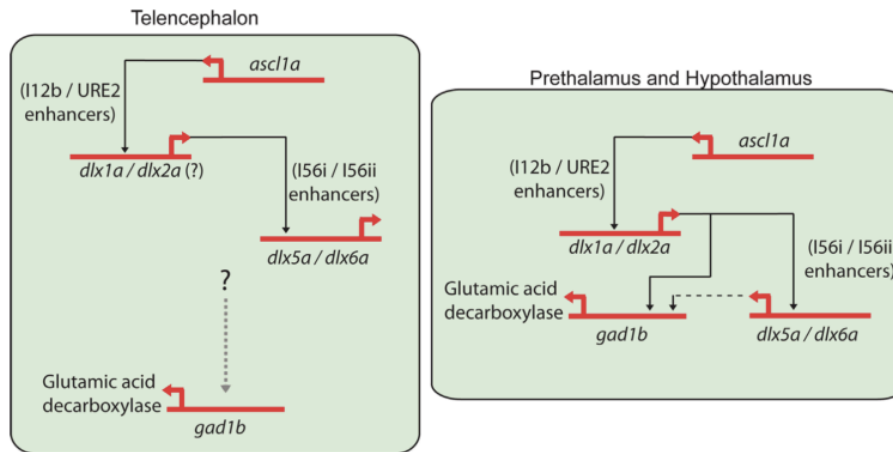


Figure 4. Model showing the regulation of the *dlx* and *gad1b* genes in the zebrafish forebrain.

As shown by the dash black line the *dlx5a/6a* genes may play a role in *gad1b* regulation in the diencephalon. (MacDonald et al. 2013)

1.9 Neurogenesis and regeneration

The neural tube is the progenitor source of all neurons and glial cells that are going to form the brain during embryonic development. Once these cells are generated by their progenitors, they almost always migrate distances to form mature neurons, approximately 100 billion in the case of the human brain. Most progenitors are multipotent early in development, where retroviral lineage studies have shown that a single infected cell can give rise to neurons, astrocytes and oligodendrocytes (Ravin et al. 2008). However, in some regions of the brain, there are committed progenitors that only produce either neurons or glia.

The molecular and cellular mechanisms involved in neural induction, fate determination and migration within the CNS are complex and being investigated. Canonical signalling pathways are important and have been well characterized for neural induction, namely the antagonism of bone-morphogenic proteins (BMP), Wnt signalling pathways and the Notch/Delta cascade, which is critical for singling out neuroblasts from pro neural clusters. The conservation of these classic pathways of neural induction illustrates the ancient origin of these developmental mechanisms (Stern 2005) .

Although neurodevelopment in embryonic stages present well studied mechanisms, adult neurogenesis, specially in mammals, has been a topic of discussion in the last decades. Some evidence support the notion of undetectable levels of neurogenesis in the human adult hippocampus (Sorrells et al. 2018; Gould 2007; Mathews et al. 2017), while many others show the existence of the birth of new neurons in the mammalian brain throughout adulthood (Altman and Das 1967; Eriksson et al. 1998; Yuan et al. 2014; Boldrini et al. 2018; Kempermann et al. 2018) . Based on evidence obtained in the mammalian brain, it is certainly a common agreement that in mammals, adult neurogenesis is very limited and also greatly affected in

neurodegenerative disease (Moreno-Jiménez et al. 2019). The molecular mechanisms behind these events still remain elusive.

The adult zebrafish however, possesses much greater rates of adult neurogenesis in multiple niches along the brain and presents a remarkable capacity to regenerate their central nervous system following mechanical or chemical insult. Compared to mammals like rodents and also birds for instance, in which constitutively active neurogenic domains are restricted to the forebrain, in this teleost fish new neurons are generated in most brain regions throughout adult life (reviewed (Kizil et al. 2012) (Schmidt, Strähle, and Scholpp 2013a)). The dorsal telencephalon is an area of the zebrafish brain that contains regions homologous to the mammalian subependymal zone of the lateral ventricle (SEZ), the subgranular zone of the dentate gyrus of the hippocampus (SGZ), as well as a neocortex-like region, and in the case of rodents neural progenitors are silent in these regions (Fig. 5).

The zebrafish adult brain contains thirteen to sixteen distinct neural stem cell niches that can generate a large number of new neurons during the entirety of its life. Neurons born in these specific proliferative zones of the adult zebrafish brain migrate tangentially to their target areas, where they will mature and integrate into the existing neuronal networks. (Schmidt R. et al, 2013). After migration, these neurons are reported to have a low incidence of cell death (Rothenaigner, Krecsmarik, Hayes, Bahn, Lepier, Fortin, Götz, Jagasia, and Bally-cuif 2011).

Both regeneration and continuous adult neurogenesis require high levels of progenitor cell proliferation. They might produce a burden on the immune system and continuous cell production may lead to cancerous growth. It is thought that this is one of the reasons why more complex vertebrates lost high regeneration responses ability throughout evolution (Pearson and Alvarado 2009).

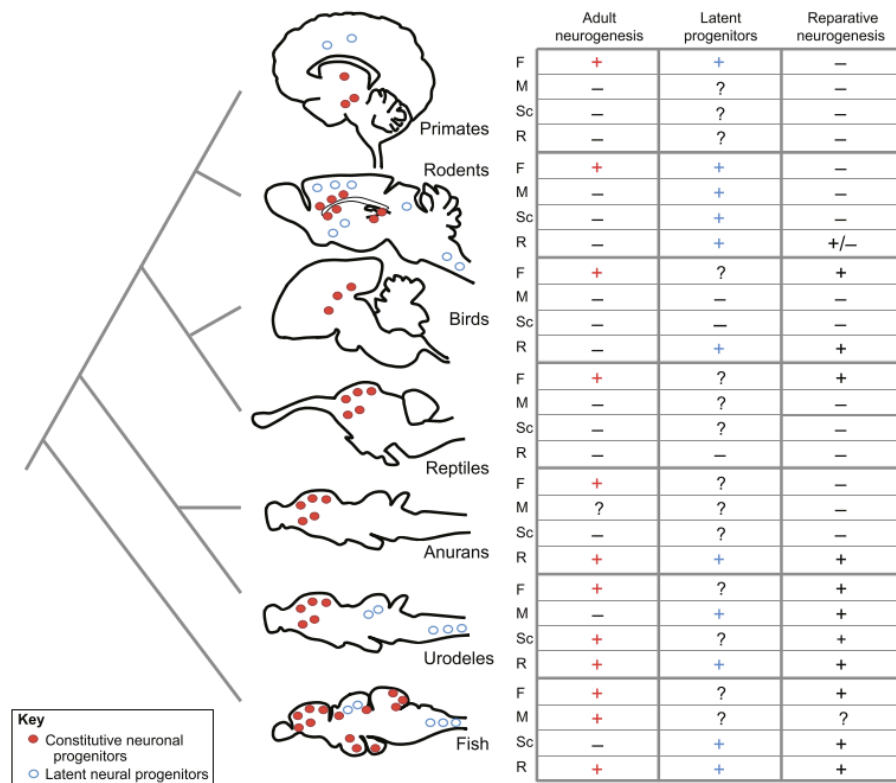


Figure 5. Phylogenetic tree of animal taxon used as models for neuronal regeneration.

The table on the right summarizes continuous neuronal progenitors regions, latent neural progenitors and reparative neurogenesis in the different central nervous system regions of vertebrates with the zebrafish brain (in the bottom) showing a high rate of continuous neuronal progenitors. F, forebrain; M, midbrain; Sc, spinal cord; R, retina. (Alunni and Bally-Cuif 2016).

In vertebrates, glia cells have important roles in maintaining homeostasis in the CNS and also in providing a response that aids in neuronal repair. In the adult zebrafish, proliferating adult stem cells also reveal a glial identity and are able to self-renew and generate new neurons (Rothenaigner, Krecksmarik, Hayes, Bahn, Lepier, Fortin, Götz, Jagasia, and Bally-Cuif 2011). Studies using conditional Cre/Lox lineage tracing in which radial glial cells and their progeny were permanently labeled, have demonstrated that radial glial cells give rise to neuroblasts that migrate to the site where an injury was applied, where they differentiate into long-lasting neurons (Kroehne et al. 2011) (Fig. 6). In the same study Kroehne and collaborators (2011) have developed a traumatic lesion assay and analyzed multiple cellular reactions to injury. Their study shows that adult zebrafish can efficiently regenerate brain lesions presenting an acute inflammatory response and gliosis, but lack permanent glial scarring.

Proliferation of glial cells and microglia in response to stab injury in the adult zebrafish telencephalon, was also analyzed by Marz and collaborators (2011). In this study, radial glial markers were up-regulated at the lesioned ventricle and co-expressed the proliferation nuclear antigen (PCNA). Parenchymal proliferation was almost inexistent in comparison to the increase in proliferation in the ventricular zone. This suggested that most of the cellular material for regeneration is derived from regions of continuous neurogenesis. Furthermore, the proliferative response was almost completely restricted to the lesioned hemisphere, indicating that signals inducing regeneration remain mainly confined within the lesioned half of the telencephalon (Viales et al. 2015).

Even though previous studies have investigated how cells migrate and neuronal repair takes place from niche progenitors in zebrafish [revised in (Fernández-Hernández and Rhiner 2015)], a detailed spatiotemporal characterization of the heterogeneity of this niche in terms of lineages, cell subtypes and fate has not yet been reported.

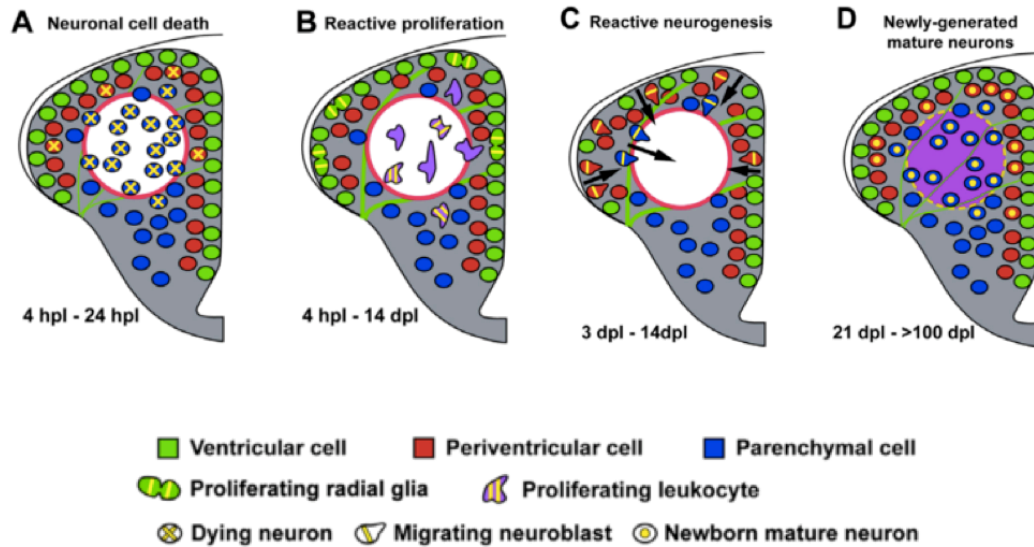


Figure 6. Important events during regeneration in the zebrafish brain.

(A) Cell death. (B) Reactive proliferation. (C) Reactive neurogenesis. The newborn neuroblasts leave the VZ and migrate towards the lesion site. (D) Many newly generated mature and active neurons are detected within the lesion site in the parenchyma and in the PVZ. (Kroehne et al. 2011).

Another well-studied case of regeneration in zebrafish and a response based on the recruitment of latent progenitors, is within the retina. Muller glia is the primary glia cell type in the retina and in zebrafish they have been shown to respond to retinal injury by hypertrophy and activation of cytoskeletal genes, for instance glial fibrillary acidic protein (*Gfap*) and vimentin⁶. Interestingly, a variety of genes encoding growth factors and cytokines, like Hb-egf, Insulin, Igf-1, IL11 and leptin are induced in the injured fish retina and can stimulate Müller glia proliferation under appropriate conditions. Only rarely do Müller glia divide following retinal injury in mammals, and when they do this often results in fibrosis and glial scarring (Wan and Goldman 2016).

1.10 Lineage tracing in development and regeneration

The formation of a complex multi-cellular embryo deriving from just one single cell has always been an intriguing process and a passionate pursuit for many biologists. Major advances were made in the field ever since one of the first milestones of embryology with the characterization of multiple genes involved in the earliest steps in embryonic pattern formation in *Drosophila* (Nüsslein-Volhard C. & Wieschaus 1980). Many decades before that however, the first efforts for conducting lineage tracing during development were made using light microscopy to study early cleavages in invertebrate embryos with ascidian eggs (Conklin E. G. 1905)

Lineage tracing consists in the identification of the progeny of a single cell or a group of cells, and provides extensive information about the number of progeny from the founder cell as well as their location, their differentiation status and a deeper understanding of tissue development, homeostasis and disease and cell-fate decisions (Kretzschmar and Watt 2012). In stem cell research, lineage tracing is an essential tool as it provides information about how the

cell behaves over time in the context of the intact tissue or organism, different to what it is capable of doing following isolation and transplantation or using *in vitro* cultures.

Axolotl are a well-known species for their ability to fully regenerate limbs, also providing a good model for the study of molecular mechanisms in regeneration. A comparison study using CRISPR/Cas9 to genetically label unique cell lineages within the developing axolotl embryo and then tracking these lineages within amputated and fully regenerated limbs, revealed that regenerated limbs are high fidelity replicas of the originals even after repeated amputations (Flowers, Sanor, and Crews 2017). Other examples of the use of lineage tracing in regeneration studies is in mouse models, where data obtained from these models engineered for lineage tracing are transforming our understanding of the changes in cell fate that underlie renal pathophysiology, the role of stem and/or progenitor cells in kidney development, and the mechanisms of kidney regeneration (Romagnani, Rinkevich, and Dekel 2015).

The essential characteristics of a good lineage tracer are that they should not change the properties of the labeled cell, its progeny or its neighbours. Ideally the label should be fully retained, passed on to the progeny of this founder cell and not transferred to unrelated cells (Solek and Ekker 2012). Ever since the first direct observations, many other different lineage tracing techniques have been developed to meet the variety of investigations such as: labeling cells with dyes and radioactive tracers, introduction of genetic markers by transfection or viral transduction, transplantation of cells and tissues, genetic mosaics, cell labeling by genetic recombination and multicolor reporters.

1.11 Cre-loxP Based Lineage Tracing

The Cre-loxP system is an important and useful tool for conditional gene expression and also for lineage tracing. The labeling of cells of interest in this technique consist of a site-specific recombinase technology used to perform deletions, insertions, translocations and inversions at targeted regions of the DNA and has been used as a common effective technique for these purposes (Sauer and Henderson 1988) (Fig. 7). The use of inducible Cre for lineage tracing with this system has been applied to many tissues and animal models, and has provided unprecedented levels of information about the organization of the stem cell compartment in postnatal tissues.(Nowak et al. 2010) (Solek and Ekker 2012) (Walker 2012) (Fig. 7).

The Cre/LoxP system for genetic recombination also permits lox-flanked target gene alteration via stage- or tissue-specific control dependent upon the regulation of Cre gene expression *in vivo*. The most commonly used modification is a fusion with the hormone-binding domain from the human or mouse estrogen receptor (ER) to obtain a CreER (Feil et al. 1997). In order to avoid interaction of the hormone-binding domains with endogenous estradiol, a glycine to arginine mutation is introduced in the ER and the affinity for estrogen is reduced, whereas affinity for hydroxy-tamoxifen is increased (Danielian et al., 1993). The resulting modified ER hormone-binding domains are named ER^T (T for tamoxifen, with a number following T indicating subsequent modifications of the domain), CreER^{T2} in the case of two modifications (Indra et al. 1999).

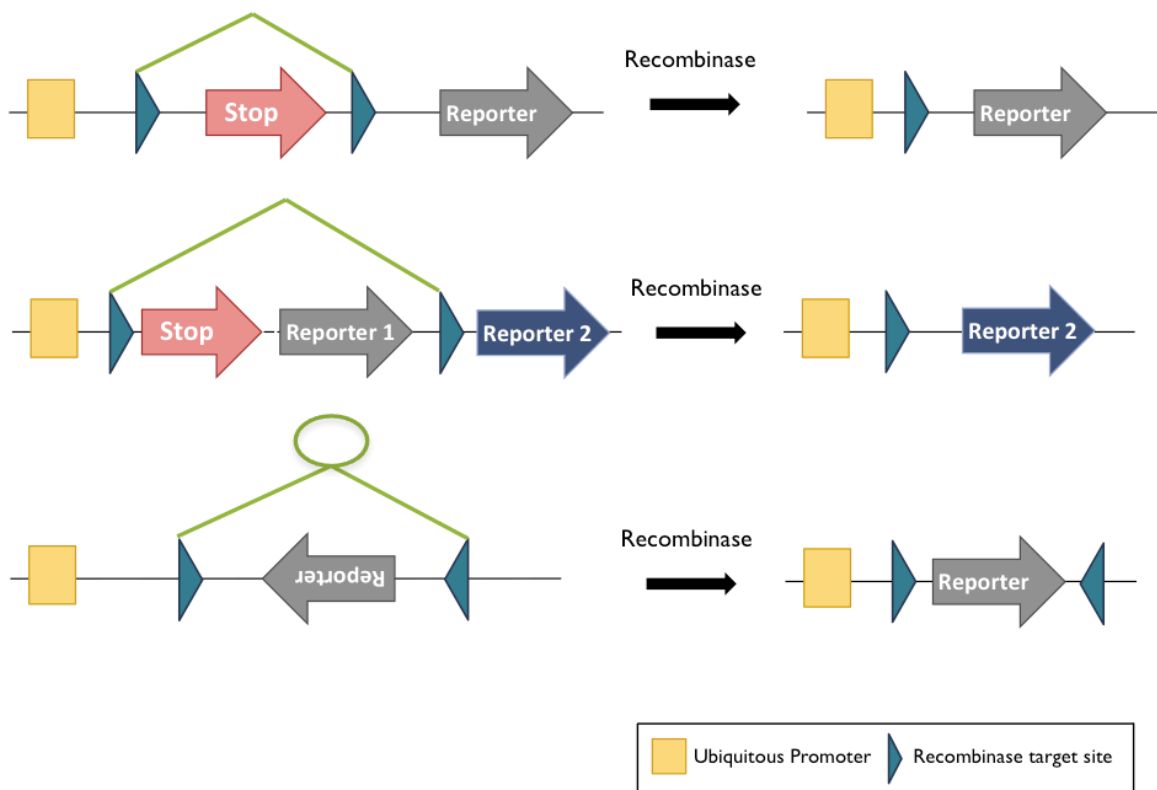


Figure 7. Cre mediated *loxP* recombination reactions at *loxP* sites.

Schematic representation of frequently used reporter alleles, including STOP cassette excision (first scheme), dual reporter (second) and reporter gene inversion (bottom). Adapted from (Solek and Ekker 2012)

Former members of our laboratory, Shengrui Feng and Dr. Cynthia Solek have generated two transgenic zebrafish lines that express the enzyme Cre-ER^{T2} under the control of the 1.4kb *dlx5/6* intergenic enhancer region (*dlx5/6CreER^{T2}*) and URE2 and I12b, two enhancers of *dlx1a/2a*, *Tg(dlx1a/2a:CreER^{T2})*. The enhancers are used to drive CreER^{T2} expression in *dlx1a/2a*- and *dlx5a/6a*- expressing cells. Fish from these lines are bred with zebrafish that ubiquitously express a floxed GFP gene followed by the mCherry gene under the control of an ubiquitin promoter, *Tg(ubiSwitch:GFP)* (*Ubi:Switch*, obtained from Dr. L. Zon, Harvard, U. (Mosimann, Kaufman, Li, Pugach, Tamplin, et al. 2011). Embryos resulting from this cross are double transgenic carrying both the ubiquitous expression of GFP and the Cre-ER^{T2} enzyme specifically in *dlx*-expressing cells.

These double transgenic fish are used for the molecular switching, allowing the permanent labeling of *dlx*-expressing cells by the administration of 4 hydroxy-tamoxifen (4OHT). The drug 4OHT is a labile metabolite of the prodrug Tamoxifen (TAM) with a 30–100-fold higher affinity for the ligand-binding ER domain than unmodified TAM (Katzenellenbogen et al. 1984). The drug 4OHT recognizes the ER^{T2} sites placed on the Cre enzyme, which activates Cre driving the enzyme back to the nuclei of the cell. An active Cre-recombinase produces an exchange of the fluorescent label from GFP to mCherry by recombining the two LoxP sites and floxing out GFP. After this molecular recombination takes place, the CreERT2 expressing cells, in this case either *dlx1a/2a* or *dlx5a/6a*, no longer express GFP but mCherry and this is passed on to the progeny of these cells, thus permanently labeling the progeny of *dlx1a/2a*- and *dlx5a/6a*-expressing cells (Fig. 8). This tool allows the observation and tracing of these cells on a time-related manner at different time point

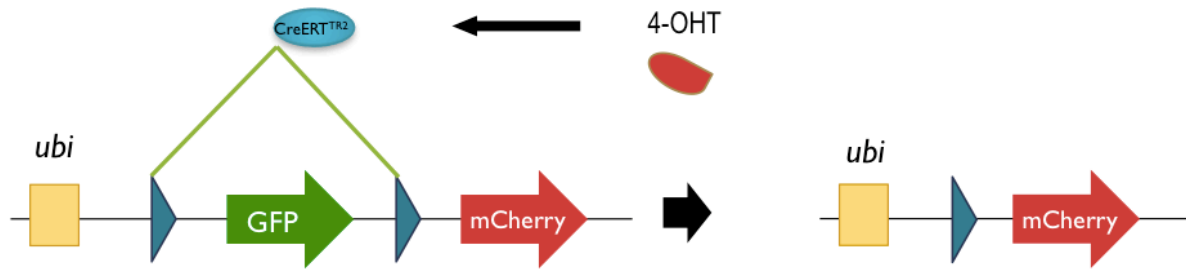


Figure 8 Representation of site-specific Cre recombination in the *Ubi* Switch transgene.

In the presence of CreER^{T2} and after recognition by 4OHT, recombination of *loxP* sites occurs and the GFP sequence is excised, leaving mCherry to be driven by the *Ubi* promoter.

The permanent genetic labeling of the Switch system ensures that the labeled cells will continue to express mCherry throughout the life of the fish and can be observed in the adult brain. A detailed lineage analysis of the *dlx1a/2a* and *dlx5a/6a* expressing progenitors in the zebrafish brain has been carried out using this approach and the transgenic lines previously designed (Solek et al. 2017). These analyses established a foundation for further characterization of the role of these transcription factors even beyond the specification of GABAergic neurons. In some instances for example, labeling larval *dlx5a/6a*-expressing cells, but not *dlx1a/2a*-expressing cells, have resulted in massively expanding, widespread clonal expansion throughout the adult brain.

The majority of GFP-expressing cells in transgenic Tg(*dlx6a*-1.4kbp*dlx5a/ dlx6a*:GFP) developing zebrafish are GABA-positive and some express various GABAergic interneuron markers (Yu et al. 2011). However lineage tracing of *dlx1a/2a* and *dlx5a/6a* expressing progenitors have shown to give rise for the most part, to populations of cells which continuous only a small proportion of GABAergic cells in the adult brain tissue and some of the cells do not acquire a neuronal phenotype suggesting that, regardless of the time a cell expresses *dlx* genes in the brain, it can potentially give rise to cells other than neurons (Solek et al. 2017).

1.13 Zebrafish as a model system to study development and regeneration

The zebrafish is bony tropical freshwater fish that has emerged as an excellent model organism to study vertebrate biology and development (Briggs 2002). One major reason for the popularity of the zebrafish as model system is that, not only it is a vertebrate and therefore closer to human than invertebrate models, it also combines several characteristics - previously found mostly in invertebrate models - that facilitate genetic analysis. The zebrafish is a highly prolific organism and can produce a few hundred eggs per spawning, their generation time is

short (2-3 months), the embryos are optically clear and their development is relatively fast (2-3 days to hatching).

Several molecular resources are also available for the zebrafish including the genome sequence a resourceful Zebrafish Model Organism Database (ZFIN) and other tools allowing the integration of high-throughput molecular analysis to developmental genetics and physiological studies. Functional studies in zebrafish are facilitated by powerful technologies such as transposon-mediated transgenesis, CRISPR-Cas9 and TALEN-mediated mutagenesis (Peng et al. 2014).

Taken together these characteristics combined with greater rates of adult neurogenesis and regenerative capability presented by the zebrafish, this is an ideal model system to study different aspects of regeneration and the role of specific molecular players on adult neurogenesis. The zebrafish also allows different injury paradigms to be used in order to induce regeneration in the brain like physical lesions, chemical injuries and transgenic approaches, each presenting advantages and disadvantages. Physical lesions promote a complex multi-cellular response that involves apoptosis, inflammation, proliferation of glial cells and increased progenitor cell activity that provides a platform to study the numerous aspects of adult neurogenesis and how they differ from developmental programs that can be explored for therapeutic applications in humans (Kizil et al. 2012).

1.14 Statement of inquiry

Complex genetic regulatory networks are responsible for proper establishment of neuronal diversity in the central nervous system during development. The establishment of new neurons also takes place in the adult zebrafish brain where multiple areas present continuous proliferation. Similar to mammals, the zebrafish brain undergoes an injury response, different from mammals however, this teleost fish presents a remarkable ability to regenerate and repair their brain after injury. This regeneration capability makes this organism an ideal model for the identification of genes involved in the establishment of new neurons not only in early stages of development, as this model has been robustly explored for, but also in adulthood where studies are still few.

The *Dlx* homeobox genes encode homeodomain transcription factors that take part in neuronal differentiation. These genes are required for the proper establishment of GABAergic neurons within the brain. The overall goal of the research presented in this thesis is to investigate the activity of *dlx* genes in adulthood, both in homeostasis and in the context of adult neurogenesis. We hope to address questions concerning: the expression of *dlx* genes in the adult zebrafish brain, their cell identity, activity during brain regeneration during an injury response, and the location and fate decisions of the progeny of *dlx*-expressing cells in the adult zebrafish brain.

We hypothesize that all four *dlx* paralogs are expressed in the adult zebrafish brain, that the majority of cells that express these genes are GABAergic neurons and that the progeny of *dlx*-expressing cells in adults will give rise to distinct cell populations. We also conjecture that *dlx*-expressing cells could have different roles in different areas of the adult zebrafish brain. Additionally, during neurogenesis following injury in the zebrafish adult brain, we predict an up-

regulation of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* genes and different patterns of expression of cells expressing these genes.

In addressing the hypothesis postulated above and by better understanding activity of *dlx* genes in adults, we hope to contribute to the general knowledge of the molecular pathways involved adult neurogenesis and regeneration in the zebrafish adult brain.

CHAPTER 2. Materials and Methods

2.1 Animal care, husbandry and strains

Adult and juvenile zebrafish were housed maintained following standard procedures previously described (Westerfield, 2000). All experiments were conducted using protocols approved by the University of Ottawa Animal Care Committee. Light cycle in the zebrafish facility was composed of 14 hours of light and 10 hours of dark. Water conditions of the fish systems were controlled and maintained at 28–30°C and pH 7.5–8.0. Zebrafish embryos were obtained from the natural spawning of adult zebrafish, collected and raised in a petri dish containing Embryo media (13mM NaCl, 0.5mM KCl, 0.02mM Na₂HPO₄, 0.04mM KH₂PO₄, 1.3mM CaCl₂, 1.0mM MgSO₄, and 4.2mM NaHCO₃). At the 5dpf larval stage, they are placed in the water system on the conditions described.

Five transgenic lines plus facility-raised wild type zebrafish were used for the experiments here described. Two reporter lines previously designed in the laboratory where the GFP is expressed under the control of *cis*-regulatory elements of *dlx1a/2a* and *dlx5a/6a*, Tg(*dlx1a/2a*:EGFP) and Tg(*dlx6a-1.4kbpdlx5a/ dlx6a*:GFP) respectively (MacDonald et al. 2010; Zerucha and Ekker 2000; Ghanem et al. 2003). Three other reporter lines were used for Cre/LoxP recombination and lineage tracing, Tg(*dlx1a/2a:CreER^{T2}*) and Tg(*dlx5a/6a:CreER^{T2}*), previously designed in our Laboratory (Solek et al. 2017) and the Tg(ubi:Switch), kindly provided by Christian Mosimann and Christian Lawrence (Mosimann, Kaufman, Li, Pugach, and Tamplin 2011).

2.2 Adult zebrafish brain lesions

Surgeries were performed as described by März (2011). Briefly, after anaesthesia by immersion in Tricaine MS-222 solution at 0.4% in system water, fish are injured by the insertion

of sterile 23 or 30g needle directly and vertically through the skull into the medial region of one telencephalic hemisphere under a dissecting microscope (Schmidt et al. 2014) (Fig. 9). After lesion, fish are immediately returned to new fish tanks. Control specimens are anesthetised just as the lesion individuals, but no injury is provoked (Fig 17). Adult zebrafish between 3 months-post-fertilization (mpf) to 1 year-post-fertilization(ypf) were used for these experiments. After lesion surgeries, adult zebrafish were maintained at the same temperature, light cycle and feeding conditions as the rest of the colony. Lesion and control specimens were euthanized at 3 or 7 days post-lesion (dpl) for analyses.

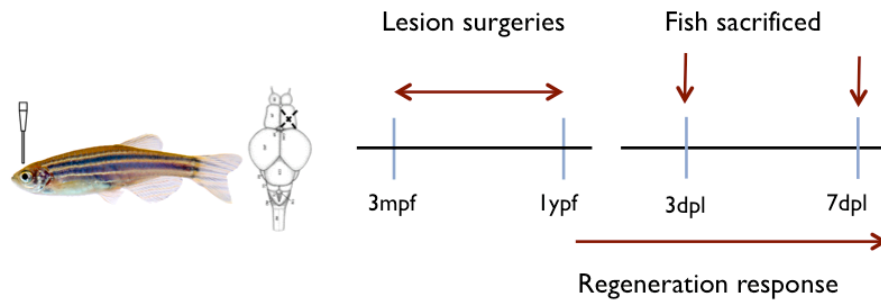


Figure 9. Experimental design for assessing the participation of *dlx* in regeneration and adult switching.

Wild type adult zebrafish received mechanical stab lesions directly through the skull in the telecephalon as shown in the left panel. Surgeries were performed on adult fish aged 3mpf to 1ypf and euthanized at either 3 or 7dpl as represented in the scheme on the right panel.

2.3 Tamoxifen preparation and adult exposure

To understand the contribution and fate of neuronal progenitors cells expressing *dlx1a/2a* during adult neurogenesis at different adult stages, adult zebrafish (3mpf – 1ypf) from a *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:UbiSwitch)* cross were induced with 4-hydroxy Tamoxifen (4OHT) for 6-8 hours each day on 7 consecutive days. The drug 4OHT was diluted in EtOH and added at a final concentration of 5 μ M directly to the water tank (125 μ l in 250 ml of water). Negative control specimens received EtOH only at the same amount (125 μ l) and frequency as treated fish. Fish were euthanized at 3, 7 or 14 days-post-treatment (dpt) for visualization of mCherry expression and cell co-localization analyses (Fig. 10.A). Treatment with 4OHT at this concentration and frequency was sufficient to provoke molecular switching and permanent labelling of the progeny of *dlx1a/2a*-expressing cells in adults.

2.4 Tamoxifen exposure in embryos

Surgeries were performed in adult zebrafish from *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:UbiSwitch)* treated with 4OHT early at developmental stages, at 3dpf (Fig 10.B). The tamoxifen treatment for these embryos proceeded like described by Solek (2017). Briefly, *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:UbiSwitch)* 3dpf embryos were placed in six-well culture plate, approximately 15 embryos per well, and induced with 4OHT for 24hours at a final concentration of 10 μ M. The embryos were placed in an incubator at 28.5°C in the dark for the 24-hours treatment period. Embryos were then washed three times with embryo medium to remove 4-OHT completely.

The screening for mCherry positive cells was carried out under a fluorescent dissecting microscope (Nikon SMZ1000) the day following 4OHT treatment and selected fish positive expressing mCherry were returned to the facility. After reaching 3mpf, these adult *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:UbiSwitch)* fish received a stab lesion under the same protocol as described before and were euthanized at 7 or 14dpl for visualization of mCherry expression and cell counting.

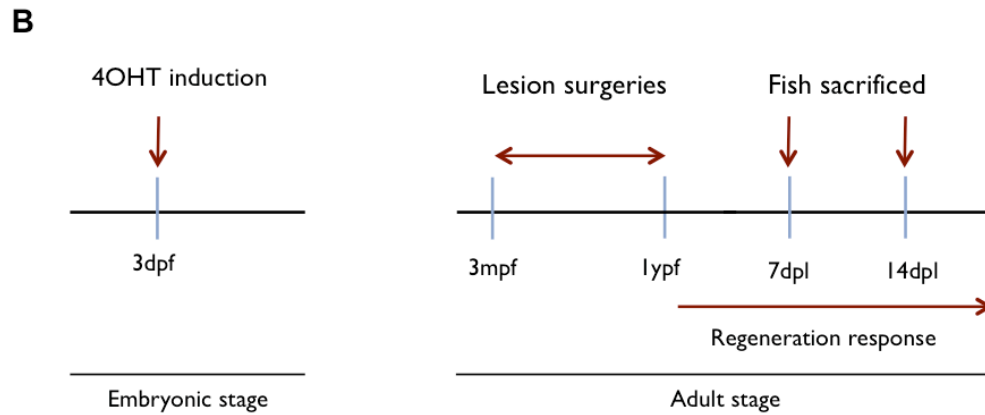
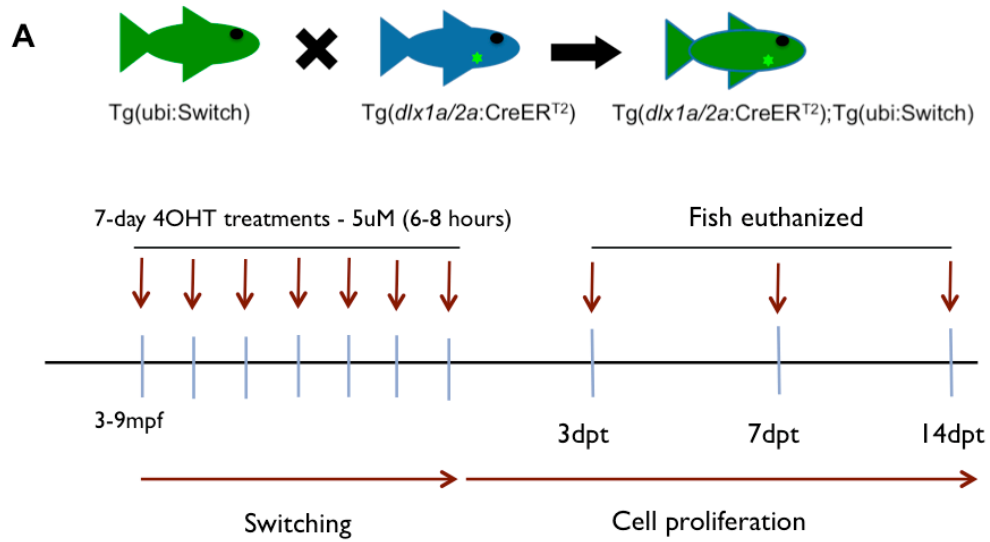


Figure 10. Experimental designs for lineage tracing of *dlx1a/2a* and participation on regeneration.

2.5 Collection of zebrafish brain tissue and sectioning

Adult fish were euthanized in immersion in Tricaine MS-222 solution at 0.8% in system water, the upper skull was removed and the whole head was fixed in 4% PFA/PBS overnight at 4 °C. Whole brain was dissected in a petri dish a under dissecting microscope (Nikon SMZ1000) and post fixed in 4% PFA/PBS for 30 min. The tissue was then washed several times with PBS and equilibrated with 30% sucrose/PBS overnight at 4 °C. Whole brains were then incubated in a solution of 1:2 30% Sucrose:OCT Compound (Tissue-Tek, VWR Canada) for 30 min and frozen inside of cryomolds in liquid nitrogen. Cryosections of 14-16 µm were obtained with a CM3050S cryostat (Leica, Concord, ON) in duplicate, triplicate or quadruplicate slides.

2.6 Immunohistochemistry (IHC), double IHC and cell counting

Sections were first rehydrated in PBST (PBS with 0.1% Tween-20), and blocked in 10% calf serum in PBST for at least 2 hours at room temperature. The primary antibodies were used at different dilutions according to the manufacturer's instructions and optimization of the protocol (Table 1). The primary antibody incubation was carried overnight at 4 °C in 1% calf serum in PBST. Sections were then washed 3 times /15 min with PBST and incubated with the secondary antibodies for 2 h at room temperature (Table 1). Sections were again washed 3 times /15 min with PBST and nuclei visualized with DAPI (Life Technologies, Burlington, ON). For controls, slides containing cryosections did not receive primary antibody incubation.

The Calbindin, Calretinin, TH, PCNA and HuC/D (which recognizes Elav family members HuC and HuD neuronal proteins) antibodies required an extra step of antigen retrieval. Sections were treated for 20 min at 85 °C in 0.01 M sodium citrate/0.05% Tween-20 solution and cooled down to RT for 15 minutes prior to blocking. Images were acquired with either a Nikon A1

Confocal microscope or a Zeiss AxioPhot Fluorescence Microscope and treated with NIS-Elements Advanced Research Software or ImageJ.

For cell counting, sections of lesioned and control individuals were matched both by anatomic similarities and number of sections acquired for each brain. Images were first equally treated with ImageJ with contrast/brightness adjustments making positive cells more distinguishable and cell counts in each section were performed by a minimum of two individuals in a blinded fashion to eliminate researcher bias.

Table 1. Antibodies used for Immunohistochemistry

Primary antibodies			
Host	Target	Catalogue - Supplier	Dilution
mouse	Calbindin	38A - Swant, Marly, Switzerland	1:450
mouse	Calretinin	6B3 - Swant, Marly, Switzerland	1:450
rabbit	GABA	A2052 – Sigma, St. Louis, MO	1:500
rabbit	Gfap	z033429-2 – Agilent, Santa Clara, CA	1:450
mouse	GFP	3E6 – Life Technologies, Burlington, ON	1:500
rabbit	GFP	A11122- Life Technologies, Burlington, ON	1:500
mouse	HuC/D	A-2127 - Life Technologies, Burlington, ON	1:200
rabbit	mCherry	ab167453 - Abcam, Toronto, ON	1:750
mouse	PCNA	ab29 – Abcam, Toronto, ON	1:450
rabbit	Sox2	ab97959 - Abcam, Toronto, ON	1:450
mouse	TH	ab152 - Millipore, Darmstadt, Germany	1:350
Secondary antibodies			
Host	Target	Catalogue - Supplier	Dilution
goat Alexa 488	mouse	Life Technologies, Burlington, ON	1:1000
goat Alexa 594	mouse	Life Technologies, Burlington, ON	1:1000
goat Alexa 488	rabbit	Life Technologies, Burlington, ON	1:1000
goat Alexa 594	rabbit	Life Technologies, Burlington, ON	1:1000

2.7 Riboprobes and In-situ hybridization (ISH)

Expression of *dlx1a*, *dlx2a*, *dlx5a*, *dlx6a* and *gad65* was determined using In situ hybridization assays with antisense mRNA probes on crysections as described (Dorsky et al., 1995). Antisense mRNA probes were labeled with digoxigenin-dNTP or dinitrophenol DNP-11-UTP and synthesized from cDNA clones, *dlx1a* (Ellies et al., 1997), *dlx2a* and *dlx5a* (Akimenko et al., 1994), *dlx6a* (Ellies et al., 1997) and *gad65* (Martin 1998). Vectors containing the cDNA clones were linearized with either BamHI, EcoRI or XhoI and the antisense riboprobes were synthesized using either the T7 or T3 polymerase as required.

Brain sections stored at -20°C were thawed at room temperature for 30 minutes before the experiment. Hybridization on slides containing sections was carried out overnight at 70°C in a humidified chamber. Slides were washed twice with Solution A (50% Formamide, 5% 20x SSC in dH₂O) and twice with TBS. Blocking with 10% FBS TBST was carried for 2 hours in RT. Detection of hybridized probes was performed with anti- DIG antibodies AP fragments (Roche, Basel Switzerland; dilution 1:1000) overnight at 4°C. After four TBST washes, staining was developed with NBT/BCIP for 6–18h (Sigma, St-Louis, MO). Images were acquired with a Zeiss AxioPhot Fluorescence Microscope.

2.8 Double Fluorescent In-situ Hybridization (dbIFISH)

For double fluorescent in situ hybridization (dbIFISH) sections were treated with 2% H₂O₂ in PBS to inactivate endogenous peroxidase followed by incubation with anti- DIG antibodies POD fragments in combination with anti- DNP POD fragments were used (Roche, Basel Switzerland; dilution 1:1000). Incubations with these antibodies was done separately at 4°C, overnight, for each of the antibodies. Staining with tyramide Cy3 solution (1:100) in

PBS/Tween and Fluorescein was done for 10 min each (Perkin-Elmer, Woodbridge, Ontario). Images were acquired with a Nikon A1 confocal microscope and/or Zeiss AxioPhot Fluorescence Microscope and treated with NIS-Elements Advanced Research Software or ImageJ.

2.9 RNA extraction, cDNA synthesis and qRT-PCR

Quantification of RNA within brain tissues of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* transcripts, was performed on a BioRad CFX96 quantitative Reverse Transcription PCR detection system using SsoFast EvaGreen (BioRad) fluorescent dye supermix and specific primers for each gene (Table 2). Primers were designed in separate exon sequences using NCBI's Primer-BLAST Program (Primer-Blast, National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD) ensuring products were free of primer dimers.

Total RNA was extracted from the dissected and isolated forebrain of each adult fish using homogenization with TriZol (Ambion) according to manufacturer protocol. Concentration of extracted RNA was obtained using NanoDrop 2000 (Thermo Scientific). Synthesis of cDNA was accomplished by reverse transcription of total RNA. From control and lesion specimens 500ng of total RNA were reverse transcribed using the Quantitect reverse transcription kit (Qiagen). Quality and purity of cDNAs was confirmed by Agarose gel Electrophoresis.

In order to assay transcripts of genes of interests by qPCR, the following conditions were used: 95°C for 30s, followed by 40 cycles of 95°C for 5s and 59°C for 5s, then a melt curve progressing from 65°C to 95°C, at 5s per 0.5°C increase. Two reference genes were used for each qPCR either *ef1a*, *ywhaz* or *rpl13a*. Data were analyzed using CFXManager (Bio-Rad) and compiled using GraphPad PRISM.

Table 2. Primers used for qRT-PCR

Gene of interest	Forward primer	Reverse primer	Fragment size
<i>dlx1a</i>	CAACTCGGTCGGTAGCCATT	GCTTGCGGATCTTTTGCCT	176 bp
<i>dlx2a</i>	GAAACGCTTTCGGCCCCTA	CCATTCGGATTTCAGGTTTCGC	96 bp
<i>dlx5a</i>	GGCTCATACTCCACAGCGTA	CATCCTTACTTCGGGCTCGG	105 bp
<i>dlx6a</i>	CAGCAGACTCAATACCTGGCA	TACCGCCTTGTTTCAACAGC	133 bp
<i>ef1a</i>	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTA GCATTAC	87 bp
<i>ywhaz</i>	TCTGCAATGATGTGTTGGAGC	TCAATGGTTGCTTTCTTGTCGTC	151 bp
<i>rpl13a</i>	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	148 bp

2.10 Hematoxylin/Eosin staining

For histological observations within sections of lesioned brain, Hematoxylin and Eosin (HE) staining was carried as previously described (Sullivan-brown, Bisher, and Burdine 2011). Briefly, cryosections-containing slides were immersed in Gill's Hematoxylin (Polysciences Inc., cat. #24243) for 5 minutes and rinsed in deionized water. Destaining was carried with acid water, deionized water washes and Scott's Tap Water. Slides were then immersed in Eosin (Electron Microscopy Sciences, cat. #14851) dye for 3 minutes, rinsed, dried at RT and sealed with coverslips.

2.11 Statistical analyses

Statistical comparison of two groups (lesion and controls) for GFP cell counting values and qRT-PCR results was conducted using an unpaired t-test using GraphPad PRISM. An alpha-value of 0.05 was defined as statistically significant. For * $p \leq 0.05$ and n.s. = not significant $p > 0.05$. Error bars represent standard error on the mean (SEM).

CHAPTER 3. Results

Results - Part 1

3.1 Expression of *dlx* genes in the adult zebrafish brain and characterization of *dlx*-expressing cells identity.

3.1.1 Expression of *dlx* genes in the adult zebrafish

The expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* in the adult zebrafish brain was first analysed in wild type fish. This provided us with novel information regarding location and domains of expression of the four different *dlx* paralogs in the adult brain. The expression of the four different *dlx* genes presented a very similar pattern with *dlx1a*, *dlx2a* and *dlx5a* appearing to be generally more abundant (Fig. 11). It was possible to visualize the expression of all four paralogs in all the subdivisions of the adult brain with distribution in regions of the forebrain and midbrain.

For all of the four paralogs the expression was consistently abundant in the ventricle region of the forebrain, especially in the dorsal, ventral and postcommissural nucleus of ventral telencephalic area (Vd, Vv and Vp) (Fig 11. B - B'''). For *dlx2a* and *dlx5a*, expression was also found within the central nucleus of the ventral telencephalic area (Vc). The anterior part of the parvocellular preoptic nucleus (PPa) was observed to be one of the regions with the most abundant expression of all four *dlx* genes in the adult zebrafish brain (Fig. 11 C-C'''). This is an area known to present continuous proliferation in adults and also to be the transition between forebrain and midbrain (Kizil et al. 2012) . The caudal and dorsal zones of the periventricular hypothalamus (Hc and Hd) presented abundant expression of all four *dlx* genes (Fig 11. D - D'''). This region of the zebrafish brain is still not mature in embryos. The hypothalamus has multiple important roles in the adult brain it is one of the areas in the zebrafish brain where there is also continuous proliferation.

These data has demonstrated for the first time that *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* are expressed in the adult zebrafish brain and the expression was consistent and similar among the four different paralogs. These observations were also consistent among different stages in adult zebrafish, ranging from 3mpf to 18mpf (N=8 for each gene).

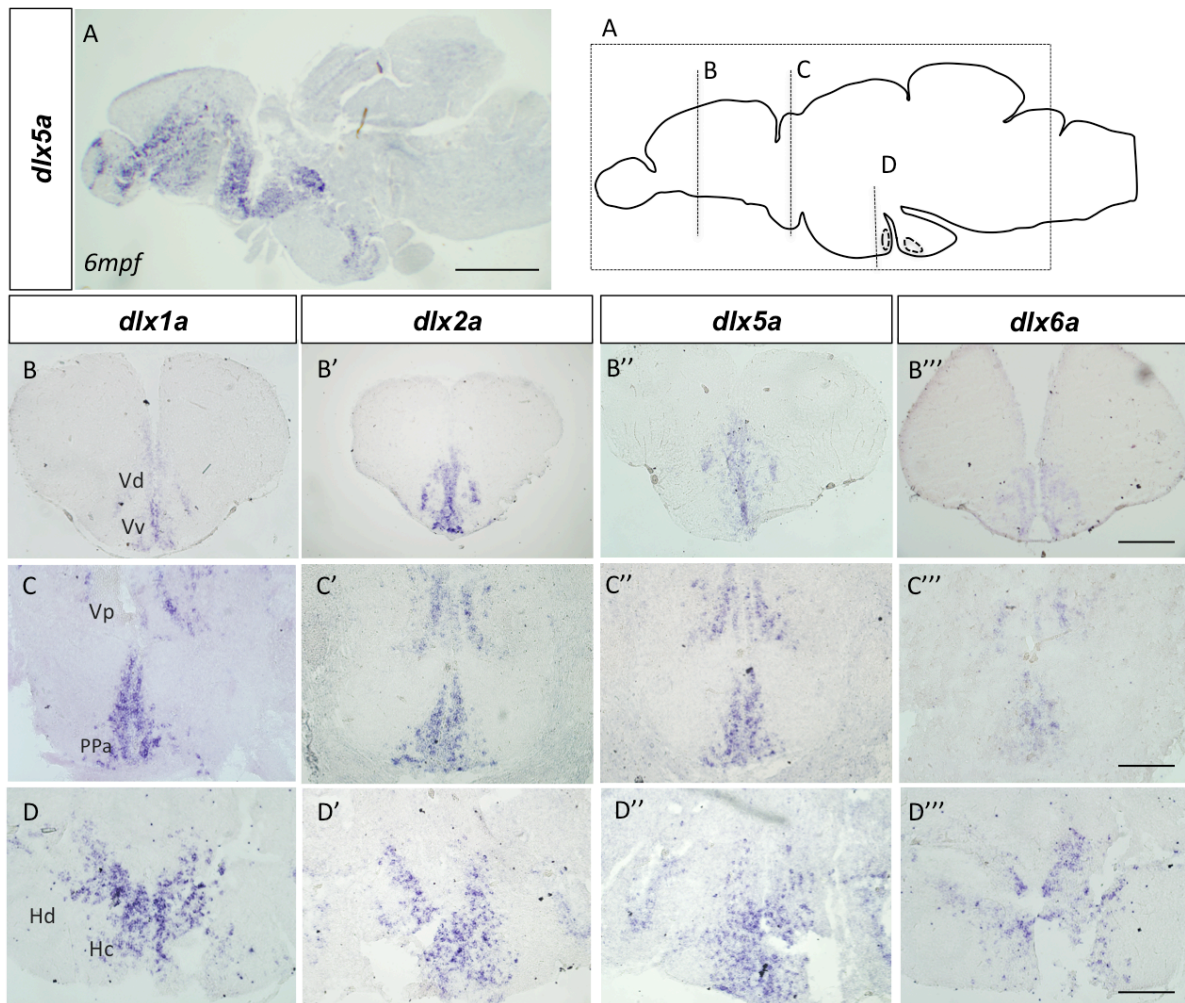


Figure 11. *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* are expressed in the adult zebrafish brain.

Schematic representation of sections depicted in the top right panel. In situ hybridization shows *dlx* expression in consecutive cryosections of one adult zebrafish brain. (A) Sagittal section showing *dlx5a* expression on a 6mpf fish. Transverse sections showing expression throughout areas of the forebrain, midbrain and hindbrain of *dlx1a* (B-D), *dlx2a* (B'-D'), *dlx5a* (B''-D'') and *dlx6a* (B'''-D''') in 1 ypf zebrafish (N=6 for each *dlx* gene). Ventral telencephalic area (V), dorsal nucleus of V (Vd), ventral nucleus of V (Vv), postcommissural nucleus of V (Vp), anterior part (PPa), caudal zone of periventricular hypothalamus (Hc) and dorsal zone of periventricular hypothalamus (Hd)

Scale bar (B-C): 400µM (D): 200µM

The expression of *dlx5a* and *dlx6a* genes was further verified with the GFP reporter line *Tg(dlx6a-1.4kbdlx5a/6a:GFP)*, created by previous members of the Laboratory (Zerucha et al. 2000; Ghanem et al. 2003), where a 3.5kb *dlx6a* fragment and the intergenic fragment containing I56i and I56ii between the *dlx5a/6a* bigene, drives the expression of GFP. In this line, GFP expression is indicative of *dlx5a* and/or *dlx6a*. As there is an absence of effective antibodies against all Dlx proteins, these lines are very important in order to visualize several aspects of development and activity in adults and specially for co-localization analyses of *dlx5a/6a*-expressing cells with different markers using double immunostaining.

Similar to what was observed with the in situ hybridization assays, in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* transgenic fish, the expression of *dlx5a/6a* was observed in different regions throughout the forebrain, midbrain and hindbrain in a very similar pattern of distribution (Fig. 12).

Immunohistochemistry with antibodies recognizing GFP, indicative of *dlx5a/6a*, revealed the presence of GFP-expressing cells throughout the different areas of the forebrain. More specifically, *dlx5a/6a* expression was found in the olfactory bulb (OB) dorsal telencephalic area (D) in the dorsal (Vd), ventral (Vv) and central (Vc) nucleus of the ventral telencephalic area. In the midbrain, expression was localized to the anterior part of the parvocellular preoptic nucleus (PPa), within the parvocellular preoptic nucleus and at the caudal and dorsal zones of the periventricular hypothalamus (Hc and Hd) (Fig. 12). Expression of the *dlx5a/6a* bigene was observed in the dorsal telencephalic area, within the periventricular grey zone of the optic tectum (PGZ) and the coporus cerebelli, different than reported by In situ hybridization. The PGZ is known to be where most of the proliferating cells are distributed in the midbrain (Ito et al. 2010).

Overall, these observations demonstrate in concordance with the observations with in situ hybridization, the expression of the bigene cluster *dlx5a/6a* is observed throughout forebrain and midbrain similarly to results obtained by mRNA expression. The exception would be the expression observed in the periventricular grey zone of the optic tectum (PGZ), where the in situ expression in this region was observed to be very weak.

Another reporter line previously generated in our laboratory where enhancers of *dlx1a/dlx2a* bigene drive expression of GFP was also used, the *Tg(URE2dlx1a/2a:GFP)* (MacDonald et al. 2010). However, expression of GFP was undetectable at the adult stage. More specifically time-course of reporter expression, starting at the juvenile stage showed that GFP was no longer detectable at around 2mpf. Immunohistochemical assays with adult zebrafish from different crosses of this reporter line showed no expression of GFP. Therefore, all results presented here concerning GFP labelling, including co-expression assays in adult zebrafish, were analyses carried out using the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* line.

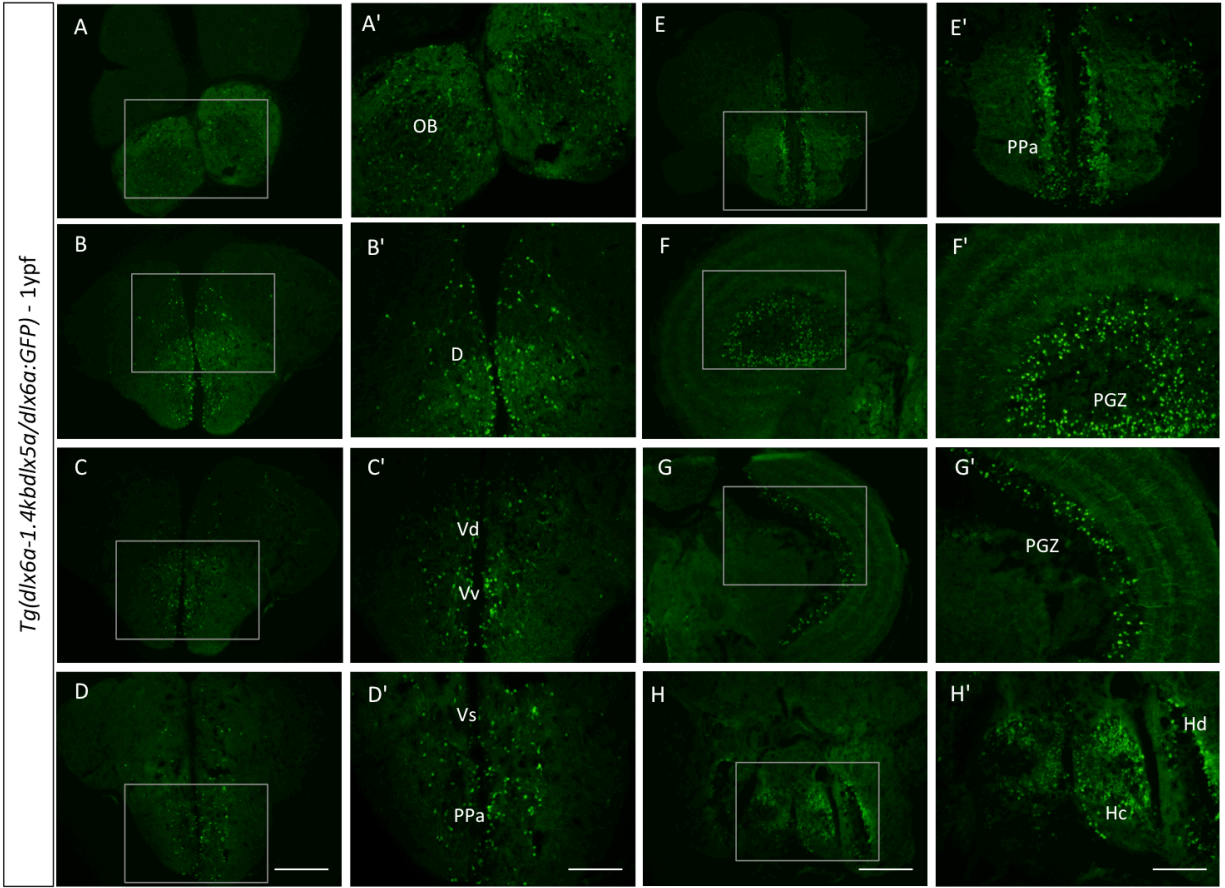


Figure 12. Immunohistochemical labeling of GFP in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* adult zebrafish.

Expression determined with a GFP antibody in transverse cryosections of *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* adults. Gray rectangles in A-H indicate area observed at higher magnification in A'-H'. Dorsal telencephalic area (D), ventral telencephalic area (V), dorsal nucleus of V (Vd), ventral nucleus of V (Vv), parvocellular preoptic nucleus, anterior part (PPa), optic tectum (TeO), periventricular grey zone of optic tectum (PGZ) and caudal zone of periventricular hypothalamus (Hc). Scale bar (A-H): 200µM (A'-H'):

3.1.2 Expression of *dlx* and neurogenic zones in the brain of zebrafish

The adult zebrafish presents high rates of continuous proliferation in multiple niches and neurogenic areas along the brain as described in previous chapters. We sought to compare the expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* with the areas in the adult brain known to have adult stem cells niches and to present continuous proliferation. In fact, many of the adult zebrafish brain domains where *dlx* is expressed are indeed neurogenic areas and some of these, also present continuous proliferation.

The following neurogenic zones with continuous proliferation presented similar expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a*: Olfactory bulb (OB), ventral nucleus of ventral telencephalic area (Vv), parvocellular preoptic nucleus, anterior part (PPa), posterior zone of dorsal telencephalic area (Dp), periventricular nucleus of posterior tuberculum (TPp) and caudal zone of periventricular hypothalamus (Hc) and dorsal zone of periventricular hypothalamus (Hd) (Fig. 13, similar observations for *dlx2a* and *dlx6a*). Expression of the *dlx5a/6a* bigene was also verified within the periventricular gray zone of optic tectum (PGZ) as verified with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line.

These observations demonstrate that all four *dlx* paralogs are expressed in almost all niches presenting continuous proliferation in the adult zebrafish brain, suggesting that these genes may participate in continuous proliferation throughout adult stages of the zebrafish brain. The observations of where *dlx* genes are expressed in this context can further help elucidate the nature and identity of these progenitor cells that are neurogenic and the molecular patterning cues in the newly formed cells that direct differentiation and proper establishment in the mature brain.

- Region with constitutive proliferation
- Neurogenic areas

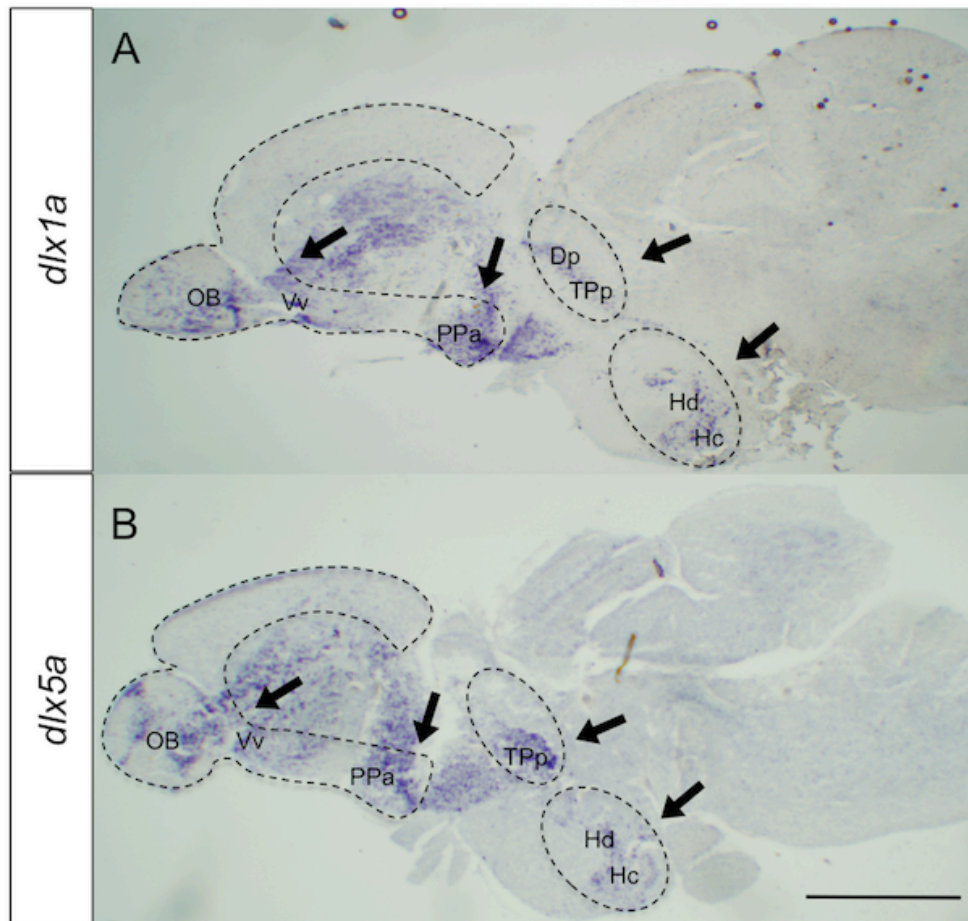
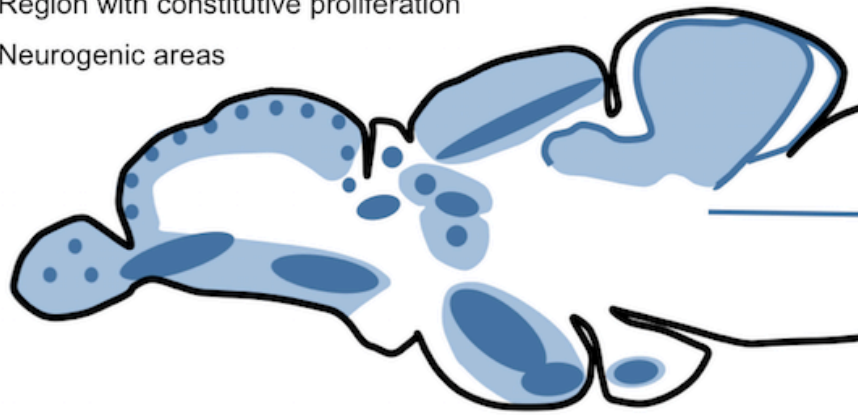


Figure 13. Comparison of *dlx1a* and *dlx5a* expression and neurogenic areas in the adult zebrafish brain.

Upper panel shows drawing of an adult brain sagittal section depicting neurogenic areas and zones with constitutive (continuous) proliferation (adapted from Kizil C. *et al*, 2011). [A-B] shows expression of *dlx1a* and *dlx5a* verified with ISH. Arrows indicate the main areas where expression of *dlx* genes matches areas with continuous proliferation. These areas are: olfactory bulb (OB), ventral nucleus of ventral telencephalic area (Vv), parvocellular preoptic nucleus, anterior part (PPa), posterial zone of dorsal telencephalic area (Dp), periventricular nucleus of posterior tuberculum (TPp) and caudal zone of periventricular hypothalamus (Hc) and dorsal zone of periventricular hypothalamus (Hd). Scale bar: 1mm

3.1.3 Identity of *dlx*-expressing cells in the adult zebrafish brain

To characterize the identity of cells expressing *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* in the adult zebrafish brain, we performed several co-localization analyses using double immunohistochemistry and double fluorescent in situ hybridization assays. Based on the relationship between *dlx* and *gad1* expressions and on regulatory roles for *dlx* genes in GABAergic neuron development, we first wanted to analyse if *dlx*-expressing cells could be GABAergic interneurons in the adult zebrafish brain. Double fluorescent ISH assays were performed combining an mRNA probe recognizing one of the four *dlx* paralogs with *gad65*, a gene encoding an enzyme that catalyzes the decarboxylation of glutamate to GABA and CO₂.

Widespread co-expression of *dlx* genes and *gad65* was observed throughout the adult zebrafish forebrain. The great majority of *dlx2* and *dlx5a*-expressing cells co-expressed *gad65* in the medial zone of the dorsal telencephalic area (Dm), dorsal nucleus of ventral telencephalic area (Vd), postcommissural nucleus of ventral telencephalic area (Vp), parvocellular preoptic nucleus, anterior part (PPa) and posterior part of parvocellular preoptic nucleus (PPp) (Fig. 14 I-L, N=4). Similar results were observed for *dlx1a* and *dlx6a* (N=3). In the midbrain, co-expression of *gad65* and *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* presented seemingly lower levels when compared to areas of the forebrain. In the ventral zone of periventricular hypothalamus (Hv) and dorsal zone of periventricular hypothalamus (Hd), only a small portion of *dlx*-expressing cells presented a GABAergic interneuron identity (Fig. 15).

These results demonstrate that the great majority of *dlx*-expressing cells in the forebrain of adult zebrafish are indeed GABAergic mature interneurons. In the midbrain and specially at the hypothalamus domain a great portion of *dlx*-expressing cells do not have a GABAergic identity indicating that those cells could still be in the cell cycle and have not yet acquired the GABAergic phenotype or that, in these areas, *dlx*-expressing cells present different identities.

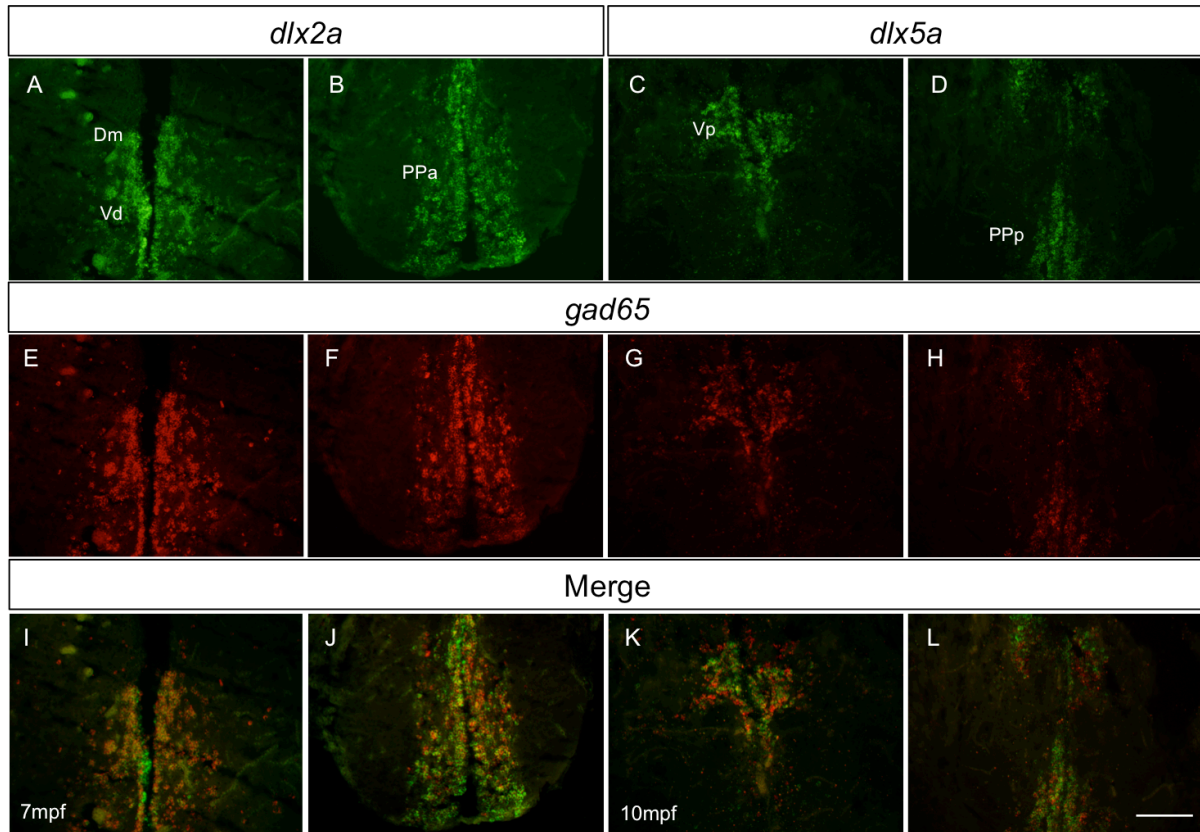


Figure 14. Co-expression of *dlx2a* and *dlx5a* with *gad65* in the adult zebrafish forebrain.

Double fluorescent in situ hybridization with transverse sections of forebrain with [A-D] expression of *dlx2a* and *dlx5a* in green and anatomical parts indicated and [E-H] expression of *gad65* in red. [I-L] Merging of images shows co-localization of dlx and gad65 in yellow. Merged images were created with ImageJ(32) software. (N=4 for *dlx2a* and *dlx5a* and N=3 for *dlx1a* and *dlx6a*). Scale bar: 200µM

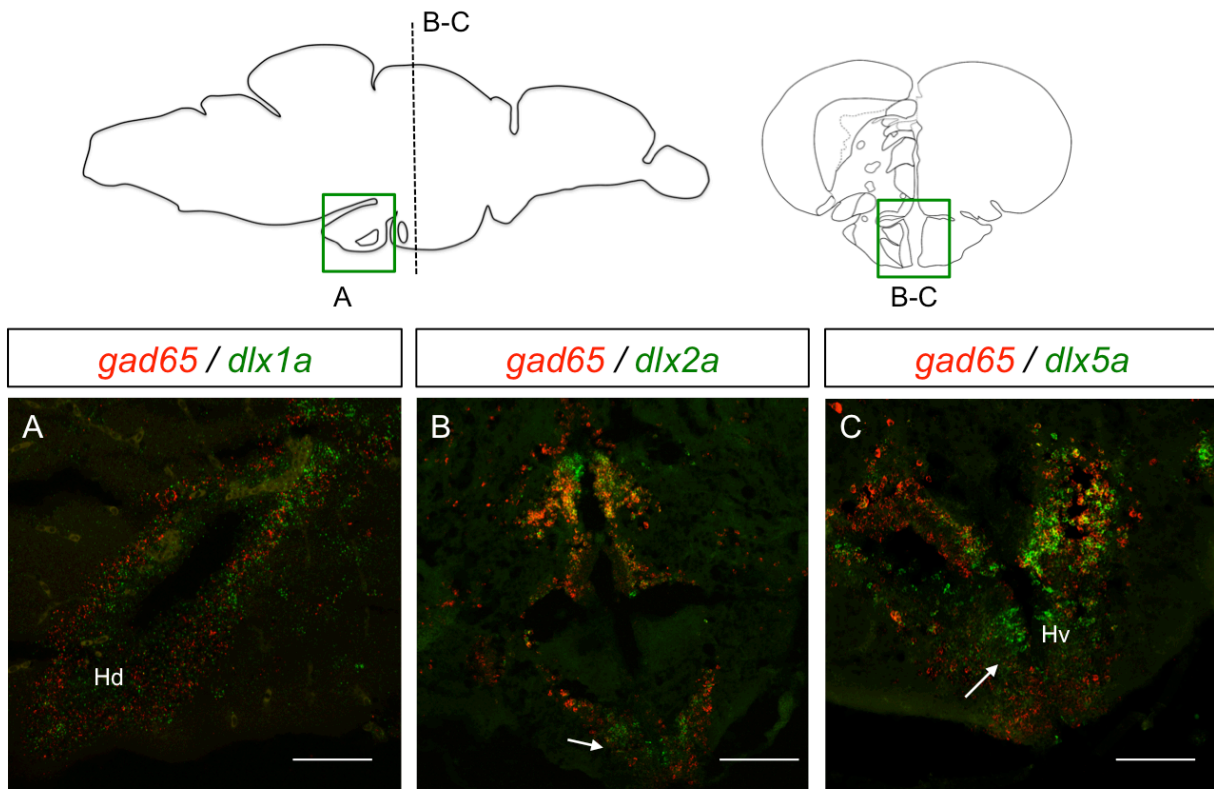


Figure 15. Co-labelling of *dlx1a* and *dlx2a* with *gad65* in the adult zebrafish midbrain.

Upper panel shows drawing of an adult brain sagittal section on the left and a transverse section on the right showing areas depicted in the images (adapted from Wilimann M., 1996). Double fluorescent in situ hybridization with sagittal (A) and transverse (B-C) sections of midbrain and expression of *dlx1a*, *dlx2a* and *dlx5a* in green and *gad65* in red. Merging of images show co-localization of *dlx* and *gad65* in yellow and white arrows indicate areas where *dlx* expression does not colocalized with *gad65*. Merged images were created with NIS-Elements Advanced Research Software. Scale bar (a and C):50µM (B):100µM

Even though all GABAergic neurons present an inhibitory function, these neurons can be morphologically, electrically and chemically heterogeneous and there are several subtypes of GABAergic neurons in the SNC. We sought to further analyse what subtypes of GABAergic interneurons these *dlx*-expressing cells could be classified into. Using double immunohistochemistry assays with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line, we performed co-localization analyzes with three of the most abundant different subtypes of GABAergic neurons, namely calbindin, calretinin and parvalbumin (Gonchar and Burkhalter 1997).

Our observations indicate that the majority of *dlx5a/6a*-expressing cells do not co-localize with these specific subtypes with a few exceptions (Fig. 16). Co-labeling with Calretinin has shown very little to almost null co-localization with GFP. Only a few *dlx5a/6a*-expressing cells appear to be Calretinin neurons in the periventricular gray zone (Fig. 16.C). In fact, within the PGZ area, a few *dlx5a/6a*-expressing cells have also shown a Calbindin and a Parvalbumin identity and IHC assays with anti-Parvalbumin have shown inconclusive results to other areas of the adult zebrafish brain.

Interestingly, we observed many *dlx5a/6a*-expressing cells co-localizing with Calbindin marker within the supracommissural nucleus of the ventral telencephalic area (Vs) and within the anterior and posterior part of parvocellular preoptic nucleus (PPa and PPp) in the diencephalon (Fig. 16.E). A few GFP positive cells also co-express the Calbindin marker within the dorsal and ventral nucleus of ventral telencephalic area (Vd and Vv), but with very low co-localization incidence. No co-localizations were observed in areas of the Hypothalamus.

These results revealed that the great majority of *dlx5a/6a*-expressing cells within the telencephalon and diencephalon of the adult zebrafish are not co-labeled by the three interneuron markers that we tested. The exception to these observations is the Vs, PPa and

PPp area of the diencephalon where many GFP positive cells co-localize with the marker for Calbindin.

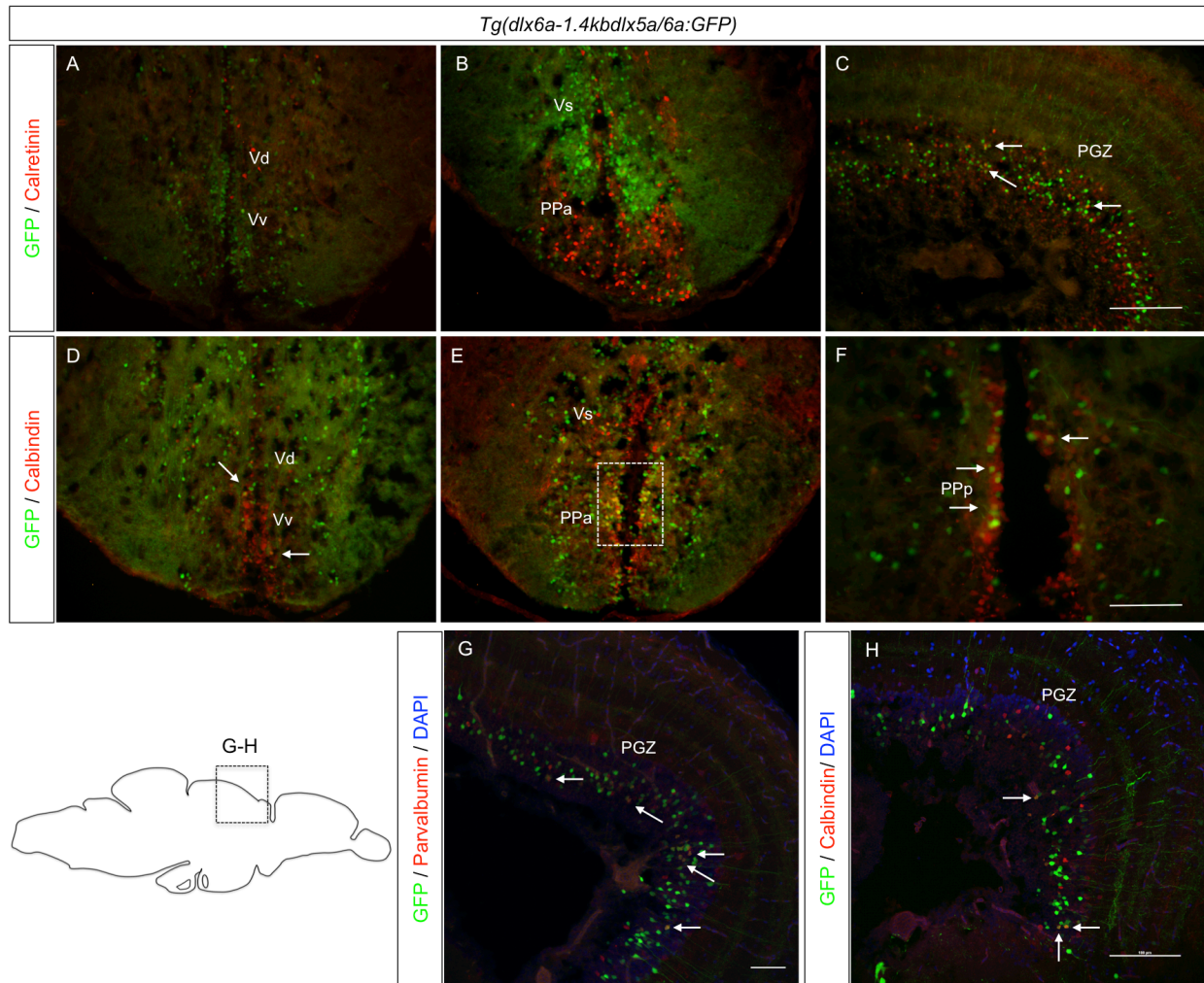


Figure 16. Immunohistochemical co-labeling of GABAergic subtypes and GFP in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* adult zebrafish.

Expression determined with a GFP antibody to stain transverse (A-F) and sagittal (G-H) sections of *Tg(dlx6a-1.4kbdlx5a/6a:GFP)*. Schematic representation of location of sagittal sections in the bottom left corner. [A-C] merged images of GFP (green) and Calretinin (red) show co-localizations of both marker in yellow. [D-F and H] merged images of GFP (green) and Calbindin (red) show co-localizations of both markers in yellow. [G] merged images of GFP (green) and Parvalbumin (red) show co-localizations of both markers in yellow. Arrows indicate co-localization events and rectangles demonstrate area with multiple co-localizations. Merged images were created with with ImageJ(32) software and NIS-Elements Confocal software. Ventral telencephalic area (V), dorsal nucleus of V (Vd), ventral nucleus of V (Vv), parvocellular preoptic nucleus , anterior part (PPa), periventricular grey zone of optic tectum (PGZ) and caudal zone of periventricular hypothalamus (Hc).

Scale bars (A-E and G-H): 200µM; (F): 100µM

We verified if GFP positive cells in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* would co-localize with cells labeled with Sox2. Antibodies against Sox2 are commonly used to label NSCs in zebrafish research (Diotel et al. 2013) (Boldrini et al. 2018). As *dlx* genes can also be considered proneural transcription factors known to promote neural proliferation (Petryniak et al. 2007) (Castro et al. 2011), we wanted to investigate if NSCs cells could also express *dlx* genes.

We observed that cells expressing *dlx5a/6a* co-localized with Sox2 in some areas of the adult zebrafish brain while expression of GFP and Sox2 were just adjacent in others. The following areas of the forebrain presented a small percentage of co-localization of the two markers: medial dorsal telencephalic area (Dm) in the dorsal and ventral nucleus of the ventral telencephalic area (Vd and Vv). Within the supracommissural nucleus of the ventral telencephalic area (Vs), we observed sparse but a higher percentage of co-localization than the domains of the telencephalon as mentioned before (Fig. 17).

The anterior part of the parvocellular preoptic nucleus (PPa) within the diencephalon, was one of the areas where many *dlx5a/6a*-expressing cells co-localized with Sox2 expression. The more rostral portions of the dorsal and ventral hypothalamus presented some co-localization of GFP and Sox2, however when analysed further, the most caudal portions of the hypothalamus seemed to reveal a high percentage of GFP and Sox2 co-localization (Fig. 18).

These data suggest that in some areas of the adult zebrafish brain a number of *dlx5a/6a*-expressing cells could be neural stem cell. This NSCs identity was verified especially in the PPa and within the hypothalamus, two areas where *dlx* expression is very abundant. This suggests that within these areas, these *dlx5a/6a*-expressing cells can have a role in promoting neural proliferation during adulthood in the zebrafish brain. On the other hand, in all other areas of the adult zebrafish not mentioned before, the great majority of *dlx5a/6a*-expressing did not co-localize with Sox2. The adjacent expression of *dlx5a/6a* to Sox2, suggest these *dlx*-expressing cells already differentiated into mature neurons.

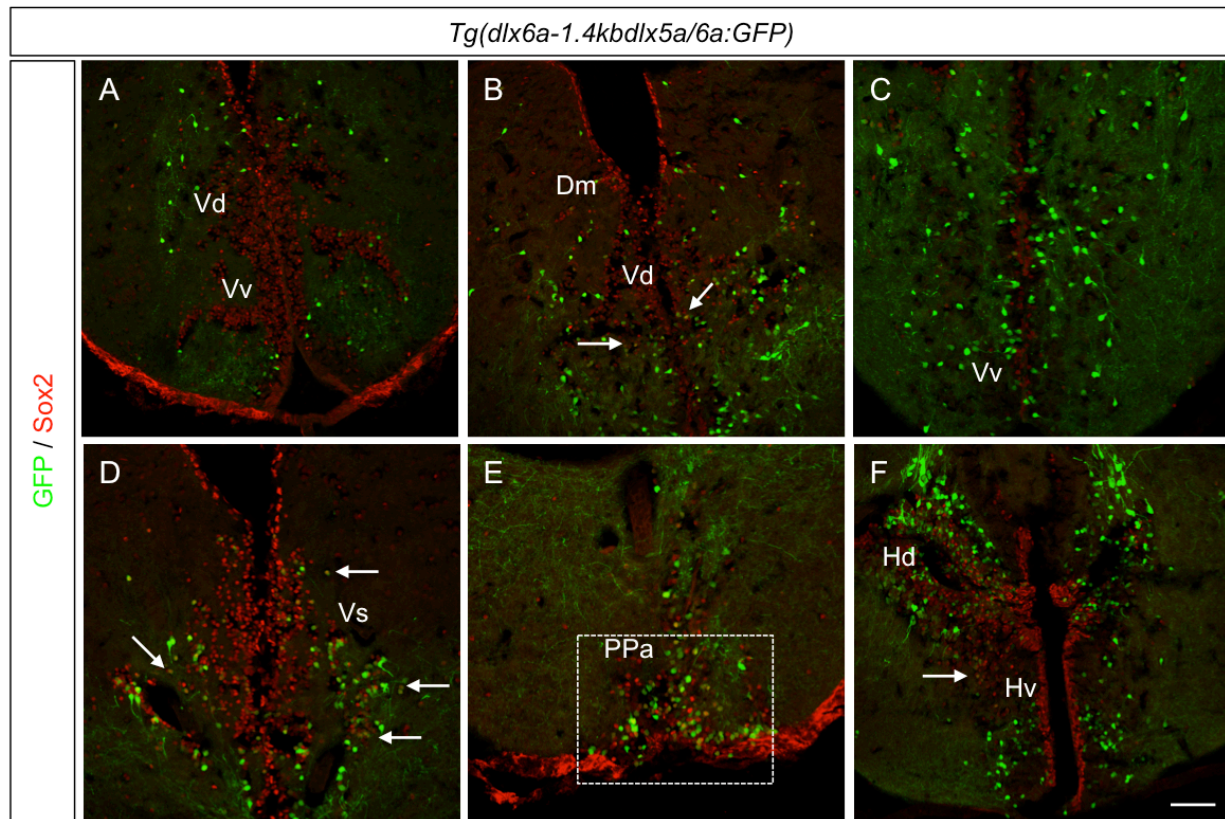


Figure 17. Immunohistochemical co-labeling of GFP in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* adult zebrafish (1ypf) and Sox2.

Expression determined using a GFP antibody to stain transverse cryosections of *Tg(dlx6a-1.4kbdlx5a/6a:GFP)*. [A-F] merged images of GFP (green) and Sox2 (red) show co-localizations of both marker in yellow. Arrows indicate co-localization events and rectangles demonstrate area with multiple co-localization. Merged images were created with NIS-Elements Confocal software. Medial dorsal telencephalic area (Dm), Ventral telencephalic area (V), dorsal nucleus of V (Vd), ventral nucleus of V (Vv), parvocellular preoptic nucleus , anterior part (PPa) and dorsal and ventral zone of periventricular hypothalamus (Hc and Hv).

Scale bar: 50µM

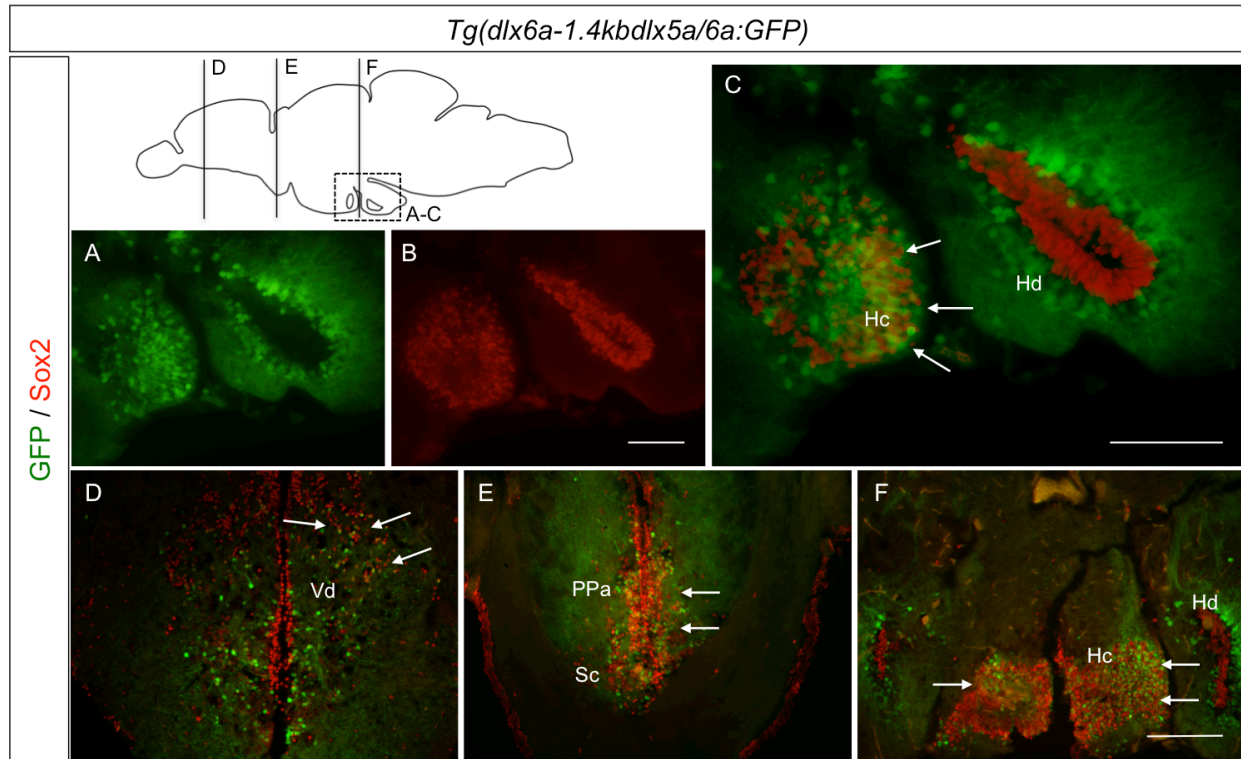


Figure 18. Immunohistochemical co-labeling of GFP in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* adult zebrafish (1ypf) and Sox2 in the Hypothalamus.

Schematic representation of sections depicted in the top left panel. Expression determined with a GFP antibody to stain transverse and sagittal cryosections of *Tg(dlx6a-1.4kbdlx5a/6a:GFP)*. Merged images of [A] GFP (green) and [B] Sox2 (red) and co-localization of both markers in [C-F] yellow (white arrows). Merged images were created with NIS-Elements Confocal software. Medial dorsal telencephalic area. Dorsal nucleus of ventral telencephalic area (Vd), Suprachiasmatic nucleus (Sc), parvocellular preoptic nucleus, anterior part (PPa) and dorsal and caudal zone of periventricular hypothalamus (Hc and Hd).

Scale bars (A-C): 50µM; (D-E): 100µM

We sought to continue exploring possible identities of cells expressing *dlx* genes in the adult zebrafish brain. The same co-localization experiments with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line were conducted with Glial fibrillary acidic protein (GFAP) in order to identify if some *dlx*-expressing cells could represent glia populations, as well as with proliferating cell nuclear antigen (PCNA) to investigate if these cells could be proliferating in the adult brain. Confocal microscopy analyses show very rare, if any, co-localization of GFP positive cells and the PCNA or GFAP markers (Fig. 19). These observations indicate that in the adult zebrafish brain, *dlx5a/6a*-expressing cells may not have a proliferating or glia cell identity. It is important to note that PCNA may not necessarily label the full population of dividing cells.

Although scarce, previous studies have shown possible associations of *dlx* genes and regulation of dopaminergic neurons (Saino-Saito, Berlin, and Baker 2003). Additionally, some evidences indicate co-expression of markers for GABAergic neurons and tyrosine hydroxylase (TH), an enzyme that catalyzes the first reaction in dopamine biosynthesis. We decided to investigate if, in adult *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* zebrafish, there was co-localization of GFP and TH in the adult brain. Once again, results show rare instances of a few single cells co-expressing GFP in and TH in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* in the forebrain (Fig. 20) (n=4). Most of the co-localization was observed in the caudal zone of the hypothalamus (Hc) (Fig. 21).

Taken together, these co-localization observations reveal that in the adult zebrafish forebrain, the majority of *dlx*-expressing cells seem to have a GABAergic neuronal identity. Other co-localizations were observed, especially at the hypothalamus of the adult zebrafish brain. Overall the results here obtained are summarized ahead (Table 3). The amount of co-localization is given by the presence and number of “+” signs within the different main brain areas where *dlx* is expressed. Null co-localization is given by “-“, very scarce co-localization is indicated by “+” and represents approximate 1 to 15% of cells, co-localization indicated by “++” represents more than 15% but less than 50% of cells and co-localization indicated by “+++”

represents high number of co-localization, higher than half of dlx-expressing cells within a determine area.

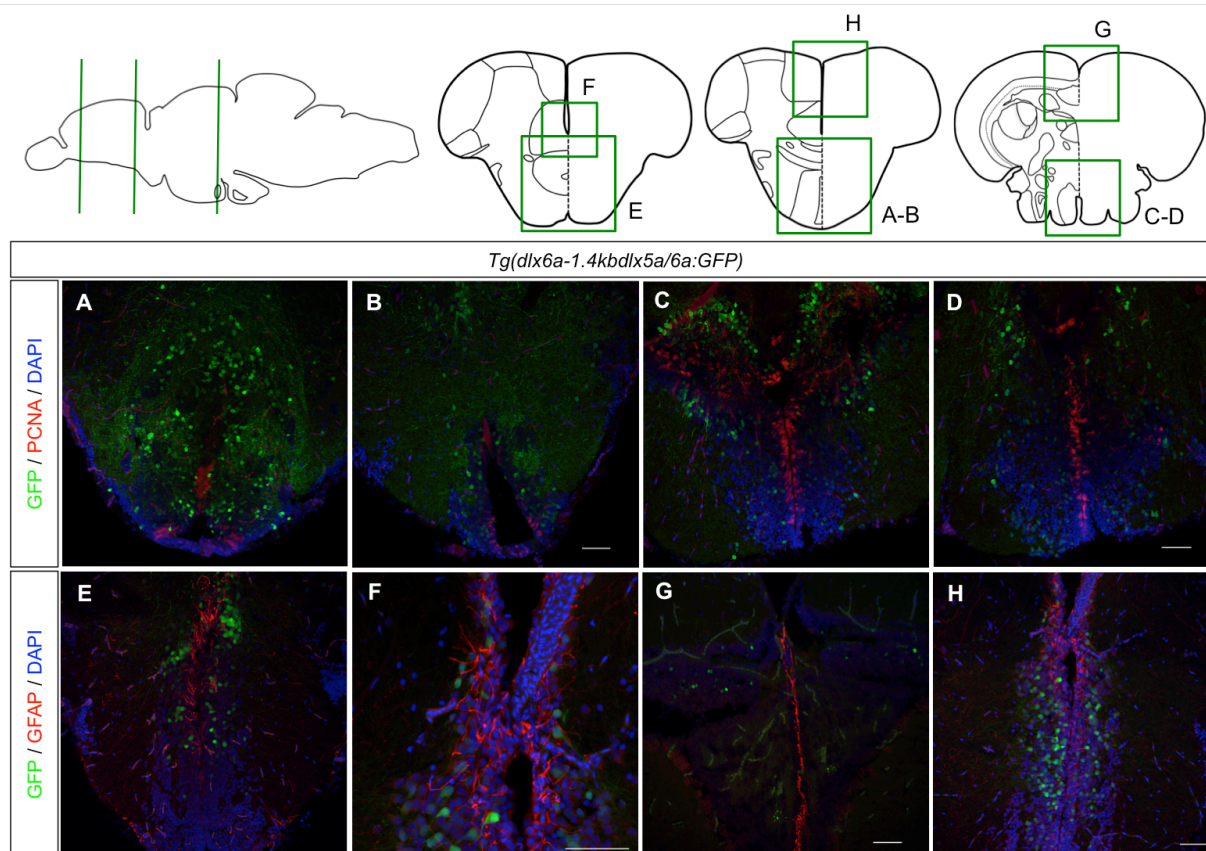


Figure 19. Immunohistochemical co-labeling of GFP with PCNA and GFAP in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* adult zebrafish.

Schematic representation of sections depicted in the top panel. Expression determined with a GFP antibody to stain transverse cryosections of *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* adults. [A-D] merged images of GFP (green), PCNA (red) and Dapi (Blue). [E-H] merged images of GFP (green), GFAP (red) and Dapi (Blue). Merged images were created with ImageJ(32) software and NIS-Elements Confocal software.

Scale bars: 50µM

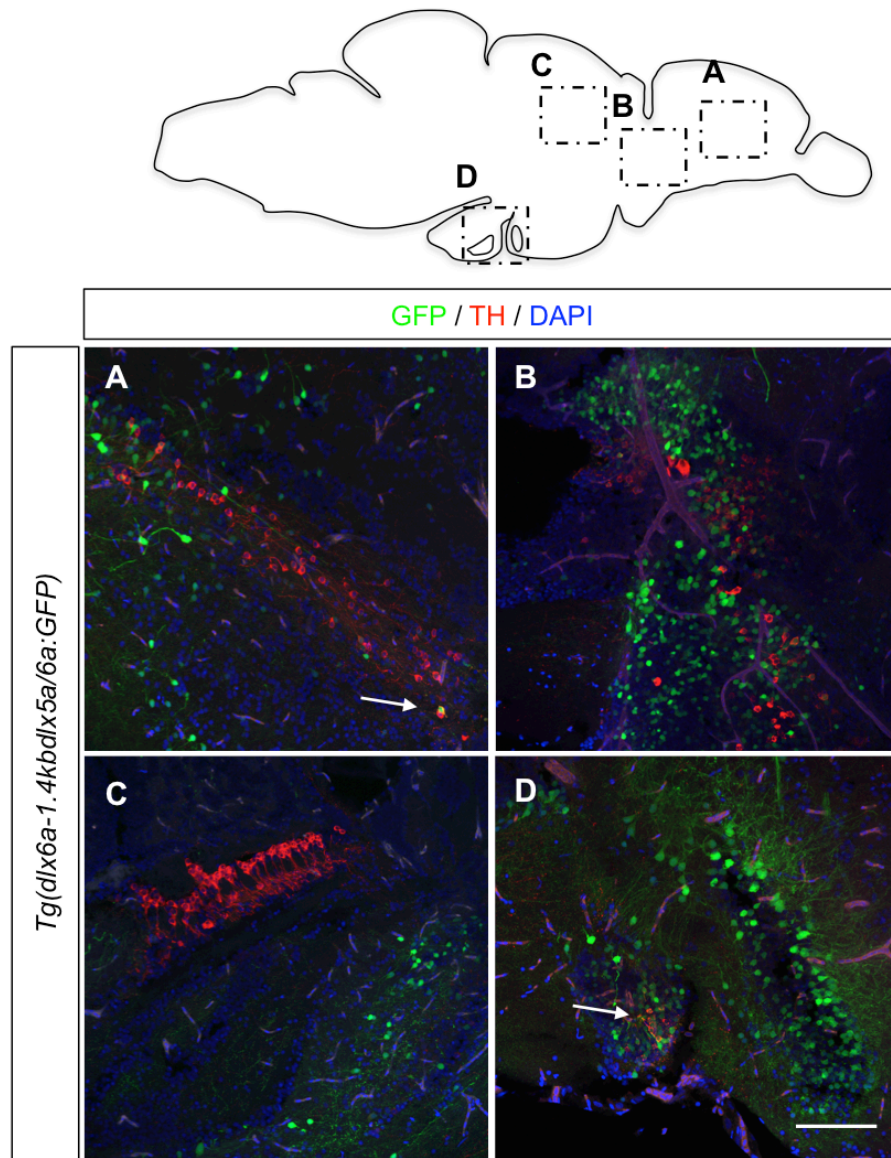


Figure 20. Immunohistochemical co-labeling of GFP and TH in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* adult zebrafish.

Schematic representation of sections depicted in the top panel. Expression determined with a GFP antibody to stain sagittal sections of *Tg(dlx6a-1.4kbdlx5a/6a:GFP)*. [A-D] merged images of GFP (green), Tyrosin Hydroxylase (red) and Dapi (Blue). Merged images were created with with ImageJ(32) software and NIS-Elements Confocal software.

Scale bar: 50µM

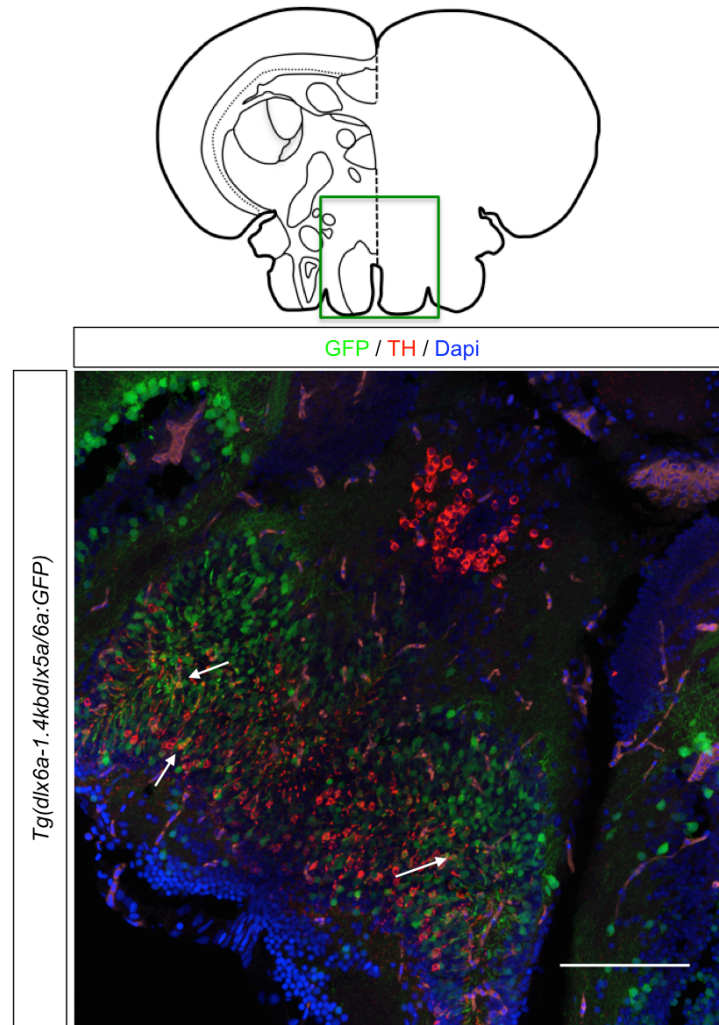


Figure 21. Immunohistochemical co-labeling of GFP and TH in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* adult zebrafish within the Hypothalamus.

Schematic representation of sections depicted in the top panel. Expression determined with a GFP antibody to stain transverse sections of *Tg(dlx6a-1.4kbdlx5a/6a:GFP)*. Merged images of GFP (green), Tyrosin Hydroxylase (red) and Dapi (Blue). Merged images were created with ImageJ(32) software and NIS-Elements Confocal software.

Scale bar: 50µM

Table 3. Co-localization of different markers and *dlx* genes

Brain areas/ Markers	gad65a [†]	Calbindin [*]	Calretinin [*]	TH [*]	Sox2 [*]	GFAP [*]	PCNA [*]
Ventral Telencephalon	+++	+	-	+	-	+	+
Dorsal Telencephalon	+++	+	-	-	+	-	-
Preoptic Area	++	++	+	-	+++	-	-
Periventricular gray zone	n.c.	+	+	-	+	-	-
Caudal zone of Hypothalamus	+	-	-	+	+++	-	-
Dorsal zone of Hypothalamus	+	-	-	-	-	-	-

n.c.:Not conclusive, results were inconclusive. Parvalbumin results were not included

[†]: Results for *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* as shown by ISH assays

^{*}: Results for *dlx5a/6a* bigene as shown by IHC assays with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line

Results - Part 2

3.2 *Dlx* expression following brain injury

3.2.1 Increased expression of *dlx* during brain regeneration.

Dlx genes are required during the development for proper establishment of neuronal populations in the central nervous system. The zebrafish presents high levels of adult neurogenesis and regeneration, as previously mentioned. Therefore we sought to explore possible roles of *dlx* in adult brain regeneration in zebrafish. In order to address this objective we have used mechanical stab lesions in the adult zebrafish brain. As the telencephalon is both an area with high rates of continuous proliferation and also one of the areas where *dlx* genes are highly expressed, this was the area where injury was provoked.

In order to verify the extent of tissue disruption and consistency of lesions applied, we have used HE staining of cryosections of lesioned individuals. At 3dpl images show the presence of blood clots that have been previously observed at early time points after lesion (black arrow Fig. 22.B) (Kroehne 2011). At 7 dpl, blood clots are no longer present and, histologically, the architecture of the lesioned hemisphere has been largely restored. At this time point there is an apparent increase in the overall number of cells, especially in the ventricular zone of the telencephalon as observed by stronger HE staining (black arrow Fig. 22.C).

Markers for proliferating cells (PCNA) and neural stem cells (Sox2) are found more abundantly within the lesion hemisphere as these cell types are involved in regeneration and adult neurogenesis. Indeed using immunohistochemistry we noticed an increase in the expression of both PCNA (gray arrows Fig. 22.D) and Sox2 (gray arrows Fig. 22.F and G), especially adjacent to the ventricular area of the forebrain. These observations indicated not only the extent and consistency of lesions but also a cellular response revealing higher rates of cell divisions and proliferation, a suiting scenario to study possible roles for *dlx* genes.

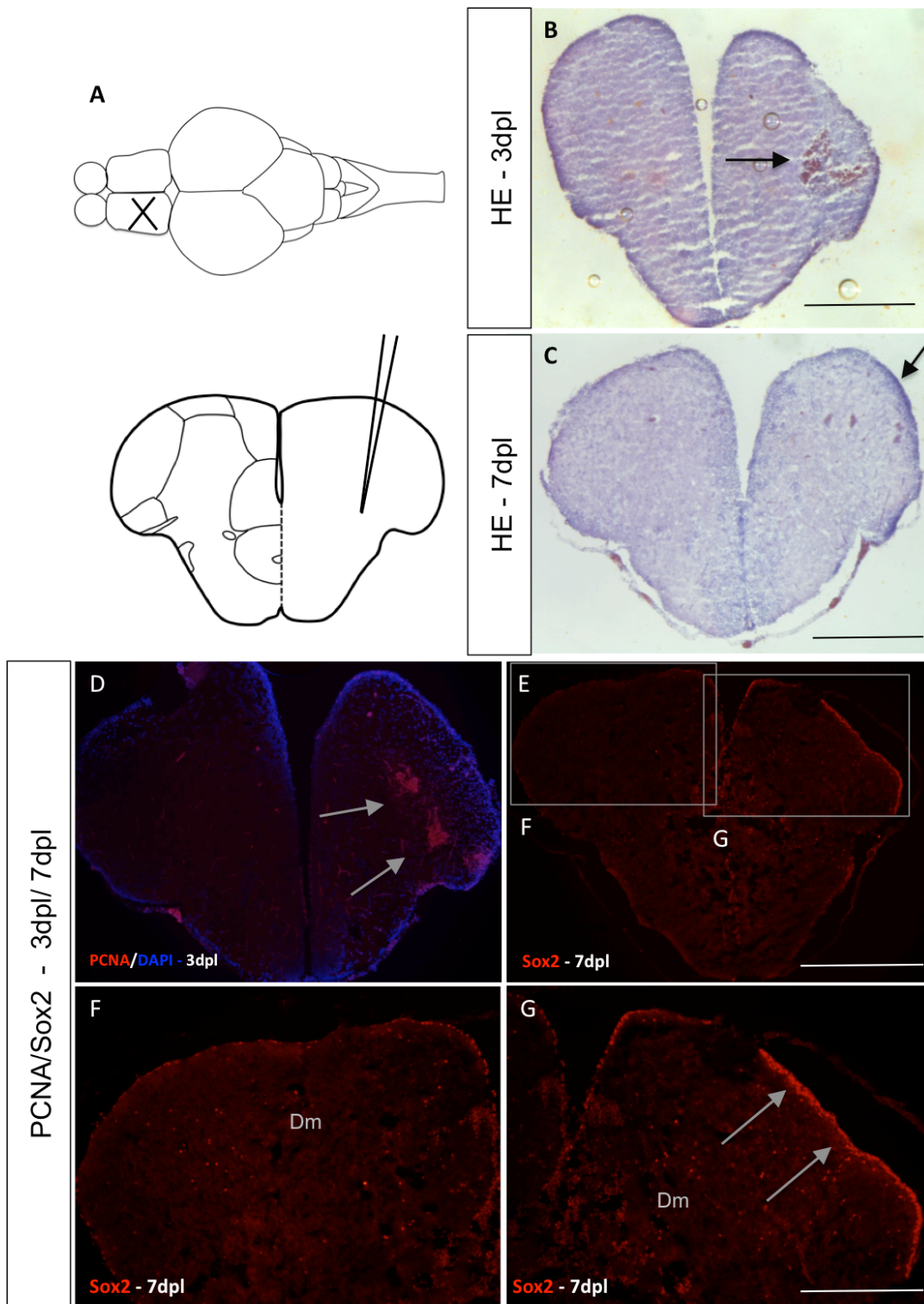


Figure 22. Stab lesion, tissue integrity and cell proliferation in adult zebrafish telencephalon.

[A] Insertion of a needle into the telencephalon is done directly through the skull in anesthetised adult fish. [B] Haematoxylin-Eosin staining shows blood clot (indicated by black arrow) within the telencephalon after lesion at 3 dpl (n=3). [C] Apparent increase in cell number revealed by higher intensity of haematoxylin staining and number of cell nuclei (indicated by a black arrow) at the lesion site at 7dpl (n=4).

[D] Immunohistochemical labeling for PCNA and Dapi staining in adult zebrafish at 3 dpl shows an increase in the number of proliferating cells at the lesion site compared to the contralateral hemisphere (n=3). [E] Immunohistochemical labeling for Sox2 in adult zebrafish at 7 dpl indicates a higher number of neural stem cells in the ventricular zone of the telencephalon (n=3). [F and G] Magnification of image shown in E shows the contralateral hemisphere in F and regenerating hemisphere in G with an increase in cells positive for Sox2. All images reveal regenerating hemisphere looks smaller when compared to the contralateral hemisphere. HE: Haematoxylin-Eosin; Dm: Medial zone of the Dorsal telencephalic area.

Scale bar (B-C): 200µM, (D-E): 200µM and (F-G):200µM

We analysed the expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* by in situ hybridization at two time points (3 and 7 dpl) after mechanical stab lesions in adult zebrafish (3mpf to 1ypf). Expression of all four paralogs did not seem to be altered at 3dpl (Fig. 23). As observed before in adults under normal conditions, the expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* remained within the dorsal (Vd) and ventral (Vv) nuclei of the ventral telencephalic area in the sections close to the lesion site (arrows Fig. 23). The expression appeared to be very similar to controls and these analyses did not reveal a possible lower or higher regulation of *dlx1a*, *dlx2a*, *dlx5a* or *dlx6a* at 3dpl.

These data could suggest that there is no up or down regulation of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* at 3 days post lesion and that these genes may not have roles in the early stages of a regeneration response in the adult zebrafish brain.

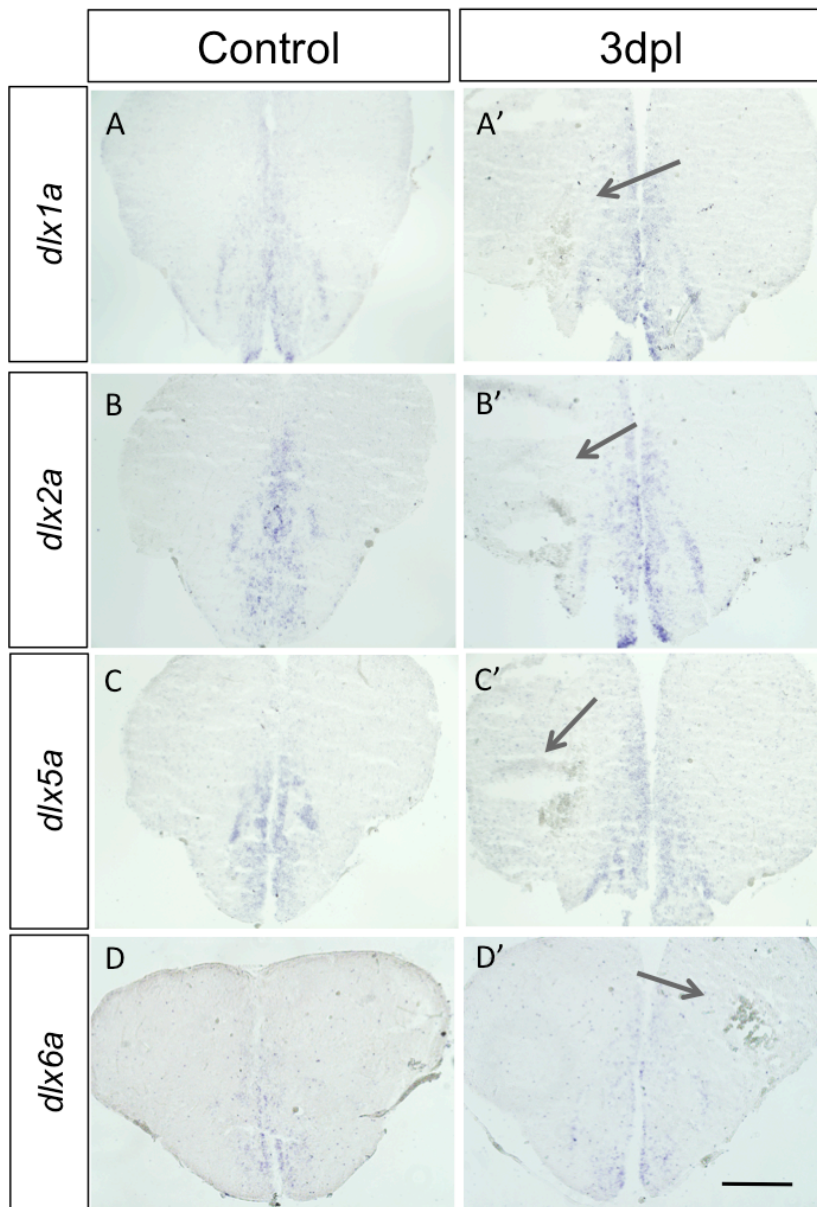


Figure 23. Expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* mRNA in transverse sections of wild type injured adult zebrafish brain at 3dpl.

Expression of the four paralogs as shown by In situ hybridization of (A-D) controls and (A'-D') injured brains in wild types adult zebrafish. Arrows in A'-D' indicate the lesion site. N=6 for each gene represents 2 biological replicates of 3 different experiments. Scale bar: 400 μ M

The second time point analysed 7dpl, is thought to present a very important stage of regeneration response and the peak of continuous proliferation after a lesion. The distribution of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* transcripts remained similar in terms of location with expression concentrated in the dorsal (Vd) and ventral (Vv) nucleus of the ventral telencephalon in the sections analysed (Fig. 24). However results suggest a up-regulation of *dlx2a* and *dlx5a* at 7dpl when compared to controls based on the intensity of the ISH signal (Fig. 24. B' and C'). The *dlx1a* and *dlx6a* paralogs presented very similar expression when compared to unlesioned counterparts.

Expression of *dlx2a* appears to be stronger at 7dpl within the dorsal nucleus of the ventral telencephalon (Vd) and slightly stronger in the medial dorsal region of the telencephalon (Vp) (Fig. 24. B'). At this time point, expression of *dlx5a* consistently presented slight increases and a more widespread expression pattern within the dorsal telencephalic area (Fig. 24. C'). Overall expression of all four paralogs is very weak in the dorsal telencephalon and ventricular zone of the telecephalon. This increase was verified by experimental repetition and biologic replicates (n= 6 for each *dlx* gene).

Although detection of mRNA by in situ hybridization is not a quantitative measure of gene expression, these data suggest that *dlx2a*, and, especially, *dlx5a* may be slightly up-regulated in the telencephalon during regeneration at 7 days-post-lesion. We did not observe differences in expression patterns of *dlx1a*, *dlx2a*, *dlx5a* or *dlx6a* at the specific site of injury for any of the two time points.

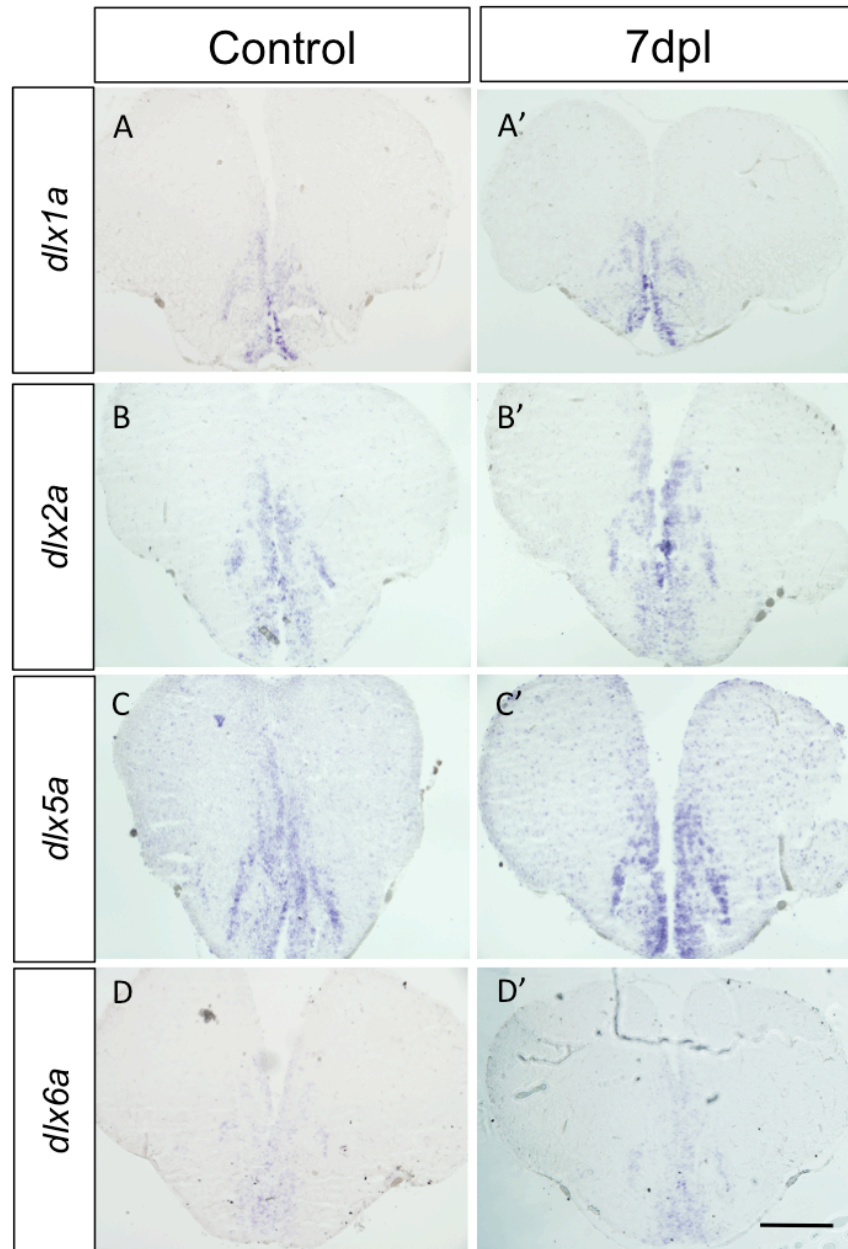


Figure 24. Expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* mRNA in transverse sections of injured adult zebrafish brain at 7dpl.

Expression of the four *dlx* paralogs in (A-D) controls and (A'-D') injured brains. (B' and C') show up-regulation of *dlx2a* and *dlx5a* compared to controls (B and C). N=6 for each gene (2 biological replicates of 3 different experiments). Scale bar: 400 μ M

Changes in *dlx* expression during brain regeneration was determined using the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line, where expression of the transgene is indicative of *dlx5a* and/or *dlx6a*. This line allowed us quantification of *dlx5a/6a*-expressing cells, by counting the GFP positive cells. The dorsal and ventral area of the ventral telencephalon is where continuous proliferation takes place and are also the region where an increase seemed more evident, therefore this area was selected for quantification by cell counting (Fig. 25. E).

Cell counting revealed an increase in the number of GFP-expressing cells in the ventral portions of the telencephalon of regenerating brains of adults (9mpf) at 3 dpl (average 266 [180-384] vs. 233 [99-396] in controls, n=6) and 7dpl (average 344 [244-408] vs. 247 [198-302] in controls, n=6). At 3 dpl this increase was not significant (*Student's t-test*, p=0.613 Fig. 25.F) while at 7dpl this number was statistically significant (*Student's t-test*, p=0.008 Fig. 25.F).

These data suggest that, at 7 dpl, when the regeneration response is pronounced in the adult zebrafish brain, the *dlx5a/6a* bigene may participate in and reflect an increased proliferation within the ventral area of the ventricle of the telencephalon. This also corroborates the results obtained by in situ hybridization with an apparent increase of *dlx5a* mRNA abundance in the regenerating brain at the same time point.

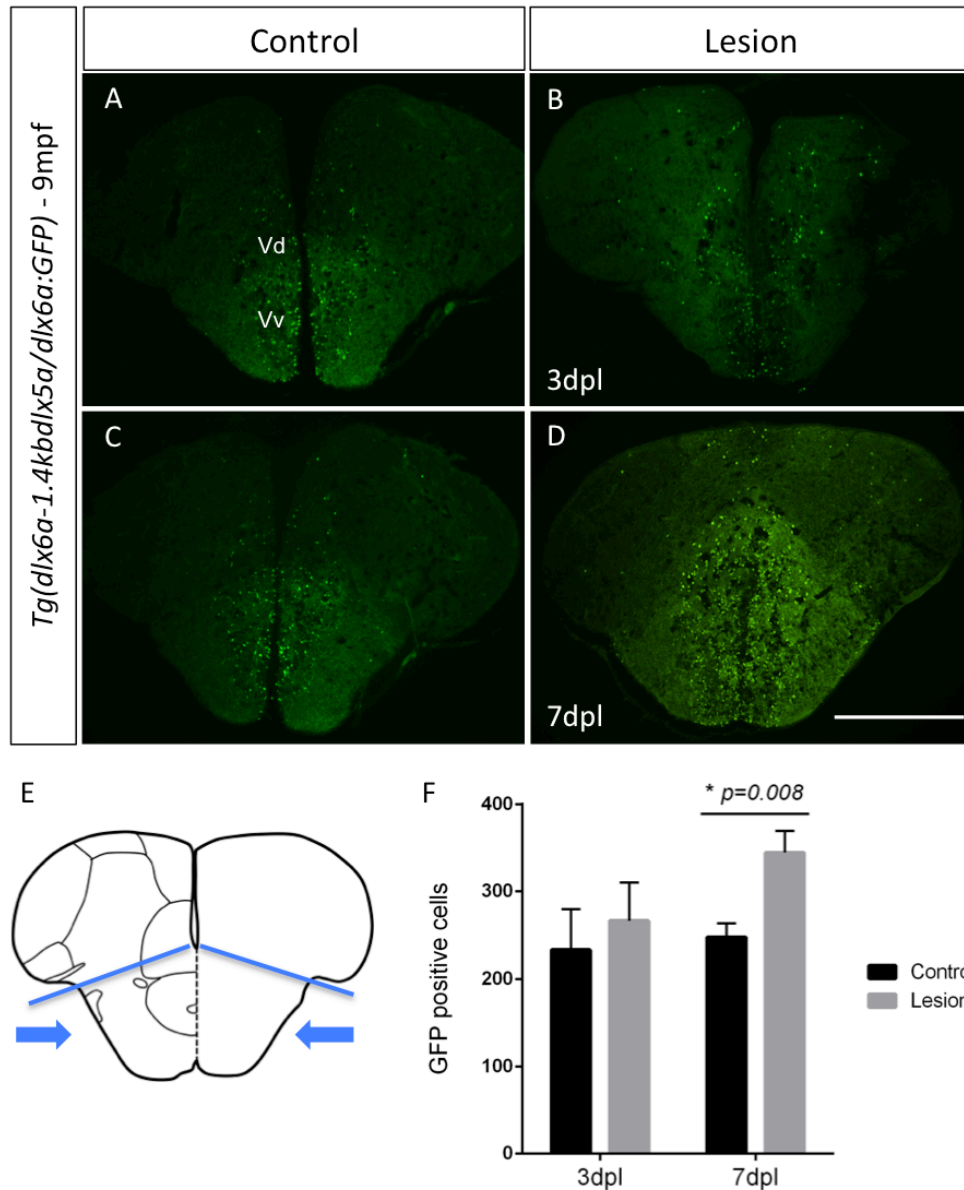


Figure 25. GFP labeling in *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* adult zebrafish at 7 dpl and cell quantification.

[A and C] *Tg(dlx5a/6a:GFP)* expression determined with a GFP antibody in controls and regenerating brain at 3 dpl [B] and 7dpl [D] . [E] Schematic representation of the telencephalon where lesion is applied and areas where cell counting was carried comprehend all GFP-positive cells bellows blue lines (area indicated by blue arrows). [F] Quantification of GFP+ cells in the regenerating brain at 3 and 7dpl. Differences reach statistical significance at 7dpl ($P=0.008$) $N=6$.

Brain regeneration triggered by mechanical lesion in adult zebrafish seemed to reveal slight increases in levels of *dlx* gene expression, particularly *dlx5a*. Possible changes in the level of expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* in the isolated telencephalon of lesioned adult zebrafish was quantified by qRT-PCR at 7dpl. We compared the changes in the expression of the four paralogs with controls of the same batch euthanized at the same time point. A single fish from different batches and experiment repetition is considered a biological replicate (n=7 for each of four *dlx* genes).

Quantification with qRT-PCR revealed slight increase in *dlx1a*, *dlx2a* and *dlx6a* expression but such increase did not reach statistical significance at 7dpl. Interestingly, there was a significant increase in *dlx5a* expression in the telencephalon of lesioned adult zebrafish at 7dpl (*Student's t-test*, n=7, p=0.008 Fig. 26). Additionally, we observed a significant decrease in expression of *dlx5a* at 3dpl (*Student's t-test*, n=8, p=0.004 Fig. 27). Expression levels at both time points were carried out using technical triplicates and normalized to the levels of two of the three following reference genes: *ef1a*, *rpl13a* and *ywhaz*.

The increase in *dlx5a* expression at 7dpl during adult brain regeneration, once again corroborates with results observed both by cell counting with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line and with mRNA presence observed by in situ hybridization in wild types. Although not statistically significant, the slight increases of all other *dlx* paralogs also confirm what was observed by in situ hybridization. At 3 dpl however, the significant decrease in *dlx5a* expression as observed by qRT-PCR contradicts with the findings obtained by cell counting. These data once again suggest that *dlx* paralogs, especially *dlx5a*, may play role during brain regeneration in the zebrafish forebrain.

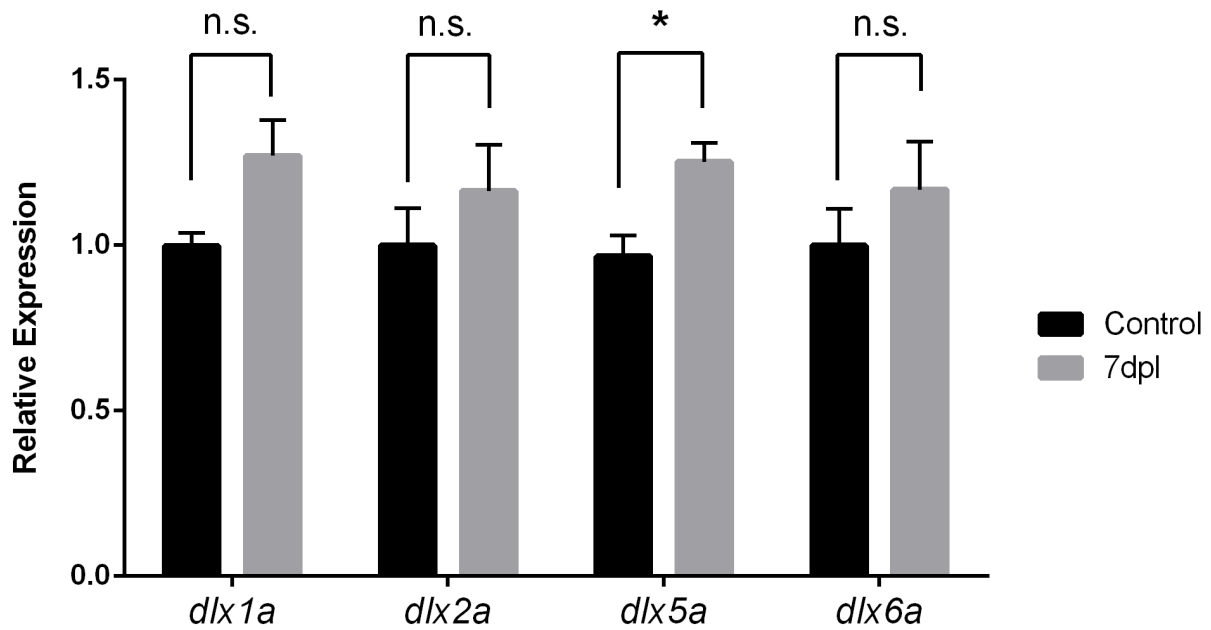


Figure 26. Changes in *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* relative expression during regeneration.

RT-qPCR analyses with RNA extracted from the telencephalon of regenerating brain at 7dpl and control specimens. No significant changes in expression levels of *dlx1a*, *dlx2a* and *dlx6a* were observed (N=7 for each gene each) but there was a significant increase in *dlx5a* expression at 7dpl ($P < 0.01$, $n = 7$).

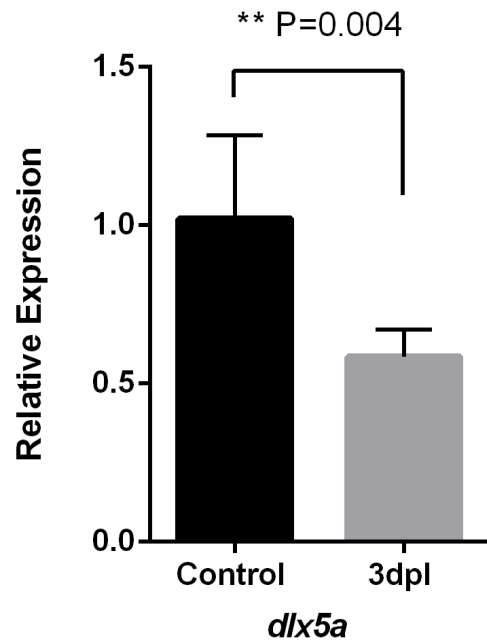


Figure 27. Changes in *dlx5a* relative expression during regeneration at 3dpl.

RT-qPCR analyses with RNA extracted from the telencephalon of regenerating brain at 3dpl and control specimens showing significant decrease in expression of *dlx5a* ($P<0.01$, $n=8$).

3.2.2 Progeny of *dlx1a/2a*-expressing cells labeled during early development, during brain regeneration in the adult zebrafish brain.

We reasoned that mCherry expressing cells from *the Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* that were induced with 4OHT during early development could proliferate and give rise to new neurons and/or also create a reserve of resident cells that would participate in the injury response. In order to explore this possibility, adult *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* fish (3-9mpf) were treated with Tamoxifen during early development (3dpf) for over 72 hours. First we verified the distribution of mCherry-positive cells at the juvenile stage (Appendix-2 Supp. Figure 1). After being raised to adulthood, these fish received the same mechanical lesion as described in the earlier sections of this chapter. Immunohistochemistry using antibodies against mCherry were performed in order to access the migration and distribution of *dlx1a/2a*-progeny after lesion, followed by cell counting of all mCherry positive cells in all cryosections acquired from each brain.

Based on our observations, the distribution of mCherry positive cells in the regenerating fish remained restricted to the olfactory bulb, forebrain and hypothalamus in the same pattern as unlesioned fish. More specifically, we observed sparse mCherry-expressing cells located in the ventral and dorsal areas of the ventral telencephalon, within the caudal zone of the dorsal telencephalic area, in the preoptic area and in the posterior part of parvocellular preoptic nucleus. In the midbrain area, mCherry positive cells were found in the lateral hypothalamic nucleus, in the dorsal and ventral zones of periventricular hypothalamus.

We observe an increase of almost 50% of mCherry positive cells in the regenerating brain at 7 dpl, even though this numbers did not reach statistical significance at this time point (average 149 [129-179] at 7 dpl and average 93 [40-142] at 14dpl vs. 109 [66-169] in controls, n=3) (Fig. 28).

These results suggest that the progeny of *dlx1a/2a*-expressing cells labeled at early developmental stages could have a role in regeneration of neurons in the adult zebrafish brain. Further analyses expanding the number of individuals and time points are necessary to verify if indeed the progeny of *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* induced with 4OHT in early development participates in the regeneration response. The same analyses were conducted with *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* induced with 4OHT at adult stages just 1 day after being subjected to the lesion (See Fig. 10.B). However results were inconclusive as the number of mCherry positive cells in these fish, controls and lesioned, was variable.

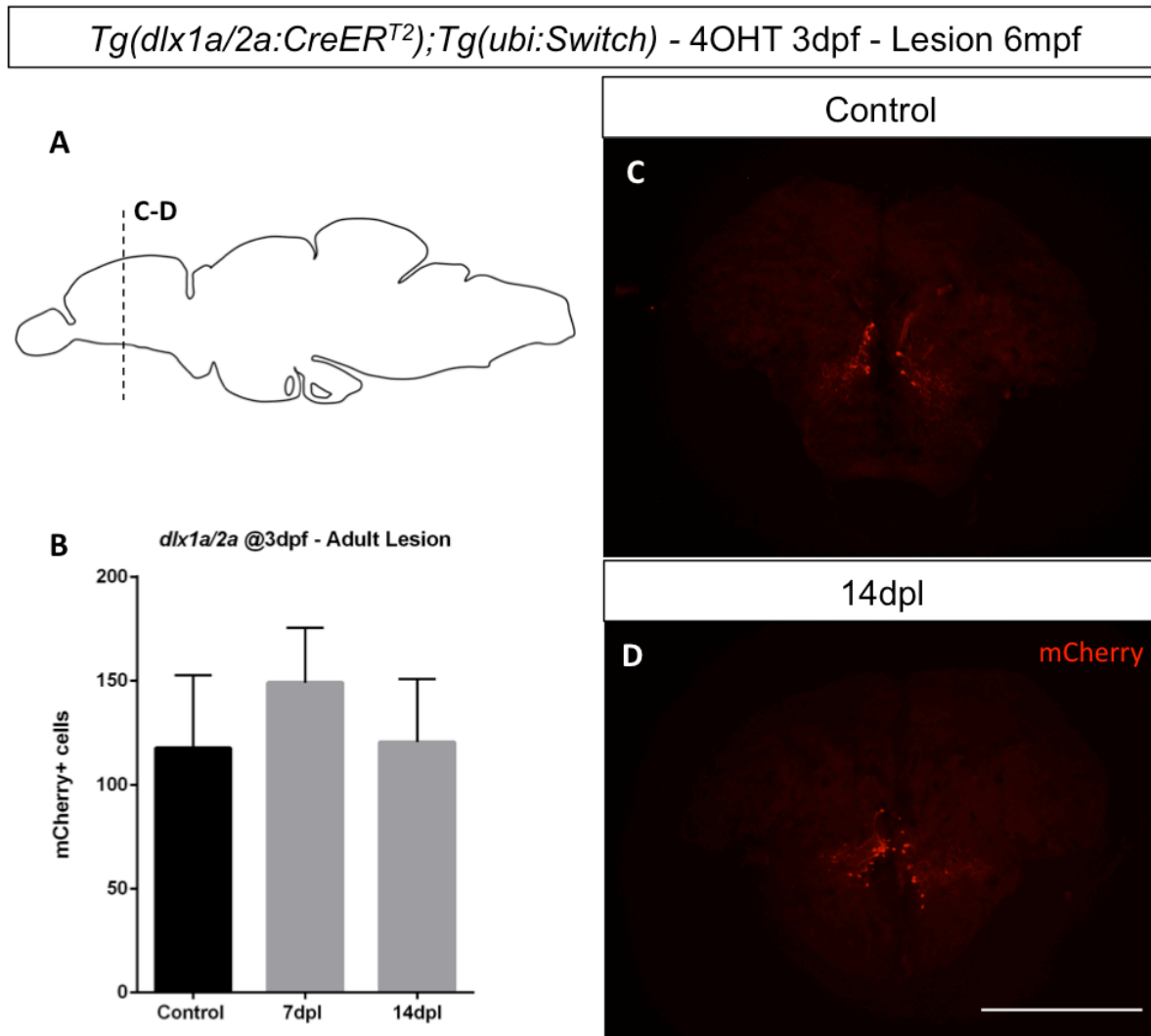


Figure 28. mCherry positive cells from *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* labeled in early development, in adult brain regeneration.

[A] Schematic representation of sections depicted in C and D. [B] Increase in number of mCherry positive cells at 7dpl but no statistical significance for both time points [C-D]. Immunohistochemistry labeling of mCherry in *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* induced with 4OHT at 3dpf, raised to adults and lesioned at 6mpf. Similar patterns and location of mCherry expression in both 7 and 14 dpl.

Scale bar: 400μM.

Results - Part 3

3.3 Lineage tracing of *dlx1a/2a*-expressing cells during adulthood

3.3.1 Lineage tracing of *dlx1a/2a*-expressing cells during adulthood

To investigate the contribution of *dlx1a/2a*-expressing progenitor cells during adult neurogenesis, adult zebrafish from Tg(*dlx1a/2a:CreER^{T2}*);Tg(ubi:UbiSwitch) ranging from 3mpf to 9mpf were induced with 4OHT as previously described. These fish were sacrificed at 3, 7 or 14 days-post-treatment (dpt). This methodology proved itself efficient as cells expressing the inducible mCherry reporter were visible at all the different time-points post treatment, co-localization with DAPI staining characterize that mCherry signal is found within cells. (Fig. 29).

The progeny of adult *dlx1a/2a*-expressing cells, mCherry positive cells were found throughout areas of the forebrain and midbrain in all different time points observed after induction. In the forebrain, mCherry positive cells were found in the olfactory bulb, the ventral and dorsal areas of the ventral telencephalic area, within the caudal zone of the dorsal telencephalic area, at the preoptic area and at the posterior part of parvocellular preoptic nucleus. In the midbrain area, mCherry positive cells were found in the lateral hypothalamic nucleus, the dorsal and ventral zones of periventricular hypothalamus (Fig. 30). These data show that the *dlx1a/2a* bigene is active during adult neurogenesis and the progeny of adult-born *dlx1a/2a*-expressing cells populate different regions of the brain in a similar pattern independent of the time point observed after treatment.

The number of mCherry positive cells, found in the adult brain was verified by cell counting and ranged from a total of 38 to 244 (average of 108 [38-244], n=10) cells throughout the whole brain counted at every third consecutive cryosection-containing slide. This variation remained consistent throughout all time points observed after treatment and was also independent of the age of the fish (3 – 9mpf) (Fig. 31).

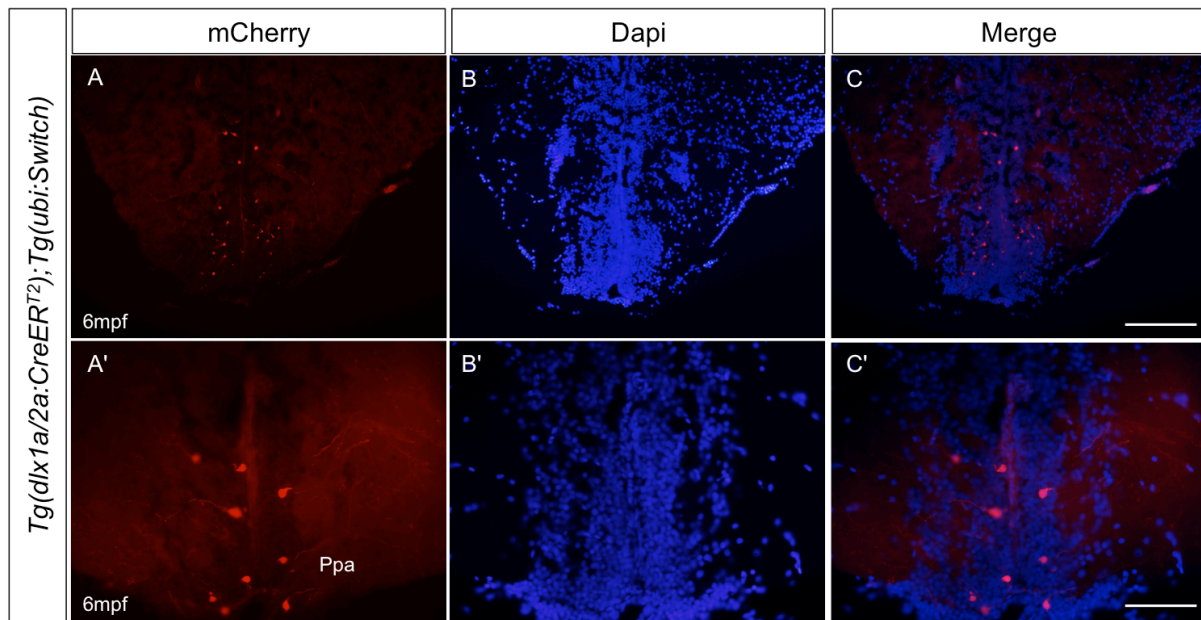


Figure 29. Immunohistochemical labeling of mCherry and DAPI in adult zebrafish (6mpf) sacrificed after 7 days post 4OHT treatment.

Detection of mCherry in *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:UbiSwitch)* adult fish is done with mCherry antibodies. [A-A'] mCherry+ cells showing neuronal projections, [B and B'] DAPI staining of cells nuclei and [C and C'] merging of mCherry with DAPI staining. Merged images were created with ImageJ(32) software. Scale bar [A-C] 200μM; [A'-C'] 400μM.

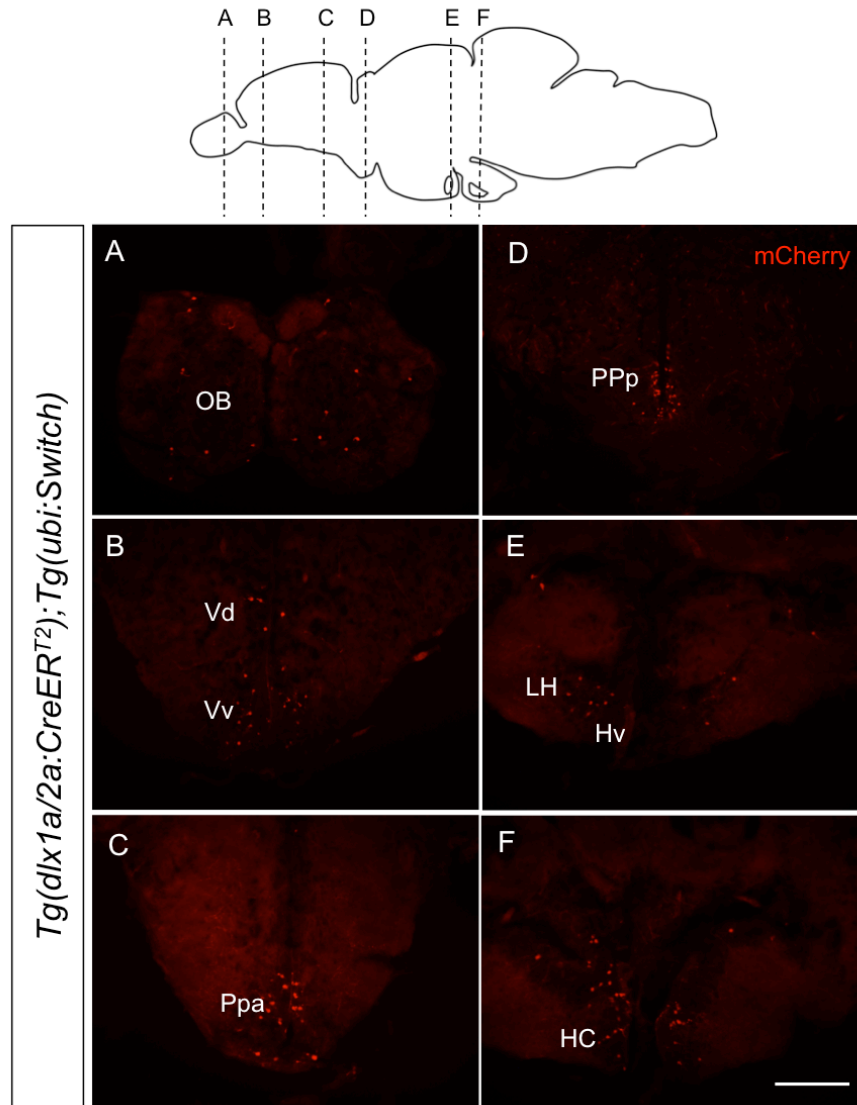


Figure 30. Immunohistochemical labeling of mCherry in different areas of adult zebrafish brain (6mpf) sacrificed after 7 days post 4OHT treatment.

Top panel depicts location of cryosections presented in A-F. OB: olfactory bulb; Vd: dorsal area of the ventral telencephalic area; Vv: ventral area of the ventral telencephalic area; Ppa: preoptic area; PPp: posterior part of parvocellular preoptic nucleus; LH: lateral hypothalamic nucleus; Hd: dorsal zone of periventricular hypothalamus; Hc: caudal zone of periventricular hypothalamus. Scale bar: 200µM

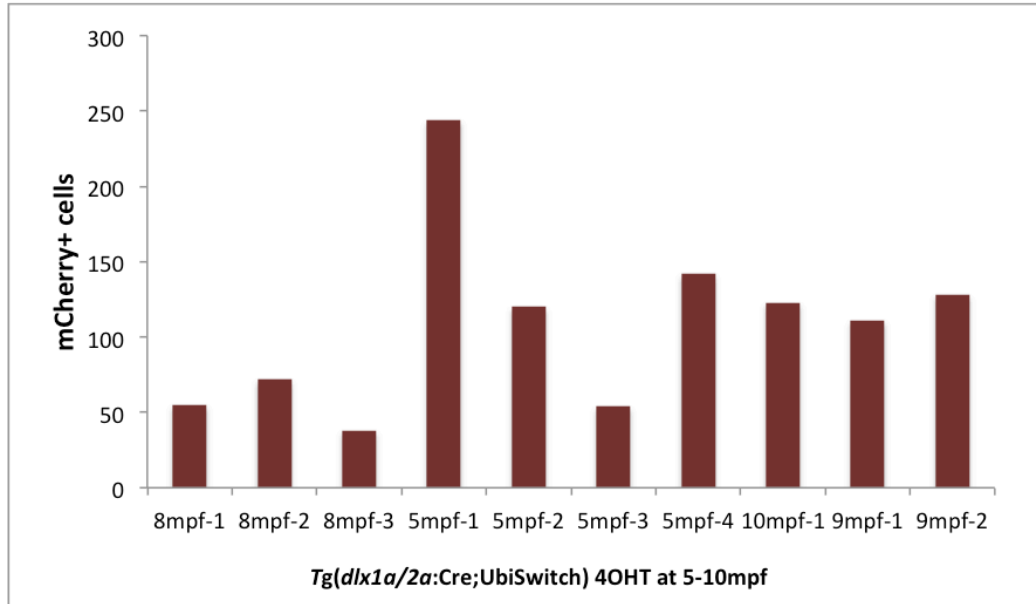


Figure 31. Number of mCherry+ cells found and the different time points in adults after switching.

Tg(dlx1a/2a:CreERT2);Tg(ubi:Switch) 5 to 9mpf fish were induced with 4OHT at different time points. The number of mCherry+ cells varied between 38 and 244 in the whole brain, n=10. 8mpf-1 to -3: 8 months-post-fertilization Sample 1, 2 and 3. 5mpf-1 to -3: 5 months-post-fertilization Sample 1, 2 and 3. 10mpf-1: 10 months-post-fertilization Sample 1. 9mpf-1 to -2: 9 months-post-fertilization Sample 1 and 2.

Although the number of mCherry-positive cells presented the same variability across the different time points observed after adult $Tg(dlx1a/2a:CreER^{T2});Tg(ubi:UbiSwitch)$ switching, some difference in staining with mCherry was observed. Later time points, such as 14dpt, showed more visible staining of what resembles neuronal projections when compared to those visualized at earlier time points after induction (Fig. 32). These cells resembled postmitotic neurons and this difference remained consistent when compared to earlier time points. This suggested the progeny of *dlx1a/2a*-expressing cells stop dividing after some time and remain within the areas described in Fig. 29.

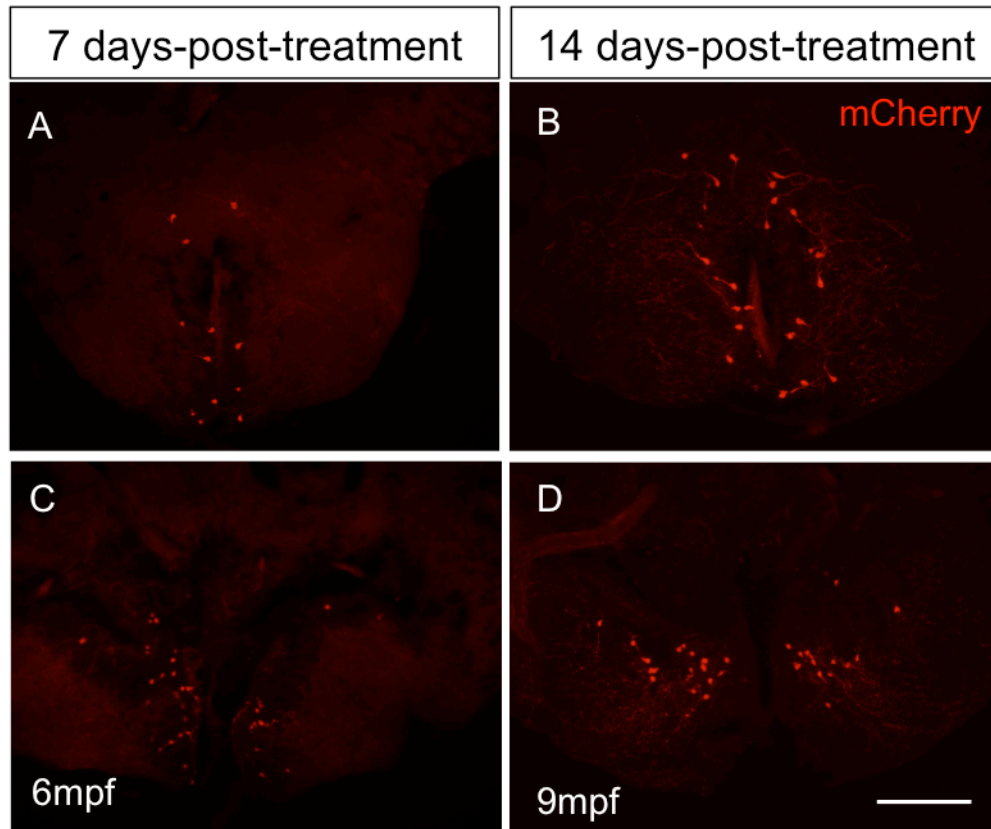


Figure 32. Differences observed with IHC labeling of mCherry in adult zebrafish brain (6mpf) at different time points post 4OHT treatment.

Cryosections of telencephalon (A–B) and hypothalamus (C–D) of adult zebrafish brain of *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:Switch)* observed at 7dpt (A and C) and 14 dpt (B and D) after labeling of cells with 4OHT at 6 or 9mpf.

Scale bar: 200μM

3.3.2 Fate mapping of *dlx1a/2a*-expressing cells during adulthood

The location and morphology of mCherry positive cells suggested the progeny of labeled *dlx1a/2a*-expressing cells acquired a neuronal fate. To understand the fate of adult progenitors expressing *dlx1a/2a* in the adult brain, the phenotype of the cells labeled with mCherry were characterized by double immunohistochemistry using the neuronal marker HuC/HuD to observe possible co-localizations with mCherry in transverse cryosections of adult zebrafish brain after 4OHT induction. The great majority of mCherry positive cells seemed to co-express HuC/HuD, particularly in the ventral areas of the ventral telencephalic area and within the caudal zone of the dorsal telencephalic area (Fig. 33). A few mCherry positive cells did not co-express HuC at the lateral recess of the diencephalic ventricle and the lateral hypothalamic nucleus (indicated by white arrows Fig. 33.D”) which suggests that within certain regions, *dlx1a/2a*-expressing cells may give rise to other cell types. Further investigations are necessary to certify the neuronal identity using other markers, as well as other cell types, using markers for oligodendrocytes, proliferating cells or neural stem cells.

To investigate if these *dlx1a/2a*-expressing cells acquired a dopaminergic neuronal fate, double immunohistochemistry using the Tyrosine hydroxylase (TH) antibody were also performed. Very few mCherry positive cells co-expressed TH (yellow arrow Fig. 34.B), and in the great majority of sections containing mCherry positive cells, no co-localization was observed, suggesting that the progeny of *dlx1a/2a*-expressing cells do not acquire a dopaminergic cell fate.

Some limitations in GABA-detection in these fish hindered the verification of a GABAergic neuronal identity for the progeny of adult *dlx1a/2a*-expressing cells. Optimizations of protocols are still necessary using either antibodies or mRNA probes that would recognize GABA and could be combined with the mCherry antibody.

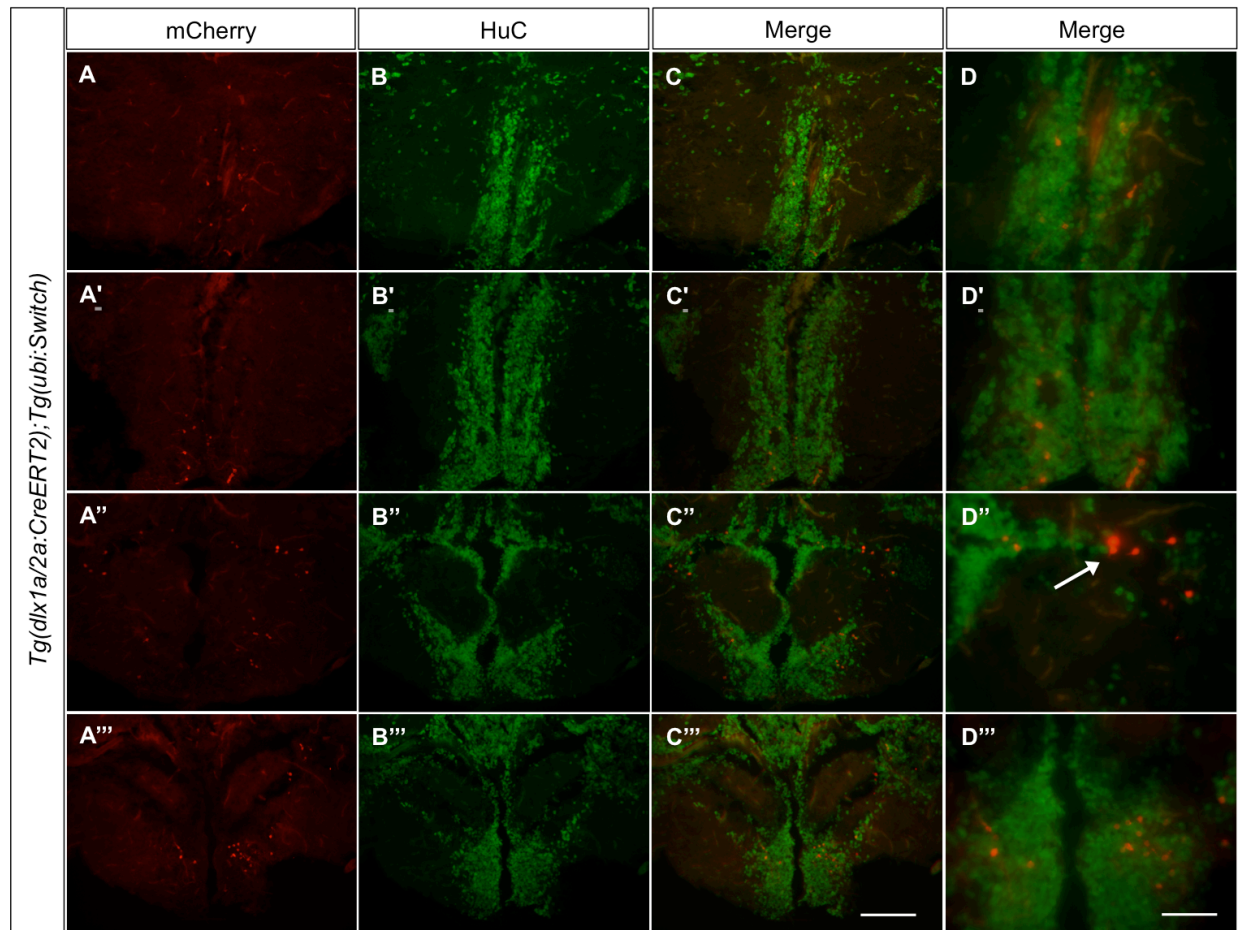


Figure 33. Co-localization of mCherry and HuC/HuD in the adult brain of *Tg(dlx1a/2a:CreERT2); Tg(ubi:Switch)* treated with 4OHT.

Double Immunohistochemical labeling of mCherry [A-A'''] and TH [B – B''']. Merged images at 20x [C – C'''] and at 40x [D – D'''] show co-localization of mCherry and HuC in yellow. White arrow [D''] show mCherry-positive cells that do not co-localize with HuC/HuD. Merged images were created ImageJ software. Scale bar (A-C'''):200µM; (D-D'''):400µM.

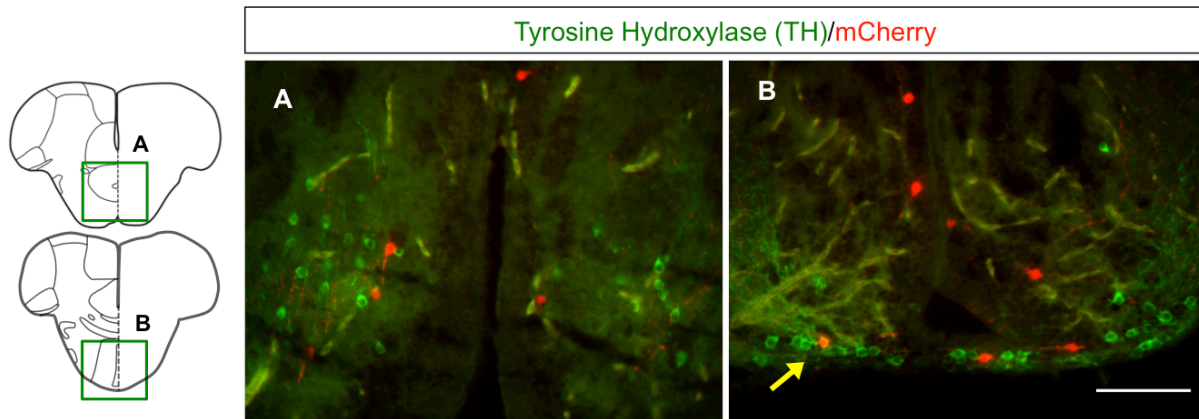


Figure 34. Co-localization of mCherry and TH in the adult brain of *Tg(dlx1a/2a:CreERT2); Tg(ubi:Switch)* treated with 4OHT.

Merged images of double Immunohistochemical labeling of mCherry and TH show only mCherry positive cell co-localizing with TH (white arrow on B). Merged images were created ImageJ software. Scale bar: 200 μ M

CHAPTER 4. Discussion

4.1 *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* are expressed in multiple domains in the adult zebrafish brain.

The activity of transcription factors underlies many different aspects of human physiology, disease and development, highlighting the importance of continuous efforts to understand their roles. These proteins can be described as multitasking regulators during brain development (Nóbrega-Pereira, 2009). As is the case of their role in limb patterning and development, the *dlx* transcription factors, seem to have multiple important roles in the CNS as well.

In this project we wanted to expand our current understanding of *dlx* activity from the context of development to adulthood, using the zebrafish as a model system. Here, we were able to demonstrate the expression of *dlx* genes in the adult brain, to characterize co-expressions of *dlx* with multiple markers and verified the possible participation of these genes during regeneration in a post-injury response. Using the Cre-LoxP system, we were also able to trace the lineage of *dlx*-expressing cells during adulthood and started to expand our knowledge of their fate determination in the adult zebrafish brain.

The first observations of *dlx2a* and *dlx5a* expression (at the time named *dlx2* and *dlx4*, respectively) in the zebrafish developing brain, indicated the onset of expression at around 13h hours-post-fertilization, about the same time the zebrafish brain is being formed (Akimenko et al. 1994). The expression of the four paralogs *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a*, is present throughout embryonic and larval stages as demonstrated by others (Ghanem et al. 2003) (Yu et al. 2011) (MacDonald et al. 2010). In adults, we demonstrate that *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* are expressed similarly among the four different paralogs and independently to varying adult stages ranging from 3mpf to 18mpf.

In the forebrain, the expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* was observed in the dorsal, ventral and postcommissural nucleus of ventral telencephalic area (Vd, Vv and Vp) and at the dorsal telencephalic area (D). These domains of expression seemed to remain consistent from embryonic development to adult stage while in the adult brain, the expression was more constricted to ventral portions of the telencephalon. For *dlx2a* and *dlx5a*, expression was also present within the central nucleus of the ventral telencephalic area (Vc).

In fact, in many areas, *dlx2a* and *dlx5a* expression seemed more abundant than *dlx1a* and *dlx6a*. These observations were not unexpected as in embryos the intensity of *dlx5a* was comparatively more uniform than *dlx6a*, which presented weaker levels of expression in the forebrain. As for *dlx1a* and *dlx2a*, extrapolating areas of the brain, only *dlx2a* transcripts were found in the neural crest, where the expression was null for *dlx1a* in this area (Ellies et al. 1997). However, we do not rule out the possibility of the results here obtained, being the result of a less effective hybridization of mRNA probes utilized for *dlx1a* and *dlx6a* in ISH assays.

The domains mentioned above (D, Vd, Vp, Vs and Vv) are part of the telencephalon in teleost fish. It is thought that most sensory systems reach the telencephalon in these species, indicating an area of the brain with high processing of different sensory inputs (Wullimann, 1996). In mammals, the telencephalon develops by the evagination of the neural tube. In contrast the telencephalon in teleost fish, is formed by eversion, or outward folding, so that the proliferative periventricular zones are not only located internally but also cover the outer surface of the telencephalon (Mueller, Vernier, and Wullimann 2006). The lateral telencephalic area is a domain considered homologous to the mammalian dentate gyrus (Gandel 2006), the only known area of the mammalian brain to present adult neurogenesis. Therefore, the telencephalon is one of the regions of the brain where continuous proliferation takes place in multiple domains.

Within the diencephalon, the anterior and posterior parts of the parvocellular preoptic nucleus (PPa and PPp), were observed to be the regions with very abundant expression of all four *dlx* genes in the adult zebrafish brain. This was observed both by ISH with all four paralogs and by IHC for GFP expression with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line. Previous studies have shown that the expression of *dlx* paralogs was almost identical in the prethalamus and preoptic area early in the development (MacDonald et al. 2013). The observations in adults, lead us to conclude that, distinct from embryos, the PPa and PPp seem to be the domains where *dlx* genes are more abundantly expressed. These are areas of the zebrafish brain with great processing of visual and olfactory inputs, once primary retinal and secondary olfactory terminals are found (Wullimann and Northcutt 1990).

Another domain of the diencephalon, the caudal and dorsal zones of the periventricular hypothalamus (Hc and Hd), in the midbrain, presented abundant expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* genes. The development of these regions of the zebrafish brain starts at 2dpf and *dlx* transcripts are also present in these areas from early development. The hypothalamus has multiple important roles in the adult brain, such as regulation of body temperature and reproduction. It is also one of the areas in the zebrafish brain where continuous proliferation takes place, indicating yet another important area of the adult zebrafish brain where specific roles of *dlx* genes should be further explored in depth.

Observations using the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line revealed expression of the *dlx5a/6a* bigene in the dorsal telencephalic area (D and DM), within periventricular grey zone of the optic tectum (PGZ) and the corpus cerebelli (CCe) more clearly than what was observed by in situ hybridization. These observations may be explained due to the fact that the GFP reporter is more sensitive and more easily detectable than mRNA transcripts with ISH. We cannot rule out the possibility of some of these observations be due to ectopic expression sometimes observed with reporter genes. However, the D, DM and PGZ all revealed weak

expression of *dlx* genes as observed by ISH. The only area of the adult zebrafish brain where no mRNA presence was detected in this work was the cerebellum, leading us to believe that within this area, the expression of GFP might be in fact ectopic. Therefore, results obtained with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line concerning the CCE are not further discussed in this chapter.

The *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line produced in our laboratory contains both the intergenic regions between the *dlx5a/6a* bigene cluster that drives the convergent expression of both genes. This construct also contains a 3.5-kb fragment of the *dlx6a* 5-flanking region including the promoter and part of the 5'UTR of *dlx6a*. Therefore, it could be argued that the expression of GFP in this line recapitulates better the expression of *dlx6a* than of *dlx5a*. However, our observations with ISH assays indicate that both genes are expressed at the same locations with *dlx5a* transcripts appearing to be more abundant.

The spatiotemporal expression of *dlx* genes in the adult brain could be indicative of multiple roles, ranging from neurogenesis to cell fate determination. As hypothesized before with studies of the inner ear in zebrafish embryos, the early expressions of *dlx* genes could specify orientation and morphogenesis, whereas their later expression could specify the fates of particular cell types (Egger et al. 1992). Indeed fate determination is an important role of *dlx* during development, and the locations of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* expression, suggest similar roles in adults.

4.2 Multiple regions of continuous proliferation in adults express *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a*.

It is important to differentiate adult neurogenesis from regenerative capability in the CNS – the latter will be discussed further in this chapter. Although the generation of new neurons is a

common event for both, the existence of adult neurogenesis does not necessarily mean a regenerative capability in the brain. Adult neurogenesis describes the process by which undifferentiated neural progenitor cells proliferate and generate mature and functional neurons that are integrated to existent circuits throughout adulthood (Schmidt, Strähle, and Scholpp 2013b) (Kempermann et al. 2018). Although present in the mammalian brain, adult neurogenesis is restricted to the subventricular zone (SGZ) of the dentate gyrus and subventricular zone (SVZ) within the hypothalamus. In amphibians and teleost fish, however, there are multiple neurogenic zones and niches along the brain presenting continuous proliferation in the adults (Alunni and Bally-Cuif 2016).

After the cloning of its genome and due to the many advantages it offers as a model system, the zebrafish has certainly become the teleost of choice for many studies, including those concerning adult neurogenesis. The zebrafish adult brain contains thirteen to sixteen distinct neural stem cell niches distributed along the brain able to generate a large number of new neurons during the entire life (Kizil et al. 2012) (Alunni and Bally-Cuif 2016).

Many of the adult zebrafish brain regions where *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* are expressed consist of neurogenic zones. Furthermore, we observed that these areas in fact presented the higher levels of *dlx* expression, namely; the olfactory bulb (OB), dorsal and ventral nucleus of ventral telencephalic area (Vd and Vv), parvocellular preoptic nucleus, anterior part (PPa), the posterior zone of the dorsal telencephalic area, the periventricular nucleus of posterior tuberculum (TPp), caudal and dorsal zone of periventricular hypothalamus (Hc).

The interplay between intrinsic and extrinsic factors plays a crucial role on ensuring the activity of adult neurogenesis niches and the generation of new neurons. Among these intrinsic players, there is a growing list of transcriptions factors which activities has been related to the important event that is adult neurogenesis (Hsieh 2012). In this context, many transcription factors are known to play roles similar to the ones already explored for the *dlx* genes during the

development, such as fate specification and differentiation. The expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* in neurogenic zones in the adult brain, lays ground for further exploration of their possible roles in the context of adult neurogenesis.

4.3 High overlapping expression of *dlx* paralogs and *gad65* in the forebrain of adult zebrafish.

One of the biggest challenges in zebrafish research is finding good quality antibodies for IHC assays. This is certainly something that posed some limitations to this project with investigations using the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line. Many of the antibodies used in assays with mice may recognize the same specific epitopes in zebrafish proteins, but this is still a challenge in many cases. As an alternative to circumvent this issue, we implemented double Fluorescent In Situ Hybridization (FISH) assays using an mRNA probe that recognizes one of the four *dlx* paralogs in combination with another mRNA probe that binds to *gad65* transcripts, which encodes an enzyme that catalyzes the decarboxylation of glutamate to GABA and CO₂.

The first indications of *Dlx* gene dependence for the specification of GABAergic neurons in the mouse was a crucial starting point for more explorations of *Dlx* activity in the brain. In these studies, homozygous *Dlx-1/2* mutant mice presented abnormal migration out of the lateral ganglionic eminences (LGE) resulting in an accumulation of partially differentiated neurons in the LGE, and a loss of normal olfactory bulb interneurons (S. A. Anderson et al. 1997). The expression of the synthesizing enzyme for GABA, glutamic acid decarboxylase (GAD), was also reduced in the neocortex of these mutants. The researchers observed that the migration of GABAergic neurons and specification into neuronal subtypes have been shown to be highly dependent on the *Dlx* homeobox genes. Since these observations, other investigations with

gene expression observations, molecular approaches and transgenes, have been elucidating the importance of *dlx* for GABAergic specification.

Given previous observations, we hypothesized that in the adult zebrafish brain, many *dlx*-expressing cells adopt a GABAergic neuronal identity, particularly within areas of the forebrain as it was observed in embryos. Indeed our observations revealed that within D, Vd, Vp, Vv, PPa and PPp regions there is a high co-expression of *dlx* transcripts and *gad65*, indicating that in the adult forebrain the great majority of *dlx*-expressing cells consist of GABAergic neurons. These observations indicate that in addition to their role in specification and development of GABAergic neurons, *dlx* genes may also have a role in maintaining neuronal identity in these regions of the adult zebrafish brain.

In the midbrain, at the ventral and dorsal zone of periventricular hypothalamus (Hd and Hv), only a small portion of *dlx*-expressing cells presented a GABAergic interneuron identity, indicating that those cells could still be in the cell cycle and have not yet acquired the GABAergic phenotype or that, in these areas, *dlx*-expressing cells will give rise to different identities.

The calcium binding proteins Calretinin, Calbindin and Parvalbumin are expressed in GABAergic and glutamatergic cortical neurons (Defelipe et al. 2014). *Dlx* enhancers in mice have been shown to be highly active in some of the major subtypes of GABAergic interneurons (Ghanem et al. 2007), suggesting that the function of these regulatory elements are important to target *Dlx* gene expression in specific populations of differentiating GABAergic neurons. Interestingly, during the early development of zebrafish, the comparison of GFP expression in the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line with markers for calbindin, calretinin and parvalbumin, revealed that a vast majority of GFP-positive cells within the telencephalon and diencephalon of 3 dpf embryos do not co-localize with any of these markers tested (Yu et al. 2011) . To further examine potential co-localization in the adult zebrafish forebrain, we used a

double immunohistochemical approach using the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line in order to evaluate whether GFP-positive cells would also be labeled with markers for these three GABAergic neuron subtypes.

Similar to what was observed during development, our results indicate that the majority of *dlx5a/6a*-expressing cells do not co-localize with these specific subtypes in the adult zebrafish brain with some exceptions. Cells expressing *dlx5a/6a* have shown very little co-localization with markers for calretinin or calbindin within the forebrain. The Vs, PPa and PPp presented a cluster of cells with high co-localization of GFP and calbindin, suggesting that *dlx5a/6a* is highly active in calbindin interneurons within this area of the diencephalon. The PGZ was the only area where co-localization was found with the three markers.

These are similar results to what was observed in zebrafish at 3dpf. The exception to these similarities were the observations in the Vs, PPa and PPp area where it seems that, in adults, *dlx5a/6a* may have an important role for calbindin interneurons. Previous work with *dlx1a/2a* zebrafish morpholino knockdown revealed the number of calbindin positive cells is remarkably reduced in these morphants (Wenqian Ma, 2011), therefore indicating these genes are involved in calbindin-positive GABAergic neuron development. We therefore suggest that in some areas of the adult brain, *dlx5a/6a* expression could also be essential to either maintain calbindin neurons or to generate these subtypes of GABAergic interneurons once co-localization was abundant in one important region of continuous proliferation and adult neurogenesis.

4.4 Neural stem cell identity for *dlx*-expressing cells in the adult brain.

The *Dlx* genes, which are essential for neuronal differentiation within the basal ganglia in mice, are also required for neocortical and olfactory bulb interneurons during development. These genes can in fact be considered pro-neural transcription factors known to promote neural proliferation (Anderson 1999) (Castro et al. 2011). Additionally, radial glia have been proposed to be the progenitors for adult neurogenesis within the CNS of non-mammalian vertebrates where proliferation is constantly taking place (*Alvarez-Buylla et al., 2001; Goetz et al., 2002; Pinto and Goetz, 2007*). Extrapolating the *dlx* role in GABAergic specification and the spatio-temporal conserved co-expression, it is crucial to explore other possible roles of these genes in the adult brain.

As observed in the results presented in this thesis, the cells that express *dlx* genes do not seem to have a glial or proliferating cell identity in the adult brain as no co-localization was observed with GFAP or PCNA markers. Interestingly, in some areas of the forebrain and midbrain, particularly in the VS, PPa, Sc and Hc, we observed high overlapping co-localization of the neural stem cell marker Sox2 and GFP in the adult zebrafish brain from the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line. These observations may suggest for *dlx* genes a role for the maintenance of neural pluripotency or in promoting neural proliferation in the adult brain.

Sex determining region Y-box 2 (SRY), also known as Sox2, is a transcription factor essential for maintaining pluripotency of embryonic stem cells and has a critical role in specifically maintaining embryonic neural stem cells (NSCs). In vivo studies with adult mice, have demonstrated that Sox2 alone is sufficient to reprogram resident astrocytes into proliferative neuroblasts in the adult mouse brain, which further develop into functionally mature neurons (Willsey et al. 2013). Studies in zebrafish have shown that Sox2 knockdown in zebrafish, resulted in a significant decrease in the number of proliferating cells at 5 days post

spinal cord injury, proposing a role for Sox2 as one of the proliferation initiators after spinal cord injury (Ogai et al. 2014). In fact, our results have also shown an increase in Sox2 expression within the lesioned hemisphere at 3dpl when compared to the contralateral one.

The overlapping localization of Sox2 and *dlx5a/6a* bigene interestingly takes place in two important neurogenic zones the PPa and Hc. The absence of co-localization of *dlx* gene expression and of GFAP lead us to believe that *dlx* would have a role within non-glia progenitor cells. Our results taken together with observations such as the ones mentioned in the previous paragraph, lead us to once again hypothesize that in some areas of the adult zebrafish brain, *dlx* genes indeed may be required for cells to become mature neurons in the adult brain.

4.5 Different roles for *dlx* genes in the hypothalamus of adult zebrafish?

Frequently in our observations, co-localizations observed in the telencephalon and diencephalon differed than those observed within the hypothalamus domain of the adult zebrafish brain. The hypothalamus is an important center for the regulation of growth, reproduction and homeostasis in the zebrafish (Kwak et al. 2018) (Bassi et al. 2016). In fact, observations in mice revealed *Dlx1/2* coordinate the balanced generation of the growth hormone-releasing hormone (GHRH) neurons promoting the growth of agouti-related protein (AgRP) neurons controlling the feeding and energy expenditure, a novel observation extrapolating the roles already known for *Dlx* in the brain (Lee et al, 2018).

Certain areas of the hypothalamus, specially the most caudal portions of Hc and Hd, were the only area of the adult zebrafish brain where very little co-localization of *dlx* transcripts and *gad65* was observed. It was also within the Hc that the highest overlapping localization of *dlx* genes and Sox2 were observed, as well as a few occasions of co-localization of TH and

GFP with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line. Additionally the expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* within the Hc and Hd, both areas presenting continuous proliferation, was abundant for all four genes.

Intriguingly, while co-localization of *sox2* and *dlx5a/6a* was abundant in the Hc, the expression of these genes was observed in adjacent patterns in the Hd, suggesting that while in some areas of the hypothalamus these genes may be involved with reprogramming of cells to become mature neurons, in other areas of the hypothalamus, *dlx* transcripts may be present in recently formed mature neurons that do not have a GABAergic identity.

The observations of some co-localization of GFP and TH in the adult hypothalamus with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line, also suggest that some of the *dlx*-expressing cells may also be other neuronal types that are not GABAergic. In summary, it appears that within the hypothalamus, *dlx* may have heterogenic roles and different than the ones proposed for the other areas of the adult zebrafish brain.

4.6 *dlx* expression following brain injury

The ability to regenerate lost or damaged tissues is an intriguing biological phenomena and the cellular and molecular mechanisms underlying this event remain to be fully elucidated. There are numerous reasons why research on regeneration should gain attention. With the growing number of individuals affected by neurodegenerative diseases, like Alzheimer's and Parkinson's for instance, we could only gain by better understanding the molecular regulators of regeneration in the SNC. Many other fields, such as cancer research, can also benefit from the studies of regeneration. Regeneration, development and cancer can all be seen as different

aspects of the same outstanding question, how is a complex large-scaled structure specified and how can the molecular signals involved be explored and extrapolated (Levin 2011).

The importance of assembling pieces of this important puzzle that is the biology of regeneration brought us to the second objective in this project. Since the zebrafish is a potent animal model for regeneration and adult neurogenesis studies, and *dlx* genes have important roles during the development and proper establishment of neuronal populations, we wanted to explore the possible participation of *dlx* genes during a regenerative response in adult zebrafish.

The most common approach to study brain regeneration is physical lesions (Kizil et al. 2012) (Viales et al. 2015) (Schmidt et al. 2014). Mechanical stab lesions with needles inserted through the skull of zebrafish were used to trigger a regeneration response. The time points chosen for analyses were based on the timeline of the regeneration response. It is presumed that at 3dpl, cell death has taken place and within the following days regeneration is more pronounced where at 7dpl, the SNC is going into great rates of reactive proliferation and neurogenesis (Kroehne et al. 2011).

First, histological staining revealed actual disruption of tissue at 3 dpl as well as repair and cell proliferation at 7dpl. As proliferating and stem cells are involved in the process of regeneration responses, we expected to see an increase in the number of Sox2 and PCNA positive cells in the injured brains and ideally, a difference in patterns of expression of these two markers within the contralateral hemispheres. We were able to see these differences with both markers. The expression of proliferating cells within the lesioned hemisphere at 3dpl was more abundant and dispersed and neural stem cells appeared to be more abundant near the ventricular zone of the lesioned hemisphere at 7dpl. Altogether, these observations indicated the actual presence of a stab lesion and cellular response and a scenario for investigating the response of *dlx* genes.

Due to the intense activity of reactive proliferation in the brain during regeneration, we expected to see changes in the patterns of expression of *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* as these genes take part on important developmental events and neuronal specification. Results with in situ hybridization however, revealed that at 3 dpl there is no up or down regulation of *dlx1a*, *dlx2a*, *dlx5a* or *dlx6a*, as observed by staining intensity. These results suggested at first that these genes may not participate in the early stages of a regeneration response in the adult zebrafish brain. This was further analysed with cell counting of GFP positive-cells with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line, where a slight increase in the number of *dlx5a/6a*-expressing cells appeared to be present in specific areas of the telencephalon at 3dpl. It is important to note that the slight difference observed with cell counting was localized to sections of the telencephalic region where the lesion was applied. It is possible that within this specific area of the telencephalon, *dlx* genes can be slightly up-regulated at this time point and such result was not evident by staining with in situ hybridization.

Quantification of RNA at 3 dpl for *dlx5a* through qRT-PCR, revealed a significant decrease in *dlx5a* expression within the injured telencephalon at 3dpl. Contrasting to analyses done by ISH and cell counting, RNA quantification was carried with the whole telencephalon of lesioned fish and this could explain the different result observed. Like mentioned before, 3dpl is a time point where cell death has taken place and regeneration response is becoming more pronounced. These observations would need further analyses, however the variable results might be a reflection of this change in scenario between neuronal loss and actual regeneration taking place at this stage of regeneration.

The second time point analysed is thought to present a very important stage of regeneration response and the peak of continuous proliferation after mechanical lesion. At 7 dpl, reactive proliferation and reactive regeneration are pronounced to repair and produce newly-generated mature neurons that start to be formed at 21dpl. We first observed stronger staining

with ISH suggesting increased expression of *dlx2a* and *dlx5a* in the telencephalon of regenerating brain at 7dpl. Both *dlx2a* and *dlx5a* expression appeared to be stronger within Vd and Vp. At this time point expression of *dlx5a*, particularly, presented slight increases and a more widespread expression pattern within the dorsal telencephalic area.

Although these increases were observed for *dlx2a* and *dlx5a*, in both cases the presence of transcripts was not found adjacent or exactly at the location of injury at the ventricular zone. For *dlx2a*, the expression seems to remain exactly at the same domains as in controls where for *dlx5a*, we observed the same scenario but also a more widespread presence of *dlx5a* transcripts at the dorsal telencephalic area. This could suggest that if these genes are participating in the regeneration response, their role could be an enhanced activity as the one in the adult brain under normal conditions.

Analyses with cell counting of GFP positive cells with the *Tg(dlx6a-1.4kbdlx5a/6a:GFP)* reporter line, revealed a significant increase in *dlx5a/6a*-expressing in specific areas of the telencephalon at 7dpl. As previously mentioned, it is not known specifically if this reporter line recapitulates the expression of *dlx5a* and *dlx6a* equally. Once again, due to the increased sensitivity of the GFP reporter and easier detection than mRNA transcripts with ISH, this could explain the higher difference observed in comparison with ISH observations.

Interestingly, RNA quantification analyses of the telencephalon of lesioned adult zebrafish corroborate the results observed by ISH and GFP expression with the reporter line. Slight increases in *dlx1a*, *dlx2a* and *dlx6a* expression were observed during regeneration at 7dpl and such increase was statistically significant for *dlx5a*. These results lead us to believe that *dlx* genes may have a role in post-injury response. Taken together with the overlapping expression of *dlx* genes and *gad65* in the adult zebrafish brain, one hypothesis is that these genes may participate in compensating for neuronal loss, specifically the loss of GABAergic neurons.

Although it is interesting to observe a pattern for *dlx5a* response to injury in the different experiments used, leading us to believe that this particular gene could have more important roles in the context of regeneration compared to the other paralogs, the distinction and comparison of each *dlx* paralogs in the regeneration response is somewhat limited. Even though it appears that *dlx2a* and *dlx5a* presented higher expression in the regenerating brain as observed by ISH, the difference was very subtle and does not allow us to make comparisons or affirmations on the importance of one *dlx* paralog over the other. Moreover, ISH should not be taken alone to draw affirmations on levels of expression. Concerning RNA quantification data, a similar scenario was observed where all four *dlx* genes presented very similar results.

One limitation that also hinders strong affirmations about each *dlx* gene individually is the fact that it was not possible to use the *Tg(URE2dlx1a/2a:GFP)* reporter line as GFP is no longer expressed after the juvenile stage in the adult zebrafish brain. Therefore, cell counting such as that carried for *dlx5a/6a*-expressing cells, could not be done for the *dlx1a/2a* bigene. Based on previous observations from developmental studies, it is very likely that if *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* present roles in adult regeneration, they would be probably very similar roles.

Traumatic physical lesions are followed by a complex multi-cellular response that involves events like: apoptosis, inflammation, proliferation of glial cells and increased progenitor cell activity. These events can lead to an increase in neurogenesis depending on the severity of the lesion, site of the trauma and competency of the progenitor cells (Bazan et al., 2005; Leker et al., 2007; Endo et al., 2007; Kaslin et al., 2008;). This leads us to consider that one of the explanations for the slight difference in *dlx* expression observed in the regenerating brain could actually be due to the severity of the lesion. It is possible that the injury was not severe enough and the increase in neurogenesis was too weak.

The adult mammalian brain harbours neural precursor cells (NSCs), which are a potential source of neurons for repairing injured brain tissue. Recent studies show that the

telencephalic ventricular zone in the adult zebrafish brain, where Vd and Vv are located, contain NPCs that share characteristics with the NPCs in the mammalian SVZ: they migrate tangentially into the OB and differentiate into mature neurons (Grandel et al., 2006; Adolf et al., 2006; Lam et al., 2009; März et al., 2010; Ganz et al., 2010; Kishimoto et al., 2011). From the results obtained in this chapter, we believe that the *dlx* genes must be further explored in the context of adult brain regeneration perhaps with different approaches such as different types of injuries.

4.7 *dlx1a/2a*-expressing cells labeled at embryonic stage and response to adult injury.

Lineage tracing, as will be further discussed ahead in this chapter, allows us to identify the progeny of cell of interests. As previously observed by former members of our Laboratory (Solek et al. 2017), there is an expansion of the lineage of both *dlx1a/2a*- and *dlx5a/6a*-expressing cells, labeled with 4OHT during development, in the adult brain. In order to explore a possible participation of the progeny of *dlx1a/2a*-expressing, labeled early in the development, during a regeneration response in adulthood, we performed stab brain lesions in adult zebrafish from *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:UbiSwitch)* treated with 4OHT at 3dpf. We sought to explore if these cells could proliferate and give rise to new neurons and/or also create a reserve of resident cells that would participate in the injury response.

Although statistical analyses revealed no significant differences, we observed an increase of almost 50% of mCherry positive cells in the regenerating brain at 7 dpl. This observation demonstrates that there is a higher number of cells marked as the progeny of *dlx1a/2a*-expressing cells (mCherry positive cells), during a regenerative response in the adult zebrafish brain. Interestingly, the results obtained also show a decrease in the number of cells

expressing mCherry at 14 dpl, almost to the same levels as controls. These results suggest that the progeny of *dlx1a/2a*-expressing labeled in developmental stages, could have a role in the regeneration of neurons in the adult zebrafish brain, especially during early stages of regeneration.

In contrast to the regeneration analyses for *dlx*-expressing cells discussed before, the results here were obtained by cell counting of all cells expressing mCherry found throughout the adult brain. Therefore, they represent the response of the whole brain to injury. The numbers of mCherry positive cells within the telencephalon at the site of injury were always too small in both controls and lesioned individuals to draw comparisons. Moreover, the images of these specific sections did not reveal a clear difference in the regenerating brain. We believe that these results may lay ground to further research analyzing other time points after lesion, the fate of mCherry positive cells in this context and a sampling increase that would allow cell counting specifically at the lesion site.

4.8 Lineage tracing of *dlx1a/2a*-expressing cells in the adult brain

In the efforts towards better understanding adult neurogenesis in the zebrafish brain, it is also of great importance to unveil the origin and identity of the neurogenic progenitor cells by genetic lineage-tracing. The proper formation of neuronal networks during development involves precise coordination of molecular mechanisms, including the expression of many transcription factors. The knowledge acquired from embryonic and larval development provides a starting point for similar studies in the adult zebrafish, where many of the same molecular mechanisms could be involved with adult neurogenesis. Studies have shed light into the fate of *dlx*-expressing cells in the developing zebrafish providing a tool kit for studies about the fate of *dlx*-

expressing cells during adulthood (Solek et al. 2017) . The conditional gene expression with the Cre-loxP system for lineage tracing allowed us to conduct lineage tracing of *dlx*-expressing cells in adults and provided us of information regarding number, location and fate decisions during adult neurogenesis.

While recombination using this system in embryos is somewhat simple, this system presents some difficulties when it comes to adult zebrafish. The first challenge is getting the 4OHT in the brain as zebrafish, as adults, have a well-developed skull and epidermal layer. Thus, the simple addition of 4OHT in the water for over 24 hours did not prove to be efficient. After trials with intraperitoneal and intracranial injections, what actually proved to be efficient was the addition of 4OHT to the water where zebrafish were swimming but in repeated over several days. This method proved to be consistently efficient for the permanent label of cells using the *Tg(dlx1a/2a:CreER^{T2});Tg(ubi:UbiSwitch)* double transgenic line and now provides a methodology that can be used for further studies. Unfortunately the same methodology as well as the other approaches tried before with injections, resulted in the negative expression of mCherry with the *Tg(dlx5a/6a:CreER^{T2});Tg(ubi:UbiSwitch)* fish, therefore adult lineage tracing at this point could not be carried out for *dlx5a/6a*-expressing cells.

The results obtained indicate that the *dlx1a/2a*-expressing cells in adults give rise to a number of cells that consistently populated the same areas of the brain, namely the OB, Vv, Vd, PPp, PPa and Hc, all known to present continuous proliferative capacities in the adult zebrafish brain. This could be one more indication of the participation of *dlx* genes in mechanisms involved in adult neurogenesis. Later time points after treatment with 4OHT, such as 14dpt, revealed more visible staining that resembles neuronal projections. These cells resembled post mitotic neurons and the morphology of mCherry positive cells was similar to patterns observed in cells labeled at the developmental stage (Appendix-2 Supp. Figure 1). Taken together these

observations suggest that during adulthood, *dlx*-expressing cells give rise to mature neurons that become post mitotic and remain within the same spatial domains.

We do not rule out the possibility that Cre-mediated recombination may not be taking place with 100% efficiency in all Cre-expressing cells. Therefore, these results could be representing a percentage of the actual progeny of *dlx1a/2a*-expressing cells found in adults.

4.9 Fate mapping of *dlx1a/2a*-expressing cells in the adult brain

One of the great features of lineage tracing is that it allows researchers to trace the fate determination of cells of interest. After indications based on morphology of the progeny of *dlx1a/2a*-expressing cells in the adult brain, we wanted to investigate if these mCherry positive cells actually acquired a neuronal fate. Co-localization of HuC/HuD analyses revealed that most mCherry cells co-localize with this marker. However the result was not completely clear as merging of images did not result in the same yellow tonality as previously observed in the work presented here with other antibodies. However, Figure 32 D'' gives an indication of a possible difference of mCherry positive cells that do co-localize with HuC/HuD or not (as indicated by an arrow).

Within the lateral recess of the diencephalic ventricle and the lateral hypothalamic nucleus, some mCherry positive cells do not co-localize with the HuC/HuD marker. This is similar to what was observed before in lineage tracing during development (Solek et al. 2017). This further confirms that the identity and possibly, the role of the progeny of *dlx1a/2a*-expressing cells in some areas of the adult hypothalamus, are distinct than what is observed in other areas of the adult zebrafish brain.

With evidence that some cells co-express markers for GABAergic neurons and tyrosine hydroxylase (TH) in the mouse forebrain (Zhang and Pol 2015), and some scarce studies indicating a role for *dlx* genes in dopaminergic neurons (Saino-Saito, Berlin, and Baker 2003), we sought to explore if mCherry positive cells could co-localize with Tyrosine hydroxylase (TH). Very rare co-expression of mCherry and TH was observed; in the vast majority of sections containing mCherry-positive cells, no co-localization was observed, demonstrating that the progeny of *dlx1a/2a*-expressing cells in its great majority do not acquire a dopaminergic cell fate.

Analyses carried out through the co-labeling of mCherry and GABAergic neurons were inconclusive. Several different approaches were attempted such as combinations of ISH and IHC and different antibodies for GABAergic neurons. Unfortunately, in most cases, there was no labeling of GABAergic neurons observed or when present, the signals were too weak and remained inconclusive. Alternatives need to be explored for the labeling of mCherry as GABAergic neurons, also further investigations are necessary to confirm or rule out other possible fates of *dlx1a/2a*-expressing cells in adulthood. These cell types can be investigated with markers for oligodendrocytes, proliferating cells, neural stem cells or glia cells.

4.10 General conclusions

The overall goal of this research was to explore a role for *dlx* genes in the adult brain and to investigate possible changes in the expression of these genes during a regeneration response. For these purposes, we have used the zebrafish as a model system, as it presents a remarkable regenerative capability in the brain. Understanding the mechanisms involved in regeneration biology of the vertebrate central nervous system presents a great potential for therapies, especially regarding human neurodegenerative disorders or acute neural injuries.

We have identified the adult brain regions where *dlx1a*, *dlx2a*, *dlx5a* and *dlx6a* genes are expressed and have confirmed that within telencephalic domains, there is high overlapping localization of the *dlx* genes with a marker for GABAergic neurons. We have also explored co-localization for *dlx* genes and have observed that in some brain regions the *dlx* genes may have a role on cells to become mature neurons in the adult brain. With investigations of the brain regeneration response after stab wound injury, we have observed a possible participation of the *dlx5a/6a* bigene, most likely, of *dlx5a*. Finally, we have also established a methodology for conducting adult lineage tracing studies and have observed the locations and possible fate decisions of *dlx1a/2a*-expressing cells in the adult brain.

Giving the important roles already described for the *dlx* genes in the CNS during development, we believe the work presented here is a valuable contribution to the knowledge of *dlx* genes function in the context of adulthood.

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APPENDIX 1 – Contributions to Manuscripts

In addition to the projects described in this Thesis, during my PhD I have contributed to two scientific manuscripts already published.

1 . Solek C.M., Feng S., Perin S., Weinschutz Mendes H., Ekker M., (2017) *Lineage tracing of dlx1a/2a and dlx5a/6a expressing cells in the developing zebrafish brain*. Developmental Biology. Jul 1;427(1):131-147. doi: 10.1016/j.ydbio.2017.04.019. Epub 2017 May 4.

My Contributions:

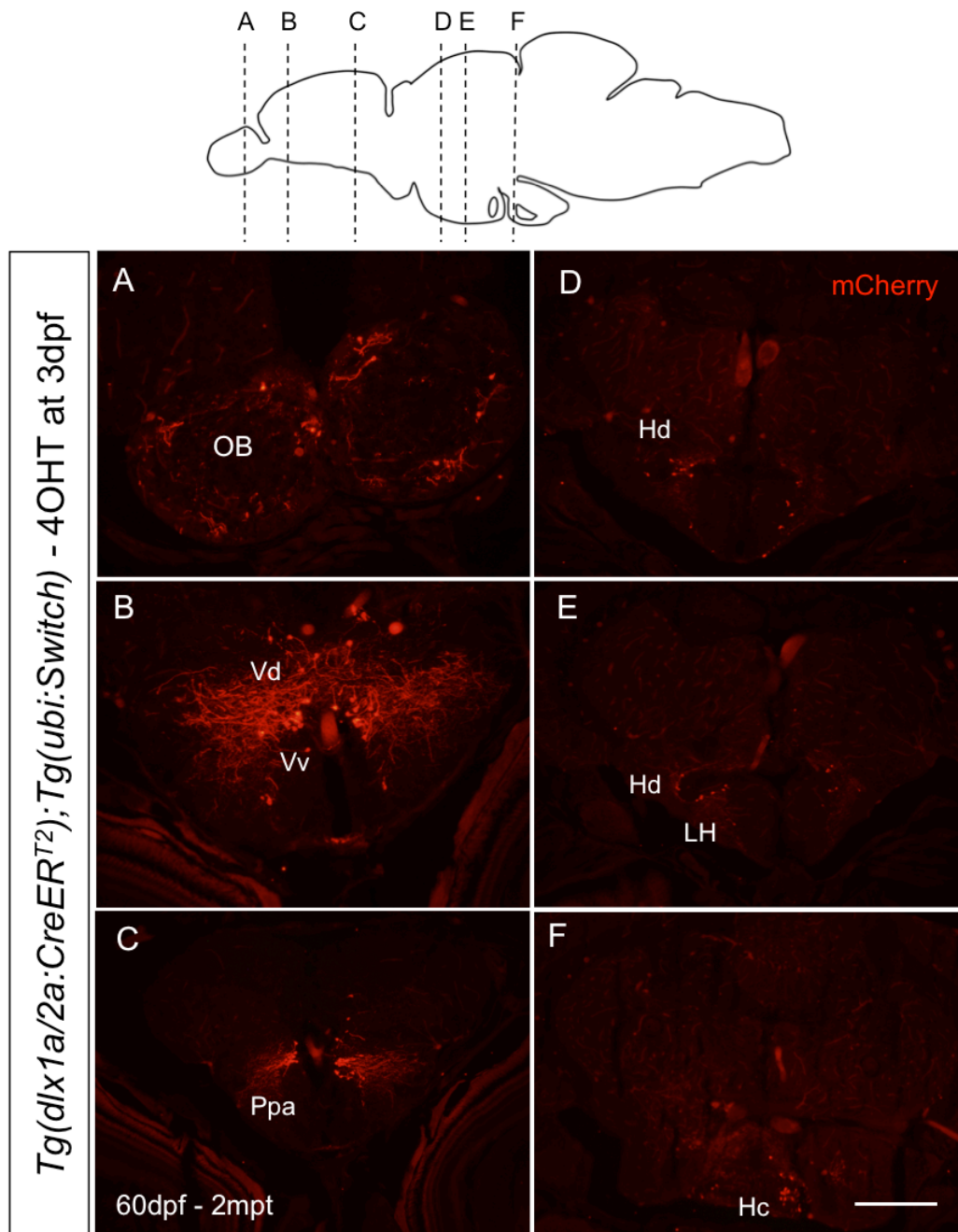
- Tamoxifen induction experiments with Tg(*dlx1a/2a:CreER^{T2}*);Tg(ubi:UbiSwitch) and Tg(*dlx5a/6a:CreER^{T2}*);Tg(ubi:UbiSwitch) zebrafish during development.
- Sectioning and sample preparation for immunofluorescence and in situ hybridization assays with zebrafish larvae
- Immunofluorescences for the detection of several different markers in zebrafish larvae.
- Critically proof-reading of the manuscript prior to publication.

2. Pena IA., Roussel Y., Daniel K., Mongeon K., Johnstone D., Weinschutz Mendes H., Bosma M., Saxena V., Lepage N., Chakraborty P., Dymment DA., van Karnebeek CDM., Verhoeven-Duif N., Bui TV., Boycott KM., Ekker M., MacKenzie A. *Pyridoxine-Dependent Epilepsy in Zebrafish Caused by Aldh7a1 Deficiency*. Genetics. 2017 Dec;207(4):1501-1518. doi: 10.1534/genetics.117.300137. Epub 2017 Oct 23.

My Contributions:

- Sectioning and sample preparation for Immunofluorescence assays with zebrafish larvae
- Immunofluorescences for the detection of Calretinin in zebrafish larvae.
- Critically proof-reading of the manuscript prior to publication.

APPENDIX 2 – Supplementary figure 1



Supplementary Figure 1. Immunohistochemical labeling of mCherry in juvenile zebrafish (60dpf) sacrificed after 2mpt post 4OHT treatment.

Detection of mCherry in Tg(*dlx1a/2a:CreER^{T2}*);Tg(ubi:UbiSwitch) adult fish is done with mCherry antibodies. Top panel indicates location of sections shown in A-F. Zebrafish embryos were induced with 4OHT at 3dpf and raised to adulthood for experiments described on section 3.2.2. Here, these fish are sacrificed at juvenile stage, showing distribution of the progeny of *dlx1a/2a*-expressing cells. OB: olfactory bulb; Vd: dorsal area of the ventral telencephalic area; Vv: ventral area of the ventral telencephalic area; Ppa: preoptic area; LH: lateral hypothalamic nucleus; Hd: dorsal zone of periventricular hypothalamus; Hc: caudal zone of periventricular hypothalamus.

Scale bar: 200µM