Dlx Gene Regulation of Zebrafish GABAergic Interneuron Development

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Abstract

The *Dlx* genes play an important role in the differentiation and migration of gamma-aminobutyric acid (GABA) interneurons of mice. GABAergic interneurons are born in the proliferative zones of the ventral telencephalon and migrate to the cortex early during mouse development. Single *Dlx* mutant mice show only subtle phenotypes. However, the migration of immature interneurons is blocked in the ventral telencephalon of *Dlx1/Dlx2* double mutant mice leading to reduction of GABAergic interneurons in the cortex. Also, *Dlx5/Dlx6* expression is almost entirely absent in the forebrain, most probably due to cross-regulatory mechanisms.

In zebrafish, the role of *dlx* genes in GABAergic interneuron development is unknown. By injecting Morpholino, we double knocked down *dlx1* and *dlx2* genes in wildtype zebrafish to investigate the function of the two genes in zebrafish GABAergic interneuron development. By comparing different subsets of GABAergic interneuron development in wildtype and *dlx1/2* morphant zebrafish forebrain, we found out that at 3dpf, 4dpf and 7dpf, double knockdown of *dlx1* and *dlx2* genes in zebrafish remarkably reduced the number of Calbindin-, Somatostatin- and Parvalbumin-positive GABAergic neurons, whereas the development of Calretinin-positive neurons is slightly affected. These results suggest that in zebrafish, *dlx1a* and *dlx2a* genes are important for the development of certain subtypes of GABAergic interneurons (Calbindin-, Somatostatin- and Parvalbumin-positive neurons) and may have minor influence on Calretinin-positive neuron development. This also suggests that different regulatory mechanisms are involved in the

development of the different subtypes of GABAergic interneurons.

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Abbreviations

ac: anterior commissure

AER: apical ectodermal ridge

CB: calbindin

CGE: caudal ganglionic eminence

CNS: central nervous system

CR: calretinin

CREs: *cis*-acting regulatory elements

DT: dorsal thalamus

Emt: eminentia thalami

GAD: glutamic acid decarboxylase

GABA: gamma-aminobutyric acid

GH: growth hormone

LGE: lateral ganglionic eminence

LIM-hd: LIM-homeodomain

LTP: long-term potentiation

LV: lateral ventricle

MGE: medial ganglionic eminence

MZ: mantle zone

NCX: neocortex

NPY: neuropeptide Y

P: pallium

PCX: palliocortex

po: preoptic region

PPSS: Somatostatin is synthesised as preprosomatostatin

PTd: dorsal part of posterior tuberculum

PTv: ventral part of posterior tuberculum

PT: posterior tuberculum

PV: parvalbumin

S: subpallium

sd: dorsal thalamus

SDF-1: cell-derived factor-1

SHFM: Split Hand/Split Foot Malformation

SOM: somatostatin

SVZ: subventricular zone

SR: sarcoplasmic reticulum

TDO: tricho-dento-osseous

TeO: tectum opticum

TnC: troponin-C

vt: ventral thalamus

VZ: ventricular zone

1 Introduction

1.1 Distal-less (*Dll*) genes

Distal-less is the earliest known gene specifically expressed in developing insect limbs whose expression is maintained throughout limb development. The signal from *Distal-less* affects the formation of imaginal discs, distinguishing the site of imaginal disc development from the general body wall (Cohen et al., 1993), thus setting the stage for limb development. *Drosophila* mutants lacking *Dll* function die as embryos because they lack the rudimentary larval limbs (Cohen and Jurgens, 1989).

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

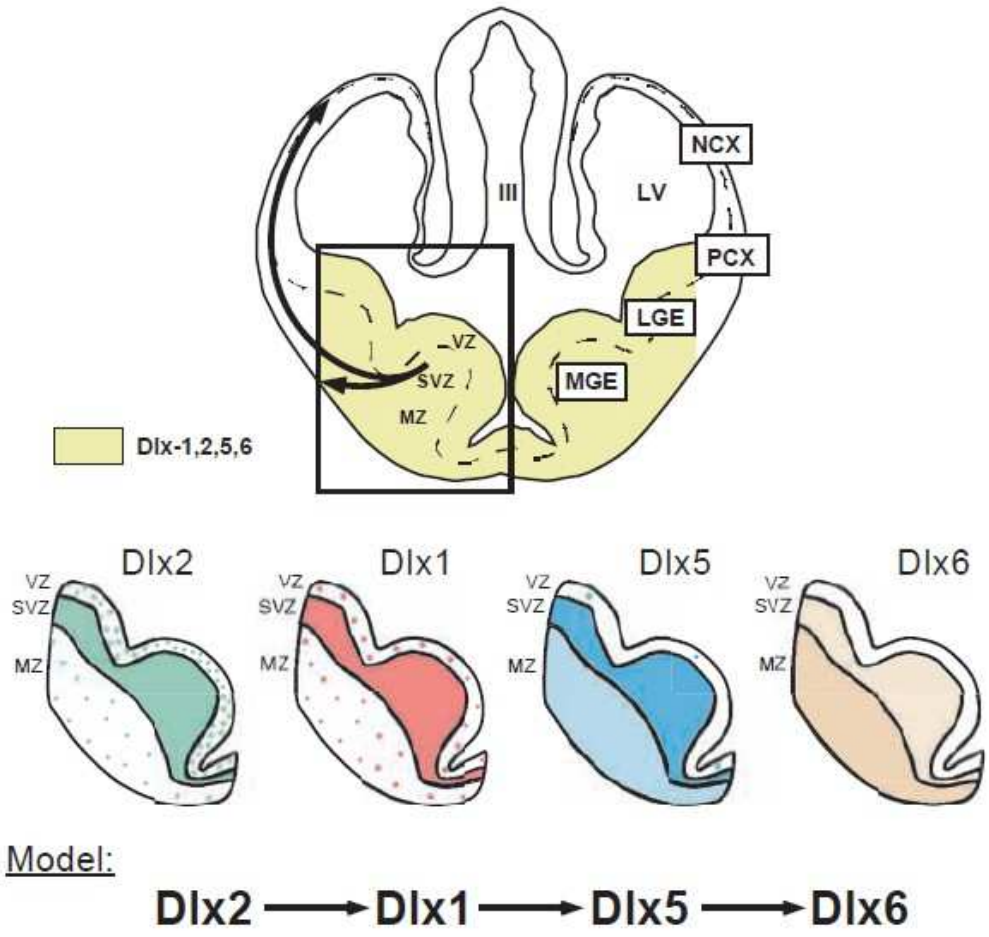
Dlx is a family of homeodomain transcription factors which are related to the Drosophila *distal-less* (*Dll*) gene (Panganiban and Rubenstein, 2002). The vertebrate *Dlx* genes are a functional diversification of paralogs (Ghanem et al., 2003). These paralogs have overlapping patterns of expression because of the *cis*-acting regulatory sequences located in the short intergenic region that separated them (Ghanem et al., 2003; Park et al., 2004; Sumiyama and Ruddle, 2003; Zerucha and Ekker, 2000). These intergenic sequences also mediate cross-regulation of *Dlx* proteins (Zerucha et al, 2000; Ghanem et al, 2003).

Gnathostoma vertebrates have six *Dlx* genes and they are closely linked in tail-to-tail pairs, *Dlx1–Dlx2, Dlx3–Dlx4* (originally called *Dlx7*) and *Dlx5–Dlx6*. They are thought to have arisen via a tandem gene duplication event followed by a series of duplication events (Simeone et al., 1994). In mammals and birds, *Dlx2, Dlx3* and *Dlx5*

are more closely related to each other than they are to *Dlx1, Dlx4* and *Dlx6* because the creation of the first linked pair of genes happened a long time before the production of the other three pairs (Neidert et al., 2001; Stock et al., 1996). Four *Dlx* genes, *Dlx1, Dlx2, Dlx5* and *Dlx6* are expressed in the forebrain in two highly restricted domains: the telencenphalon and the diencephalon (Eisenstat et al., 1999; Puelles et al., 2000; Zerucha et al., 2000). *Dlx* genes display a spatio-temporal expression in which *Dlx2* is expressed the earliest in the progenitors, followed by *Dlx1*, *Dlx5* and *Dlx6* (Liu et al., 1997; Eisenstat et al., 1999; Zerucha et al., 2000)(Figure 1.1).

Fig. 1.1. Expression domains of *Dlx1, Dlx2, Dlx5* and *Dlx6* during mouse brain development. Schema of a transverse section through the E12.5 mouse telencephalon showing the combined expression of *Dlx* transcripts. Most cells in the subpallial telencephalon express *Dlx1*, *Dlx2*, *Dlx5* or *Dlx6* at some stage of their differentiation. The arrows indicate the migration from the subpallium to the pallium (cortex) (Marin and Rubenstein, 2001). The boxed region on the left is used in the middle section to show the expression of *Dlx2, Dlx1, Dlx5* and *Dlx6. Dlx2* is primarily expressed in undifferentiated cells; it is expressed in scattered cells in the ventricular zone (green dots), in most cells in the subventricular zone (uniform green) and in scattered cells in the mantle zone (green dots). *Dlx6* is primarily expressed in differentiated cells in the mantle zone (uniform peach). *Dlx1* (red) and *Dlx5* (blue) are expressed in intermediate patterns. (Bottom) A hypothesized genetic and biochemical pathway that

proposes the sequential role of *Dlx2*, *Dlx1*, *Dlx5* and *Dlx6* at different stages of differentiation. Telencephalic regions are as follows. Pallium: neocortex (NCX) and palliocortex (PCX). Subpallium: lateral ganglionic eminence (LGE). Medial ganglionic eminence (MGE). Stages of differentiation: ventricular zone (VZ); subventricular zone(SVZ); mantle zone (MZ). LV, lateral ventricle (ventricle of telencephalon); III, third ventricle (ventricle of the diencephalon). Cited from Panganiban and Rubenstein, 2002.



1.3 *Dlx* genes in zebrafish

In zebrafish, there are eight known *dlx* genes (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997; Stock et al., 1996). Six show similar bigene genomic arrangements as in the mouse: *dlx1a/dlx2a; dlx3b/4b*; and *dlx5a/6a* clusters orthologous to *Dlx1/2; Dlx3/4; Dlx5/6*, respectively (Quint et al., 2000). Two additional genes, *dlx2b* and *dlx4a*, are thought to be duplicates of ancestral *Dlx2* and *Dlx4*, respectively, after the teleost-specific genome duplication event (Amores et al., 1998).

In the mouse, the CREs (*cis*-acting regulatory elements) I56i located in the intergenic regions of *Dlx5/Dx6* (Zerucha et al., 2000) and I12b in *Dlx1/Dlx2* (Ghanem et al., 2003), respectively, show comparable regulatory activities in the subpallial telencephalon and diencephalon (Ghanem et al., 2003). The CREs URE1 and URE2 were found located ~17 kb (URE1) and~12 kb (URE2) upstream of the *Dlx1* ATG start site. The zebrafish *dlx* genes share a similar genomic arrangements as in the mouse, including the presence of the regulatory elements I12a and I12b in the *dlx1a/2a* intergenic region, upstream regulatory element 2 (URE2) upstream of *dlx1a*, I56i and I56ii between *dlx5a* and *dlx6a* (Zerucha et al., 2000; Ghanem et al., 2003). Five *dlx* genes (all but *dlx3b, dlx4b,* and *dlx4a*) are expressed in the zebrafish forebrain with very similar expression domains in the telencephalon and diencephalon (Akimenko et al., 1994; Ellies et al., 1997).

The first two *Dlx* genes expressed during zebrafish development are *dlx3* and *dlx7* during gastrulation (Akimenko et al., 1994; Ellies et al., 1997). The zone of

expression of *dlx3* and *dlx7* during gastrulation will develop into the prospective olfactory and otic placodes (Akimenko et al., 1994; Ellies et al., 1997). Later during development, additional *dlx* genes, *dlx4* and *dlx6*, are expressed in the olfactory placodes and in the developing otic vesicle and inner ear (Akimenko et al. 1994; Ekker et al. 1992; Ellies et al. 1997)(Zerucha and Ekker, 2000)(Table 1.1).

Table 1.1 Summary of *dlx* genes expression in the zebrafish (Akimenko et al., 1994; Panganiban and Rubenstein, 2002).

Expression	**Gene**
Ectodermal stripes in gastrula	*dlx3b* *dlx4b*
Ventral forebrain	*dlx1a* *dlx2a* *dlx5a* *dlx6a* *dlx2b*
Olfactory placodes	*dlx3b* *dlx4b* *dlx5a* *dlx6a*
Migrating neural crest	*dlx2a*
Visceral arches	*dlx1a* *dlx2a* *dlx3b* *dlx4b* *dlx5a* *dlx6a* *dlx4a*
Dorsla otic vesicle	*dlx3b* *dlx4b* *dlx5a* *dlx6a*
Pectoral fin buds	*dlx1a* *dlx1a* *dlx3b* *dlx4b* *dlx5a* *dlx6a* *dlx2b* *dlx4a*
Median fin fold	*dlx1a* *dlx1a* *dlx3b* *dlx4b* *dlx5a* *dlx6a* *dlx2b* *dlx4a*

1.4 *Dlx* gene function

The *Dlx* proteins are known as DNA-binding transcriptional regulators, which control large numbers of downstream effector genes. Genetic approaches were used to show that the *Dlx* genes regulate the development of ectodermal tissues derived from neural plate, the differentiation of GABAergic neurons, patterning of the branchial arches skeleton. *Dlx* genes are also involved in bone and cartilage formation and limb development (Panganiban and Rubenstein, 2002).

Overlapping patterns of expression for the *Dlx* genes cause a problem in determining their respective roles for gene function may be partially redundant. The phenotypes of mice with targeted mutations in *Dlx* genes are consistent with partial functional redundancy. The most prominent method to study the functions of the *dlx* genes in zebrafish is the use of knockdown technologies, such as injecting morpholino oligonucleotides.

1.4.1. **Neurogenesis and neuron specification**

Neurogenesis in vertebrates occurs by the regulated withdrawal from the cell cycle of a homogeneous population of progenitor cells in the neural tube (McConnell, 1981). Subpallial neurogenesis is under the control of the bHLH, achaete-scute family, *Mash1* proneural factor (Casarosa et al., 1999). *Mash1* acts as neural determinant through regulation of the Notch pathway, it also confers ventral neuronal identity through regulation of *Dlx* and GAD67 (the rate-limiting GABA synthesis enzyme) (Casarosa et al., 1999; Poitras et al., 2007; Fode et al., 2000; Parras et al., 2002).

In the subpallium, the lateral ganglionic eminence (LGE) generates the striatal projection neurons (Deacon et al., 1994). A fate mapping study of the Nkx2.1-lineage in the mouse telencephalon demonstrated the medial ganglionic eminence (MGE) gives rise to many different projection neurons of the globus pallidus, septum, and nucleus basalis (Xu et al., 2008). The other group consists of projecting neurons whose cell bodies can be found in a series of nuclei in the subcortical telencephalon, including the medial septum (also designated as Ch1 cholinergic group) (Mesulam et al., 1983), the vertical and horizontal limbs of the nucleus of the diagonal band (Ch2 and Ch3) (Mesulam et al.,1983), and the basal magnocellular complex (designated as Ch4) (Mesulam et al.,1983), which comprises cholinergic neurons scattered through the magnocellular preoptic nucleus, substantia innominata, ventral pallidum, and nucleus basalis (Mesulam et al, 1989).

It has been shown that *Dlx1*$^{-/-}$/*Dlx2*$^{-/-}$ mutants have reduced GAD expression, a block in tangential migration, abnormal neurite morphogenesis and decreased neuronal survival (Anderson et al,1997a,b; Cobos et al., 2007; Long et al., 2007, 2009 a,b); *Dlx1*$^{-/-}$ mutants have selective postnatal loss of a subset of cortical interneurons (Cobos et al., 2005); Mice lacking *Dlx5* have a reduction in the size of the olfactory epithelium, and olfactory neurons fail to innervate the olfactory bulb (Levi et al., 2003; Long et al., 2003).

Dlx genes are crucial regulators for GABAergic differentiation. *Dlx1* and *Dlx2* double mutant mice lose the majority of GABAergic neocortical, hippocampal, and olfactory bulb interneurons due to defects in neuronal maturation

and migration (Anderson et al., 1997a; Cobos et al., 2005, 2007; Long et al., 2007, 2009 a,b). *Dlx1* and *2* mutants also have a block in the differentiation of basal ganglia GABAergic projection and interneurons (Anderson et al., 1997b; Marin et al., 2000; Yun et al., 2002). Thus *Dlx1* and *Dlx2* are essential for GABAergic neuron formation throughout the forebrain.

Over-expression assays have indicated that *Dlx2* can induce *Dlx5* expression, *Dlx1, Dlx2* and *Dlx5* can induce the expression from a *Dlx5/6-lacZ* enhancer/reporter construct, and *Dlx2* and *Dlx5* can induce *GAD65/GAD67* expression (Stuhmer et al, 2002). Dlx proteins cooperate with other proteins like Necdin to activate the transcription of target genes and promote GABA neuronal phenotype (Kuwajima et al, 2006). In addition, other homeodomain-containing factors expressed in the subpallium such as Gsh1 and Gsh2 are required for the formation and maintenance of the LGE progenitor population (Toresson et al, 2001), and appear to regulate *Dlx* expression and thus GABAergic interneuron differentiation.

In-depth analysis has shown that CREs, *I56i* and *I56ii*, in the *Dlx5/Dlx6* intergenic region act as individual enhancers and may participate in *Dlx* gene regulation during the development of specific populations of GABAergic interneurons (Ghanem et al., 2007; Ghanem et al., 2008).

1.4.2 Bone development

It has been demonstrated that *Dlx5* and *Dlx6* are expressed in all anlagen of the endochondral skeleton (Zhu et al, 2009). At later stages of skeletogenesis, *Dlx5*

and *Dlx6* are expressed in the post-mitotic prehypertrophic and hypertrophic zones (Bendall et al, 2003; Hsu et al, 2006; Ferrari et al, 2002). *Dlx5* and *Dlx6* are also expressed in the perichondrium/ periosteum in the long bones as well as ribs and vertebrae (Bendall et al, 2003; Simeone et al, 1994; Chen et al, 1996; Acampora et al, 1999). *Dlx5* single and *Dlx5/6* double knockout mice have demonstrated requirements for *Dlx5* and *Dlx6* during chondrogenesis (Bendall et al, 2003) However, it has been demonstrated that *Dlx5* is required for chondrocyte hypertrophy and that *Dlx6* has a redundant function in this tissue since the requirement for *Dlx5* and *Dlx6* function during chondrocyte hypertrophy can be satisfied with *Dlx5* alone (Zhu and Bendall, 2009).

Previous study indicated that in both *Msx1*$^{-/-}$ and *Dlx5*$^{-/-}$ mice, frontal bone development was adversely affected. The frontal bones of *Msx1* and *Dlx5* double mutant mice showed even more severe defects than either *Msx1*$^{-/-}$ or *Dlx5*$^{-/-}$ mice (Chung et al, 2010). In addition, *Dlx5* overexpression accelerates osteoblast differentiation of primary osteoblast cultures derived from chick calvariae (Tadic et al, 2002). *Dlx5* null mice display significant craniofacial and sensory capsule skeletal defects as well as delayed calvarial ossification and less well organized diaphyseal cortical bone (Depew et al., 1999; Acampora et al, 1999).

Humans with a four base deletion in the coding region of *Dlx3* have a hereditary disorder, tricho-dento-osseous (TDO) syndrome (Price et al.,1998) that affects morphogenesis of teeth and craniofacial skeleton (Lichtenstein et al, 1972) and is characterized by increased bone density (Haldeman et al., 2004; Price et al., 1998;

Price et al., 1999).

1.4.3 Limb development

In invertebrates, *Dlx* function is best understood in the development of appendages, particularly of the limbs. All of the vertebrate *Dlx* genes are expressed in the apical ectodermal ridge (AER) of the limb bud, which regulates the patterned outgrowth of the limb. In the CNS, compound *Dlx* mutants have limb defects while limb development in the *Dlx1, Dlx2* and *Dlx5* single mutants seems to be normal(Acampora et al., 1999; Depew et al., 1999; Qiu et al., 1997; Qiu et al., 1995). It has been demonstrated that *Dlx1*$^{-/-}$ /*Dlx2*$^{-/-}$ mutants have normal limbs, but *Dlx2*$^{-/-}$ /*Dlx5*$^{-/-}$ mice have split distal limb defects, similar to ectrodactyly syndromes seen in humans (Robledo et al., 2002). This syndrome is called Split Hand/Split Foot Malformation (SHFM), can be caused by mutations in SHFM1, a locus which is closely linked to the human *Dlx5* and *Dlx6* genes (Crackower et al., 1996).

1.4.4 Patterning of the craniofacial skeleton

It is known that in *Dlx5/6* double null mice the lower jaw skeleton is altered in both size and shape (Beverdam et al. 2002; Depew et al. 2002). In *Dlx1/2*$^{-/-}$mice, the maxillary process-derived elements are more strongly affected than those of the mandible (Qiu et al. 1997; Depew et al. 2005). RCAS is a replication-competent retroviral vector system that allows sustained misexpression of a gene of interest in avian cells (Gordon et al, 2009). RCAS-*Dlx2*- and RCAS-*Dlx5*-infected avian

embryos exhibit changes in skeletal development in the upper jaw. Analysis of the distribution of RCAS-*Dlx2*-infected cells suggests that *Dlx2* induces aggregation of undifferentiated mesenchyme, which subsequently develops into the ectopic skeletal elements (Gordon et al, 2010). These results suggest that *Dlx* factors mediate localization of ectomesenchymal subpopulations within the pharyngeal arches and define where skeletogenic condensations will arise.

1.5 *Dlx* targets

A variety of genes have been identified as targets of *Dlx* regulation, including the *Dlx* genes themselves. For example, Dlx1, Dlx2 and Dlx5 all can activate transcription from the mouse *Dlx5/Dlx6* and zebrafish *dlx5a/dlx6a* intergenic enhancers in tissue culture cells (Zerucha et al., 2000) and in slices of embryonic mouse brain (Stuhmer et al., 2002). *Dlx1*, *Dlx2* and *Dlx5* can also activate a glutamic acid decarboxylase enhancer (Condie et al, 1997). *Dlx2* also is thought to regulate *Wnt1* directly in the developing telencephalon (Iler et al., 1995). Arx transcription factor has been proved as a reliable Dlx2 target gene during embryonic development (Colasante et al, 2008). Dlx3 has been implicated directly in the activation of several genes, including those encoding a human chorionic gonadotropin subunit in the placenta (Roberson et al., 2001) and profilaggrin in differentiating keratinocytes (Morasso et al., 1996). *Dlx4* (previously *Dlx7*) activates both GATA1 and MYC in hematopoietic cells (Shimamoto et al., 1997). Ectopic *Dlx4* also can inhibit apoptosis via upregulation of expression of intercellular adhesion molecule 1 (Shimamoto et al.,

2000). During bone formation, *Dlx5* represses osteocalcin (Ryoo et al., 1997). Genes encoding collagen 1A1 (Dodig et al., 1996) and bone sialoprotein (Benson et al., 2000) were found to be activated by *Dlx5* during bone differentiation.

1.6 Neuronal migration in the forebrain

Neurons are most frequently born at a distance from the place where they finally become integrated in a specific neuronal circuit. They travel from their birth place to their final position in the brain.

In the subpallial telencephalon lateral ganglionic eminences (LGE) and medial ganglionic eminences (MGE) give rise to multiple neuronal populations with disparate migratory patterns. During mid-embryonic stages, the LGE gives rise primarily to cells that migrate radially in the striatum, whereas the MGE give rise to cells that migrate tangentially towards the striatum, neocortex and hippocampus, where they differentiate as GABAergic interneurons (Anderson et al., 2001; Lavdas et al., 1999; Pleasure et al., 2000; Wichterle et al., 1999; Wichterle et al., 2001).

There are two major modes of migration in the CNS: radial migration, in which cells migrate from the progenitor zone towards the surface of the brain where they differentiate into glutamatergic neurons; and tangential migration, in which cells migrate orthogonally to the direction of radial migration into the cerebral cortex where they differentiate into GABAergic interneurons (Marin and Rubenstein, 2003)

1.7 GABAergic neurons

Gamma-amino butyric acid (GABA)-ergic neurons comprise about 20% of all neurons within the cerebral cortex and hippocampus and about 95% of neurons within the striatum (Danglot et al., 2006; Rymar et al., 2004; Wonders and Anderson, 2006). GABAergic interneurons are generated in the basal ganglia and migrate across the pallium/subpallium border to populate the cerebral cortex (Anderson et al., 1997a; Wichterle et al., 2001). It is known that GABAergic interneurons modulate neuronal activity and synaptic plasticity and perform key inhibitory functions within neural systems (Freund and Gulyas, 1997). Disruption of GABAergic neuron function may cause several disorders such as schizophrenia (Lewis et al, 2006; Lewis et al, 2008), autism (Acosta et al., 2003) and anxiety (Mohler et al., 2002).

GABAergic interneurons can be subdivided by molecular markers which include Ca2+-binding proteins, such as calbindin (CB), calretinin (CR), and parvalbumin (PV), neuropeptides, such as somatostatin (SOM) and neuropeptide Y (NPY). Expression of these markers within GABAergic interneuron subtypes varies between regions, yet about 85% of interneurons can be classified by largely non-overlapping expression of PV, CR, and SOM in the cortex and PV, CR, and CB in the hippocampus (Freund and Buzsaki, 1996; Gonchar, 2008; Gonchar and Burkhalter, 1997; Jinno and Kosaka, 2006; Kubota and Kawaguchi, 1994; Miyoshi et al., 2007). In this report, we used molecular markers to distinguish different subsets of GABAergic interneurons.

1.8 Calbindin

Calbindin (CB) is a member of EF-hand family of Ca2+-binding proteins. It was originally described as a 27-kD protein induced by vitamin D in the duodenum of the chick (Kojetin et al, 2006). Calbindin and its mRNA have a wide distribution in the central nervous system (Frantz and Tobin, 1994), including in hypothalamic supraoptic (SON) and paraventricular nuclei (Sanchez et al, 1992; Abe et al, 1992; Arai et al, 1994). It has up to six high affinity Ca2+-binding sites. Gain-of-function experiments have shown that Calbindin-D_{28K} overexpression induces neuronal differentiation and Calbindin-D_{28K} facilitates neuronal differentiation via up-regulation of genes such as *NeuroD*, *Pax6,* and *Mash1*(Miettinen et al, 1992*).*

Calbindin is expressed in both GABAergic (e.g. local circuit neurons) and glutamatergic cortical neurons (e.g. projection neurons) (Celio, 1990) and is expressed in 50% of neurons that express orexin receptors and is never detected in neurons that lack orexin receptors (Korotkova et al., 2003). It has been shown that, in rat, Calbindin is expressed in gyrus dentatus and hippocampal CA1-CA3 region (Miettinen et al., 1992)

1.9 Calretinin

Calretinin (CR) is a member of the calcium-binding protein EF-hand family first identified in the retina (Rogers, 1987). It has a function in intracellular calcium buffering, messenger targeting, and is involved in processes such as cell cycle arrest, and apoptosis (Camp and Wijesinghe, 2009).

Calretinin is expressed in distinct neuronal populations including in the retina, granule cells of the cerebellum, and brainstem auditory neurons. Immunohistochemistry revealed that 83% of calretinin-containing neurons were immunoreactive for GABA and they are located in the dentate gyrus and CA1-3 subfield. Most of the GABA-negative calretinin-immunoreactive neurons were located in the hilus of the dentate gyrus and in stratum lucidum of the CA3 subfield (Miettinen et al., 1992).

In zebrafish, CR is a marker of various neurons, including numerous cells in the optic tectum, torus semicircularis, secondary gustatory nucleus, reticular formation, somatomotor column, gustatory lobes, octavolateral area, and inferior olive, as well as of characteristic tracts of fibers and neuropil (Castro et al., 2006).

Experiments have revealed that Calretinin plays an important role in the modulation of intrinsic neuronal excitability and the induction of long-term potentiation (LTP) (Schurmans et al., 1997). Furthermore, selective knockout of Calretinin in mice suggests a putative role for Calretinin in the maintenance of calcium dynamics (Camp and Wijesinghe, 2009).

1.10 Somatostatin

Somatostatin (SOM) was originally characterized as a hypothalamic peptide with a direct inhibitory activity on the secretion of growth hormone (GH) by the anterior pituitary gland (Brazeau et al., 1973).

Somatostatin was shown to have significant influences on digestive system

physiology. It modulates almost all gastrointestinal functional activities, such as motility, secretion, and absorption (Walsh., 1994; Shulkes, 1994). It is also known that Somatostatin accelerates granule cell movement near the birthplace in the EGL and in a stage-specific manner (Elina and Hitoshi, 2002).

Somatostatin is contained in mucosald cells distributed with different densities along the entire gastrointestinal tract, in neurons intrinsic to the submucosal and myenteric plexuses, and in pancreatic islet cells (Walsh, 1994; Costa et al., 1977). Somatostatin is synthesised as preprosomatostatin (PPSS), and the cDNA of three preprosomatostatins has been identified including PPSS, PPSS2 and PPSS3 (Argenton and Zecchin, 1999). It has been shown that PPSS2 is expressed in the pancreas, whereas PPSS1 is expressed in both the pancreas and the central nervous system (Devos et al, 2002).

1.11 Parvalbumin

Parvalbumin (PV) is an 11-kDa cytosolic protein and belongs to the EF-hand family of Ca2+ binding proteins (Kawasaki et al, 1998). It co-exists with GABA in cortical neurons. Immunostaining experiment has shown that Parvalbumin was exclusively present in hippocampal interneurons. Most of them were located within or close to the granule cell layer of the dentate gyrus and the CA1–CA3 pyramidal cell layers (Keilhoff et al, 2004). In adult rats, parvalbumin is present in ~50% of interneurons in the neocortex and is expressed in both Chandelier cells and in some basket cells (Xu et al, 2003).

PV has been shown to accelerate myocardial relaxation *in vitro*

PV is associated with several Ca2+-mediated cellular activities and physiological processes. It has been suggested that PV might function as a ‘‘Ca2+ shuttle’’ which transports Ca2+ from troponin-C (TnC) to the sarcoplasmic reticulum (SR) (Baylor and Hollingwort, 2003). PV may contribute to the performance of rapid, phasic movements by accelerating the contraction–relaxation cycle of fast-twitch muscle fibers. It has also been shown that PV deficiency alters the neuronal activity, a key mechanism leading to epileptic seizures (Arif, 2009).

2. Objectives of this project

As described above, it is known that the *Dlx* genes play an important role in the differentiation and migration of gamma-aminobutyric acid (GABA) interneurons of mice. Single *Dlx* mutant mice show only subtle phenotypes. However, the migration of immature interneurons is blocked in the ventral telencephalon of *Dlx1/Dlx2* double mutant mice leading to a reduction of GABAergic interneurons in the cortex. In zebrafish, the role of *dlx* genes in GABAergic interneuron development is unknown. The main objective of this project is to study *Dlx* gene function in zebrafish GABAergic interneuron development.

GABAergic neurons are remarkably diverse and can be subdivided by morphology, electrophysiology and molecular markers. There are several molecular markers for GABAergic neurons. They include Calcium-binding proteins: CB (calbindin), CR (calretinin), PV (parvalbumin); Neuropeptides: VIP (vasointestinal peptide), SOM (somatostatin), NPY (neuropeptide Y). It is possible that *Dlx* genes have different regulatory roles on different subtypes of GABAergic neurons. In order to know whether this is true, we used molecular markers to subdivide GABAergic neurons and investigate the role of *Dlx* genes.

Since *Dlx1/Dlx2* double mutant in mice is leading to reduction of GABAergic interneurons in the cortex, I focused on the regulation function of *dlx1a*/*dlx2a* in zebrafish GABAergic interneuron development.

In summary, the objectives of this project are:

■ Study *Dlx* genes function in zebrafish GABAergic interneuron development.

● Investigate whether morpholino knockdown of *dlx1a/dlx2a* affects GABAergic neuron development in the zebrafish brain.

■ Identify which subtypes of GABA cells are regulated by *Dlx* genes.

● Investigate whether molecular markers label GABAergic neurons by using double immunohistochemistry with GAD (antibody used to mark GABAergic cells).

● Investigate the influence of double knockdown of *dlx1a/dlx2a* on different subtypes of GABAergic interneurons using immunohistochemistry.

3. Research Methods

3.1 Animals and Tissue Preparation

Animals used in this study were developing zebrafish embryos/larvae (*Danio rerio*) ranging from 3 days post fertilization (dpf) to 7dpf. Embryos and larvae were fixed in 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) and dehydrated in methanol for storage.

3.2 Morpholino oligonucleotide microinjection

Morpholino oligonucleotides (MO) are synthetic oligonucleotides composed of chains of about 25 subunits that are similar to DNA and RNA oligonucleotides, except that they have a morpholine ring rather than a ribose ring. The MO is targeted to sequence 5' of the translation start site of mRNA and inhibits the progression of the initiation complex and leads to the inhibition of translation.

MOs were injected into one-cell stage wild type zebrafish embryos at a concentration of 4ng/nl. The following translation blocking morpholinos were used: *dlx1a* (Sperber et al. 2008), *dlx2a* (Sperber et al., 2008).

The amount of morpholino injected is estimated with the calibration micrometer slide.

For each immunostaining, approximately 100 morpholino injected zebrafish larvae were obtained and about 75 of them were used for sectioning and immunohistochemistry stain.

3.3 Frozen section

First, 3dpf, 4dpf and 7dpf zebrafish larvae were fixed in 4% paraformaldehyde/PBS at room temperature. Then zebrafish were equilibrated in 30% sucrose/PBS at 4°C for 1-2 days prior to sectioning. Zebrafish samples were put into section container with Tissue Freezing Medium (Triangle Biomedical Sciences), 3 fishes per bolt, face up and eyes horizontally. After this, samples were frozen in -80°C and moved to -20°C 10 min before sectioning. Zebrafish samples were sectioned in 10 µm transverse sections using crytostat (Leica CM1850) at -20°C. Sections were attached on Superfrost/Plus slides (Fisher Scientific) and stored under -20°C if not use instantly.

At last, slides were washed in PBS to get rid of section media before immunohistochemistry.

3.4 Immunohistochemistry

Zebrafish were fixed and stored and cryoprotected overnight in 20% sucrose at 4 °C. The larvae were then sectioned using a cryostat in the transverse plane at a thickness of 10 µm, mounted onto coated slides, and stored at −20 °C until further use.

GABA cells and subtypes of GABA cells were identified using an array of commercially available antibodies (Table 3.1).

The slides were soaked in PBS 10min for 3 times to get rid of sectioning media. Then the sections were blocked in BSA/PBS for 2 hours at room temperature. Diluted

primary antibody was added to sections and kept at 4°C overnight. Then slides were washed three times 10min each in PBST. For detection, the sections were incubated with the secondary antibodies in dark at room temperature for 2 hours. Prior to mounting on slides, the tissue was rinsed with PBST, placed in a mounting medium and then viewed using fluorescent microscope.

Each immunostain panel represents about 75 larvae: 15 sectioned larvae were used for immunohistochemistry at each time and 5 times experiments for every marker.

Table 3.1. Primary and Secondary Antibodies Used for Immunohistochemistry

Primary	Antigen	Dilution	Host	Source
GAD65/67	gad	1:400	rabbit	Millipore
GAD65/67	gad	1:400	mouse	Biomol
CB	calbindin	1:400	rabbit	swant
CR	calretinin	1:400	mouse	swant
PV	parvalbumin	1:400	mouse	sigma
SOM	somatostatin	1:200	rat	chemicon
Secondary				
Alexa 488(green)	mouse Rabbit	1:300	goat	invitrogen
Alexa 594(red)	mouse Rabbit Rat	1:300	goat	invitrogen

3.5 Immunohistochemistry optimization

Zebrafish were sectioned and immunohistochemistry performed at each stage with each antibody after various dilutions.

Table 3.2 Immunohistochemistry conditions optimization

Stages of zebrafish tested	2dpf, 3dpf, 4dpf, 5dpf and 7dpf
Various dilutions tested	Rabbit anti-GAD 1:300 1:400 1:500 1:1000
	Mouse anti-GAD 1:300 1:400 1:500 1:1000
	Rabbit anti-CB 1:300 1:400 1:500 1:1000
	Mouse anti-CR 1:300 1:400 1:500 1:1000
	Mouse anti-PV 1:300 1:400 1:500 1:1000
	Rat anti-SOM 1:100 1:200 1:300 1:400 1:500
Section thickness tested	8µm, 10µm and12µm

4. Results

4.1 Expression of Calbindin and GAD is remarkably reduced in 3dpf *dlx1a/dlx2a* morphant zebrafish compared with 3dpf wildtype zebrafish brain.

In 3dpf wildtype zebrafish forebrain, CB-positive cells are scattered around anterior commissure (ac) and preoptic region (po) (Figure4.1A). They can also be found in dorsal part of posterior tuberculum(DT), ventral thalamus (vt), dorsal thalamus (sd) and dorsal part of posterior tuberculum (PTd) (Figure 4.1 G). GAD+ cells occupied vt, ad sd and po (Figure 4.1 B), they also gathered around tectum opticum (TeO), PTd and DT (Figure 4.1 H). CB+ and GAD+ cells overlap in ac, po (Figure 4.1 C) and vt ,PTd, sd, DT (Figure 4.1 I).

In 3dpf *dlx1a/dlx2a* morphant zebrafish forebrain, it is hard to see CB+ cells in ac, po, DT, vt, sd or PTd where they are found in wildtype zebrafish brain.(Figure 4.1 D J). Although some red color appears in vt, sd (Figure 4.1 E K) and ac, TeO (Figure 4.1 K), the staining is more diffuse compared with the GAD expression in wildtype zebrafish and could not be considered as real GAD expression. The strong expression in TeO of morphant in panel E is strong background stain which is a common problem occurs in immunohistochemistry and may be caused by primary and secondary antibody cross-reactivity.

Thus, the expression of Calbindin and GAD is reduced in 3dpf *dlx1a/dlx2a* morphant zebrafish compared to 3dpf wildtype zebrafish brain.

4.2 Expression of Calbindin and GAD is remarkably reduced in 4dpf *dlx1a/dlx2a* morphant zebrafish compared to 4dpf wildtype zebrafish brain.

In 4dpf wildtype zebrafish forebrain, CB+ cells scattered around ac (Figure 4.2 A G) and vt, sd (Figure4.2 G). GAD+ cells appeared in ac (Figure 4.2 B H), vt, sd and TeO (Figure 4.2 H). CB+ and GAD+ cells both appeared in ac, vt in 4dpf wildtype zebrafish (Figure 4.2 C I).

In 4dpf *dlx1a/dlx2a* morphant zebrafish forebrain, there are no CB+ cells that appear in ac, vt or sd, the areas where CB is expressed in wildtype zebrafish brain (Figure 4.2 D J). Despite some diffuse expression in sd (Figure 4.2 E K), the expression of GAD is hardly be seen in regions where GAD is expressed in wildtype zebrafish. These facts indicate reductions of CB and GAD expression in 4dpf *dlx1a/dlx2a* morphant zebrafish compared to wildtype zebrafish at the same development stage.

4.3 Expression of Calbindin and GAD is remarkably reduced in 7dpf *dlx1a/dlx2a* morphant zebrafish compared with 7dpf wildtype zebrafish brain.

In 7dpf wildtype zebrafish forebrain, CB+ cells appear in vt, ac, sd. and TeO (Figure 4.3 A, G). GAD expression can be seen in sd (Figure 4.3 B E). CB and GAD are co-expressed in sd (Figure 4.3 C I).

In 7dpf *dlx1a/dlx2a* morphant zebrafish forebrain, CB+ cells can only be seen in eyes but not in brain (Figure 4.3 D J) and there is barely any GAD expression in 7dpf *dlx1a/dlx2a* morphant zebrafish brain (Figure 4.3 E K). It is clear that expression of

Calbindin and GAD is reduced in 7dpf *dlx1a/dlx2a* morphant zebrafish compared with 7dpf wildtype zebrafish brain.

4.4 Expression of Calretinin is slightly reduced while GAD expression is significantly reduced in 3dpf *dlx1a/dlx2a* morphant zebrafish compared with their expression in 3dpf wildtype zebrafish brain.

In 3dpf wildtype zebrafish forebrain, there is an abundance of CR+ cells that occupy the dorsal part of posterior tuberculum (PTd), ventral part of posterior tuberculum (PTv), DT and TeO (Figure 4.4A G). GAD+ cells could be found in PTv (Figure 4.4 B H), PTd, DT (Figure 4.4 B). CB and GAD are co-expressed in PTv (Figure 4.4 C J), PTd, DT (Figure 4.4 C).

In 3dpf *dlx1a/dlx2a* morphant zebrafish brain, although their number is smaller than in wildtype zebrafish brain, CR+ cells can clearly be observed in PTv, eminentia thalami (Emt),. (Figure 4.4 D K) and PTd, DT (Figure 4.4 D). GAD+ cells can barely be seen in the brain of morphant zebrafish (Figure 4.4 E K). These results show that in 3dpf *dlx1a/dlx2a* morphant zebrafish, expression of Calretinin is slightly reduced while GAD expression is reduced compared to expression in 3dpf wildtype zebrafish brain.

4.5 Expression of Calretinin is slightly reduced while GAD is significantly reduced in 4dpf *dlx1a/dlx2a* morphant zebrafish compared with their expression in 4dpf wildtype zebrafish brain.

In 4dpf wildtype zebrafish forebrain, there is an abundance of CR+ cells in PTd, PTv, DT and pallium (P) (Figure 4.5A G). GAD+ cells gathered in TeO, PTv, PTd and ac. (Figure 4.5 B H). CB and GAD are co-expressed in PTv and PTd (Figure 4.5 C I).

In 4dpf *dlx1a/dlx2a* morphant zebrafish forebrain, CR+ cells can be seen in PTv, PTd, P and DT (Figure 4.5 D J) but both the cell number and the staining intensity are lower than in wildtype zebrafish. GAD+ cells can barely be seen in the brain of morphant zebrafish (Figure 4.5 E K). CB and GAD are co-expressed in PTv and Emt (Figure 4.5 F L). These results show that in 4dpf *dlx1a/dlx2a* morphant zebrafish, expression of Calretinin is slightly reduced while the number of GAD + cells is remarkably reduced compared with 4dpf wildtype zebrafish brain.

4.6 Expression of Calretinin and GAD is not markedly reduced in 7dpf *dlx1a/dlx2a* morphant zebrafish compared with wildtype zebrafish brain.

In 7dpf wildtype zebrafish forebrain, there is an abundance of CR+ cells in PTd, PTv, DT, P and TeO (Figure 4.6 A G). GAD expressed in PTd, PTv, DT and P(Figure 4.6 B H). CR and GAD co-expressed in PTd, PTv, DT and P (Figure 4.6 C I).

In 7dpf *dlx1a/dlx2a* morphant zebrafish forebrain, there are some CR+ cells scattered in PTv, PTd (Figure 4.6 D J), P and DT (Figure 4.6 J) but both the cell number and labeling intensity are lower than in wildtype zebrafish. Expression of GAD can be seen in PTd, PTv, DT and P in morphant zebrafish (Figure 4.6 F L) yet the expression intensity is less than it in wildtype zebrafish. Thus, in 7dpf *dlx1a/dlx2a* morphant zebrafish, expression of Calretinin and GAD is reduced but still can be seen

compared with their expression in 7dpf wildtype zebrafish brain.

4.7 Expression of both Somatostatin and GAD is reduced in 3dpf *dlx1a/dlx2a* morphant zebrafish compared with their expression in 3dpf wildtype zebrafish brain.

In 3dpf wildtype zebrafish forebrain, SOM+ cells gathered in P, S, DT and Emt (Figure 4.7 A G). And GAD+ cells can be seen in the same areas as SOM+ cells (Figure 4.7 B H).

In 3dpf *dlx1a/dlx2a* morphant zebrafish brain, neither SOM nor GAD expression can be clearly observed in S, P and DT (Figure 4.7 D E J K) which indicates that the expression of SOM and GAD is reduced in *dlx1a/dlx2a* morphant zebrafish compared with their expression in 3dpf wildtype zebrafish brain.

4.8 Expression of both Somatostatin and GAD is reduced in 4dpf *dlx1a/dlx2a* morphant zebrafish compared with their expression in 4dpf wildtype zebrafish brain.

In 4dpf wildtype zebrafish forebrain, SOM+ cells gathered in subpallium (S) and Emt (Figure 4.8 A G). GAD+ cells gathered in Emt (Figure 4.8 B H), P, S and DT (Figure 4.8 B). SOM and GAD co-expressed in Emt (Figure 4.8 C I) and S (Figure 4.8 C).

In 4dpf *dlx1a/dlx2a* morphant zebrafish forebrain, both SOM and GAD expression are much less than in wildtype zebrafish (Figure 4.8 D E J K). These

indicate that expression of SOM and GAD is reduced in *dlx1a/dlx2a* morphant zebrafish compared with their expression in 4dpf wildtype zebrafish brain.

4.9 Expression of both Somatostatin and GAD is reduced in 7dpf *dlx1a/dlx2a* morphant zebrafish compared with their expression in 7dpf wildtype zebrafish brain.

In 7dpf wildtype zebrafish forebrain, SOM+ cells gathered in S and Emt (Figure 4.9 A G) and P, DT (Figure 4.9 G). GAD+ cells gathered in the same areas as SOM+ cells (Figure 4.9 B H C I).

In 7dpf *dlx1a/dlx2a* morphant zebrafish forebrain, there is no clear expression of SOM (Figure 4.9 D J), nor is there GAD expression (Figure 4.9 E K). These observations indicate that expression of SOM and GAD is reduced in *dlx1a/dlx2a* morphant zebrafish compared with their expression in 7dpf wildtype zebrafish brain.

4.10 Expression of Parvalbumin is reduced in 3dpf *dlx1a/dlx2a* morphant zebrafish compared with their expression in 3dpf wildtype zebrafish brain.

In 3dpf wildtype zebrafish forebrain, PV-positive cells are scattered in ac, TeO, PTd and DT (Figure 4.10 A D).

In 3dpf *dlx1a/dlx2a* morphant zebrafish, there is no clear expression of PV in the brain (Figure 4.10 D J). These observations indicate that expression of PV is reduced in *dlx1a/dlx2a* morphant zebrafish compared with expression in 3dpf wildtype zebrafish brain.

4.11 Expression of Parvalbumin is reduced in 4dpf *dlx1a/dlx2a* morphant zebrafish compared with expression in 4dpf wildtype zebrafish brain.

In 4dpf wildtype zebrafish forebrain, there is an abundance of PV+ cells in TeO, DT, PTd and PTv (Figure 4.10 B H).

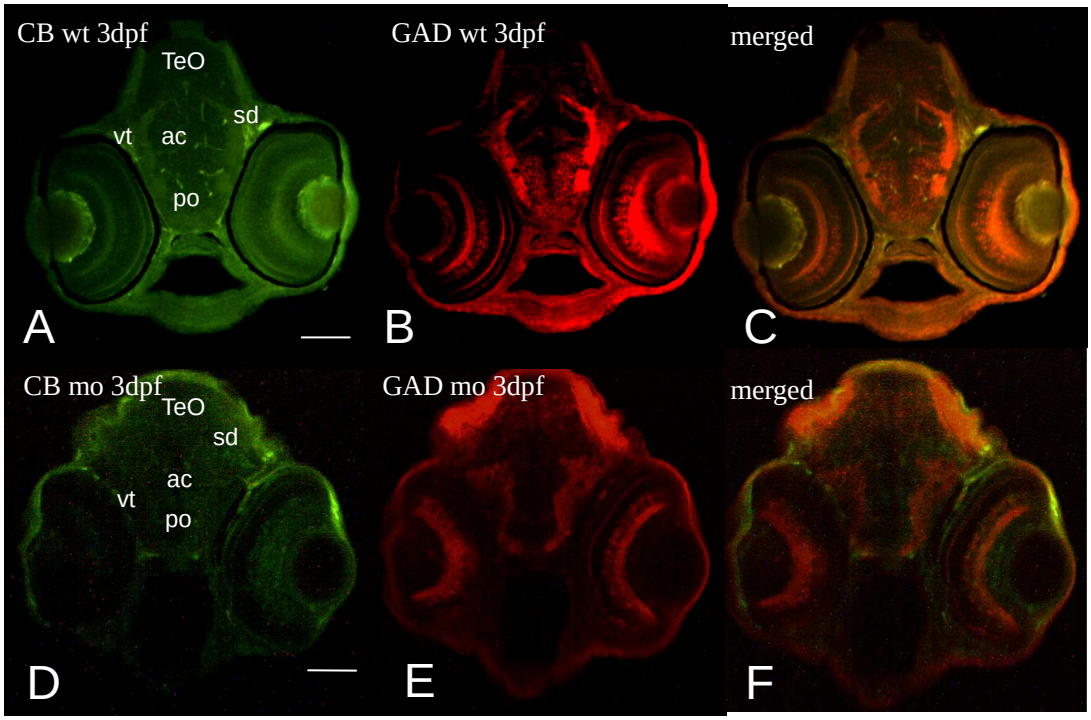
In 4dpf *dlx1a/dlx2a* morphant zebrafish, there is no clear PV expression in the brain (Figure 4.10 E K). These indicate that expression of PV is reduced in *dlx1a/dlx2a* morphant zebrafish compared with expression in 4dpf wildtype zebrafish brain.

4.12 Expression of Parvalbumin is reduced in 7dpf *dlx1a/dlx2a* morphant zebrafish compared with expression in 7dpf wildtype zebrafish brain.

In 7dpf wildtype zebrafish forebrain, there is an abundance of PV+ cells in TeO, DT, PTd and PTv (Figure 4.10 C I).

In 7dpf *dlx1a/dlx2a* morphant zebrafish, there is no clear PV expressed in the brain (Figure 4.10 F L). This suggests that expression of PV is reduced in *dlx1a/dlx2a* morphant zebrafish compared with expression in wildtype zebrafish brain at the same developmental stage.

Figure 4.1 Calbindin and GAD expression in 3dpf wildtype and *dlx1a/dlx2a* morphant zebrafish brain by immunohistochemistry on 3dpf zebrafish brain transverse sections (10μm). A, G) anti-Calbindin (CB) immunohistochemistry on 3dpf wildtype zebrafish brain. B, H) anti-GAD65/67 immunohistochemistry on 3dpf wildtype zebrafish brain. C, F, I, L) merged images of both CB and GAD expression. D, J) anti-Calbindin immunohistochemistry on 3dpf *dlx1a/dlx2a* morphant zebrafish brain. H, K) anti-GAD65/67 immunohistochemistry on of 3dpf *dlx1a/dlx2a* morphant zebrafish brain. Green represents CB expression, red represents GAD expression and yellow represents overlap of CB and GAD expression. Panel A-F and G-L are two pairs of larvae which showing the most representative phenotype of the experimental group they are in, basically they representing the same planes of brain and similar phenotype. ac: anterior commissure, DT: dorsal thalamus, po: preoptic region, PTd: dorsal part of posterior tuberculum, sd: dorsal region of the subpallium, vt: ventral thalamus, TeO: tectum opticum. Neuroanatomical atlas refered to Mueller and Mullimann, 2003. Dorsal is to the top. Scale bar= 80μm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vt
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po
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po
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GAD mo 3dpf
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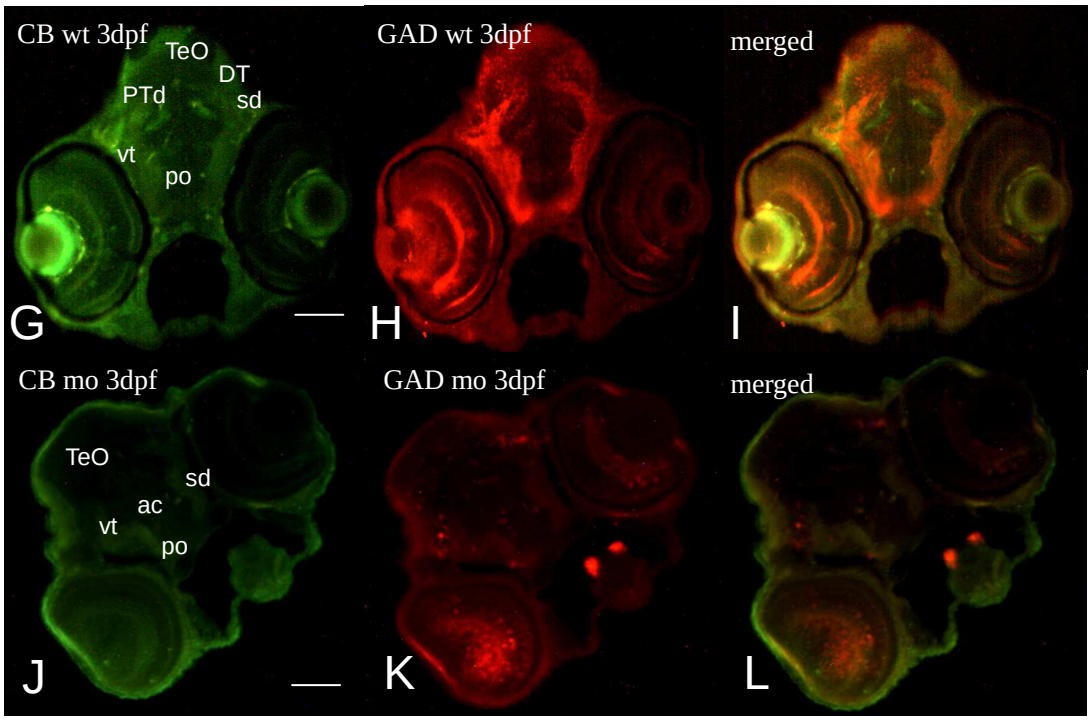
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TeO
DT
PTd
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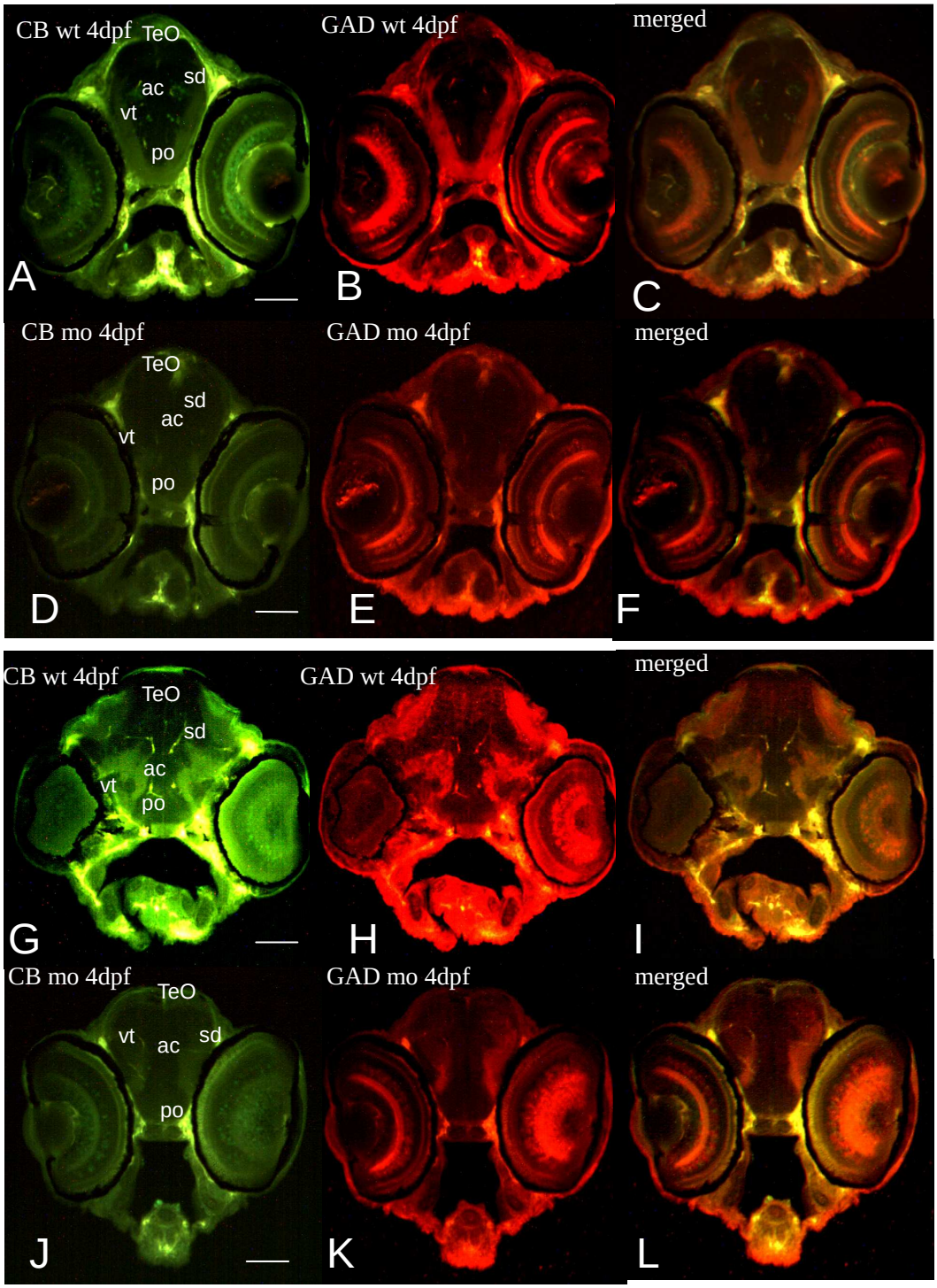
GAD wt 3dpf
H
merged
I
CB mo 3dpf
TeO
sd
ac
vt
po
J
GAD mo 3dpf
K
merged
L

Figure 4.2 Calbindin and GAD expression in 4dpf wildtype and 4dpf *dlx1a/dlx2a* morphant zebrafish brain by immunohistochemistry on zebrafish transverse sections (10µm). A, G) anti-Calbindin immunohistochemistry on 4dpf wildtype zebrafish brain. B, H) anti-GAD65/67 immunohistochemistry on 4dpf wildtype zebrafish brain. C, F, I, L) merged images of both CB and GAD expression images. D, J) anti-Calbindin immunohistochemistry on 4dpf *dlx1a/dlx2a* morphant zebrafish brain. E, K) anti-GAD65/67 immunohistochemistry on 4dpf *dlx1a/dlx2a* morphant zebrafish brain. Green represents CB expression, red represents GAD expression and yellow represents overlap of CB and GAD expression. Panel A-F and G-L are two pairs of larvae which showing the most representative phenotype of the experimental group they are in. Panel A-F representing the upper planes of the larvae brain while panel G-L representing the lower one. Dorsal is to the top. Scale bar= 80µm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G
GAD wt 4dpf
H
merged
I
CB mo 4dpf
TeO
vt
ac
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J
GAD mo 4dpf
K
merged
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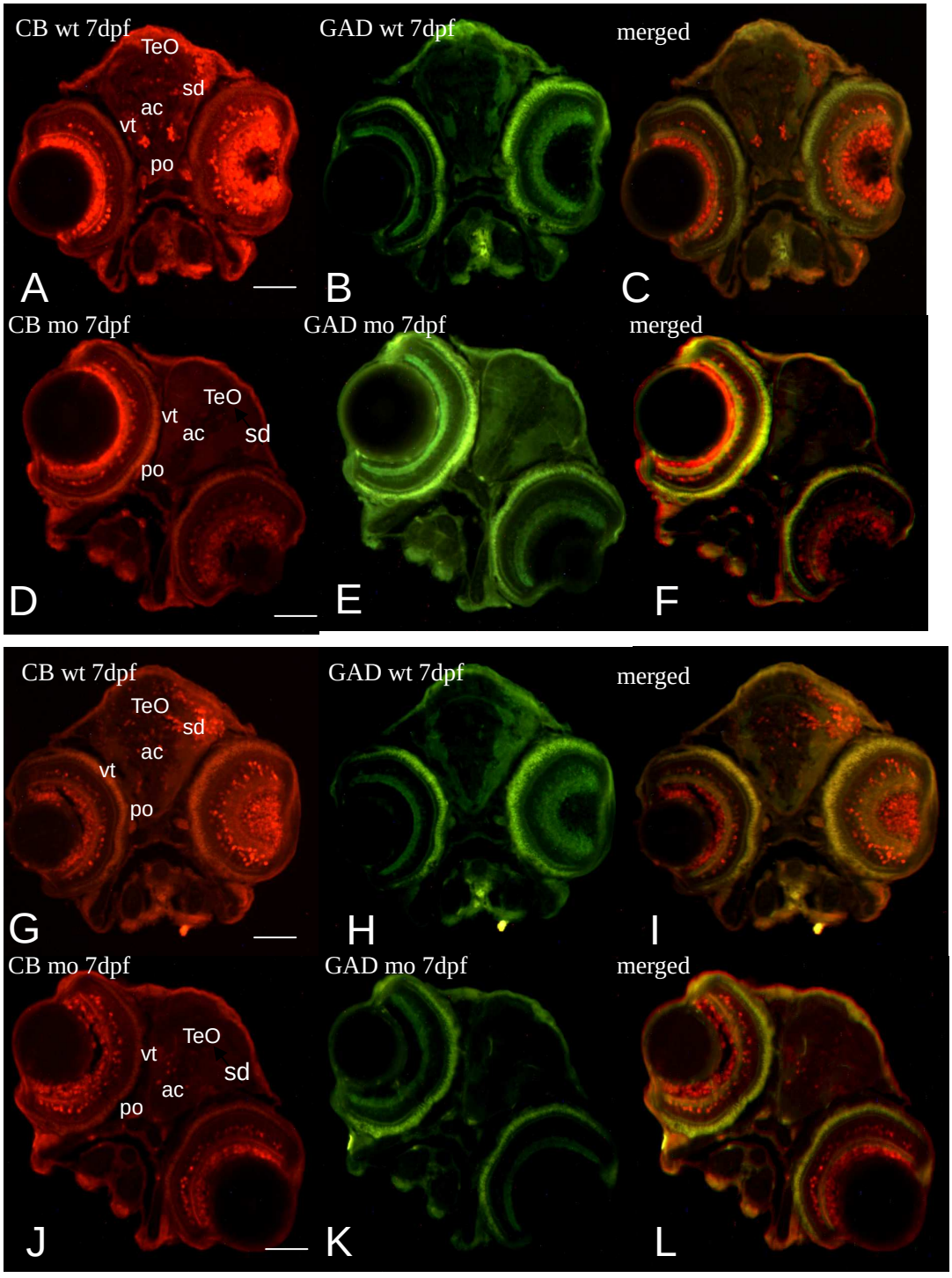
Figure 4.3 Calbindin and GAD expression in 7dpf wildtype and *dlx1a/dlx2a* morphant zebrafish brain by immunohistochemistry on zebrafish brain transverse sections (10μm). A, G) anti-Calbindin (CB) immunohistochemistry on 7dpf wildtype zebrafish brain. B, H) anti-GAD65/67 immunohistochemistry on 7dpf wildtype zebrafish brain. C, F, I, L) merged images of both CB and GAD expression. D, J) anti-Calbindin immunohistochemistry on 7dpf *dlx1a/dlx2a* morphant zebrafish brain. E, K) anti-GAD65/67 immunohistochemistry on 7dpf *dlx1a/dlx2a* morphant zebrafish brain. Panel A-F and G-L are two pairs of larvae which showing the most representative phenotype of the experimental group they are in, basically they representing the same planes of brain and similar phenotype. Green represents GAD expression, red represents CB expression and yellow represents overlap of CB and GAD expression. Dorsal is to the top. Scale bar= 80μm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vt
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G
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CB mo 7dpf
TeO
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GAD mo 7dpf
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merged
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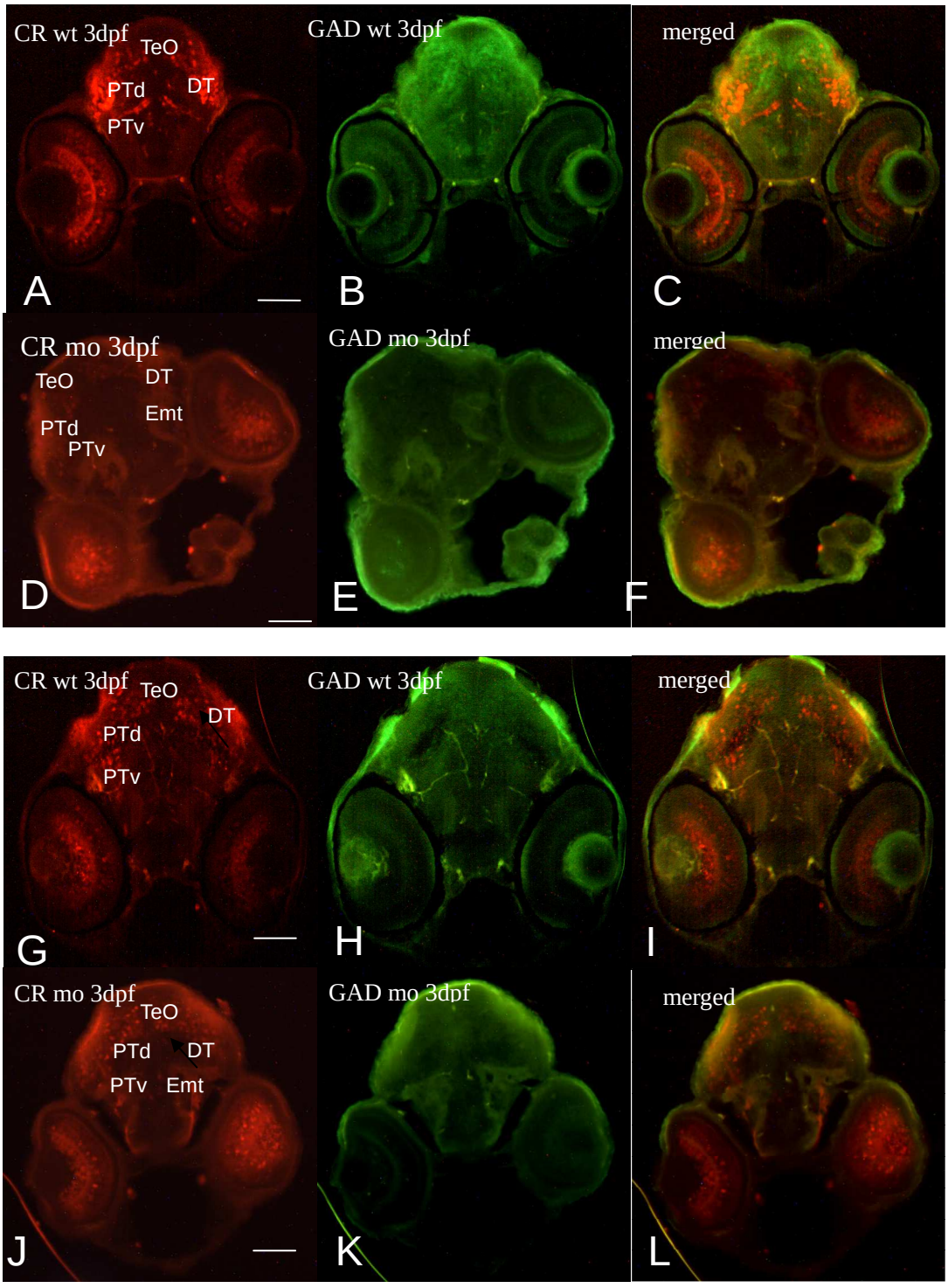
Figure 4.4. Calretinin and GAD expression in 3dpf wildtype and *dlx1a/dlx2a* morphant zebrafish brain by immunohistochemistry on zebrafish brain transverse sections (10µm). A,G) anti-Calretinin (CR) immunohistochemistry on 3dpf wildtype zebrafish brain. B,H) anti-GAD65/67 immunohistochemistry 3dpf wildtype zebrafish brain. C, I) merged images of both CR and GAD expression in. 3dpf wildtype zebrafish brain D,J) anti- Calretinin immunohistochemistry on 3dpf *dlx1a/dlx2a* morphant zebrafish brain. E,K) anti-GAD65/67 immunohistochemistry on 3dpf *dlx1a/dlx2a* morphant zebrafish brain. F,L) merged images of both CR and GAD expression 3dpf *dlx1a/dlx2a* morphant zebrafish brain. Panel A-E and F-J are two pairs of larvae which showing the most representative phenotype of the experimental group they are in. Panel A-F representing the upper planes of the larvae brain while panel G-L representing the lower one. Red represents CR expression, green represents GAD expression, yellow represents overlap of CR and GAD expression. PTd: dorsal part of posterior tuberculum, PTv: ventral part of posterior tuberculum. Dorsal is to the top. Scale bar = 80µm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GAD mo 3dpf
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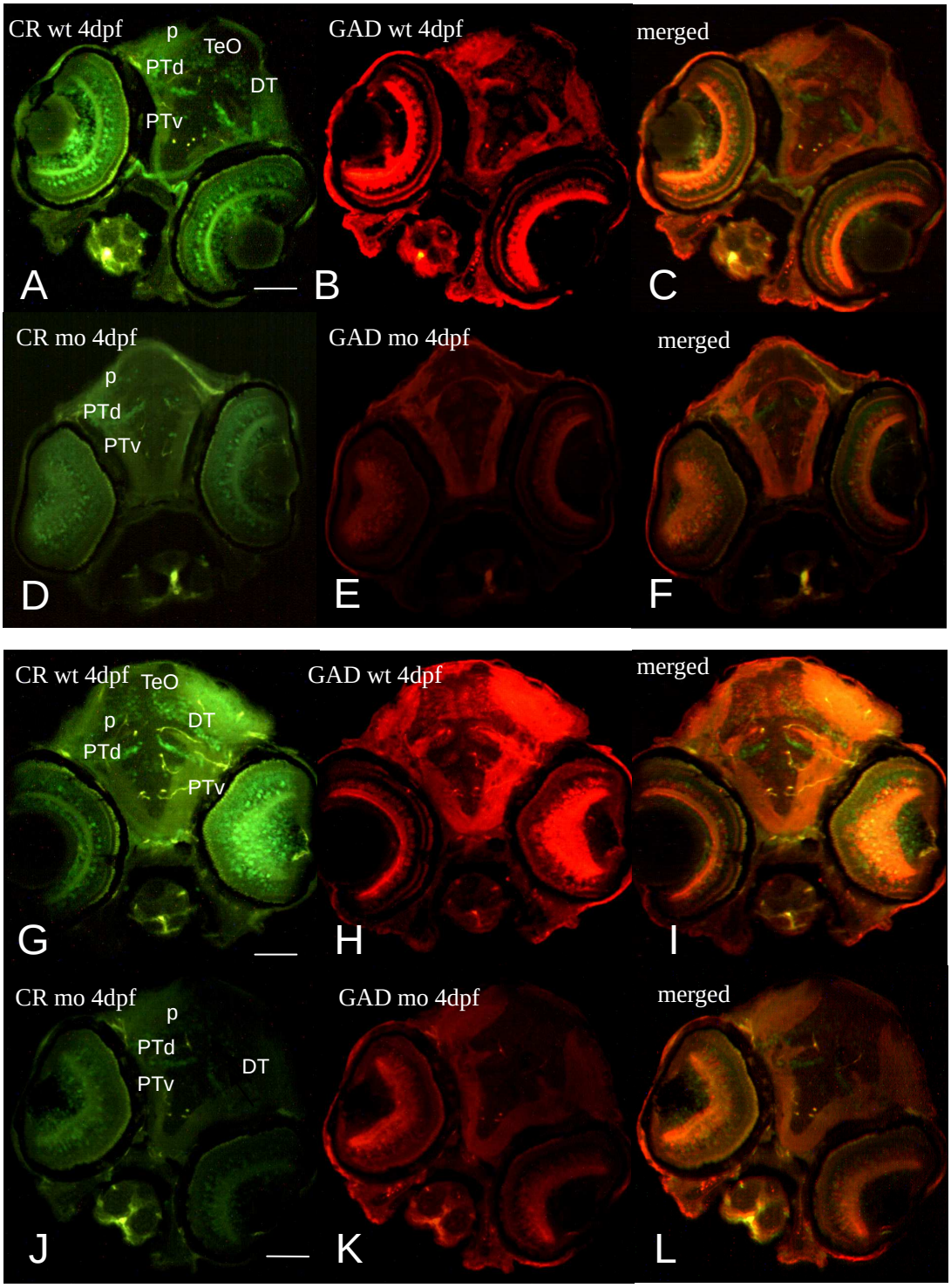
Figure4.5. Calretinin and GAD expression in 4dpf wildtype and *dlx1a/dlx2a* morphant zebrafish brain by immunohistochemistry on zebrafish brain transverse sections (10μm). A,G) anti- Calretinin (CR) immunohistochemistry on 4dpf wildtype zebrafish brain. B,H) anti-GAD65/67 immunohistochemistry 4dpf wildtype zebrafish brain. C, I) merged images of both CR and GAD expression in. 4dpf wildtype zebrafish brain D,J) anti- Calretinin immunohistochemistry on 4dpf *dlx1a/dlx2a* morphant zebrafish brain. E,K) anti-GAD65/67 immunohistochemistry on 4dpf *dlx1a/dlx2a* morphant zebrafish brain. F,L) merged images of both CR and GAD expression 4dpf *dlx1a/dlx2a* morphant zebrafish brain. Panel A-F and G-L are two pairs of larvae which showing the most representative phenotype of the experimental group they are in, basically they representing the same planes of brain and similar phenotype. Green represents CR expression, red represents GAD expression and yellow represents overlap of CR and GAD expression. P: pallium. Dorsal is to the top. Scale bar = 80μm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GAD mo 4dpf
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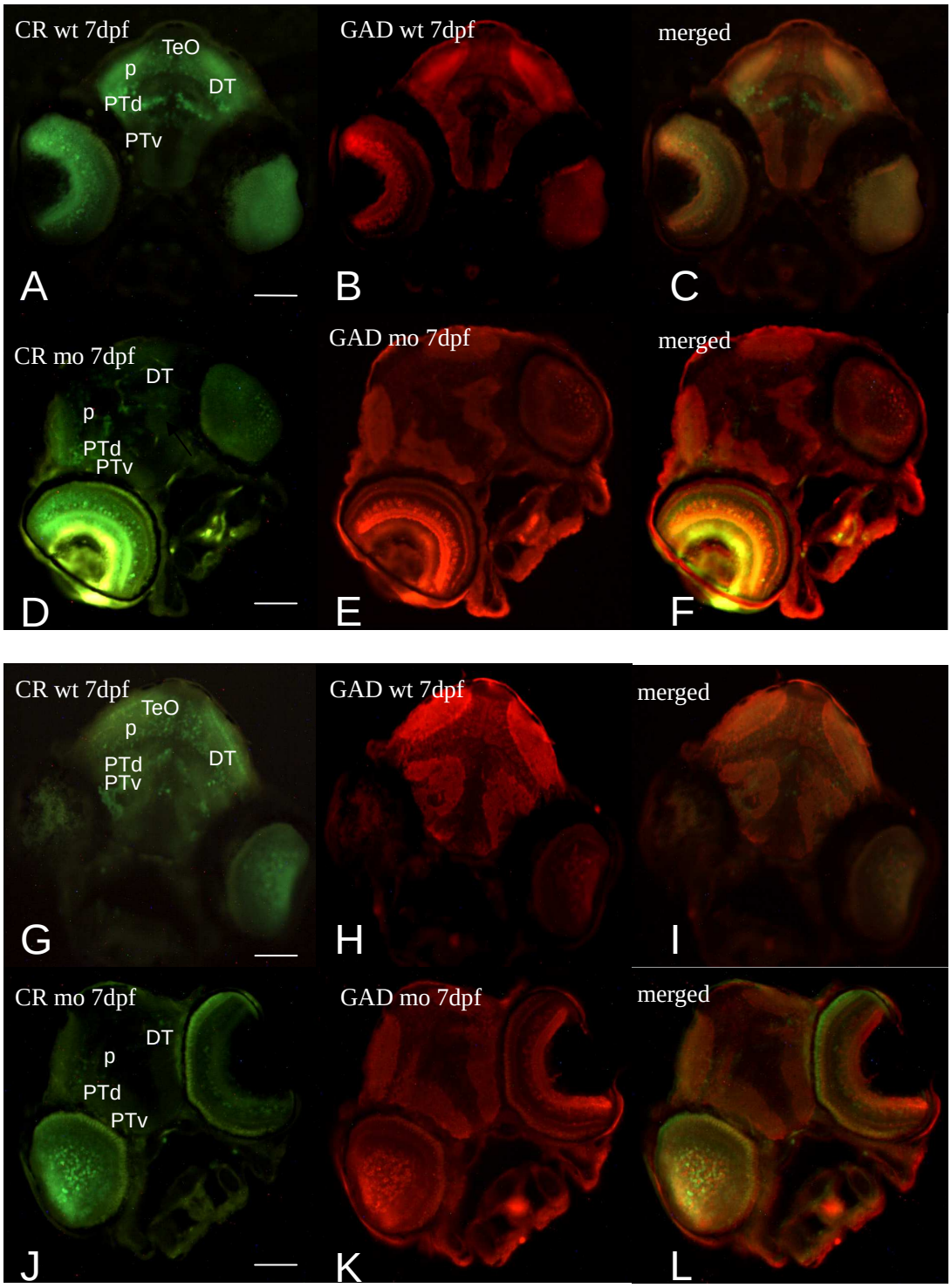
Figure 4.6. Calretinin and GAD expression in 7dpf wildtype and *dlx1a/dlx2a* morphant zebrafish brain by immunohistochemistry on zebrafish brain transverse sections (10µm). A,G) anti-Calretinin (CR) immunohistochemistry on 7dpf wildtype zebrafish brain. B,H) anti-GAD65/67 immunohistochemistry 7dpf wildtype zebrafish brain. C, I) merged images of both CR and GAD expression in. 7dpf wildtype zebrafish brain D,J) anti-Calretinin immunohistochemistry on 7dpf *dlx1a/dlx2a* morphant zebrafish brain. E,K) anti-GAD65/67 immunohistochemistry on 7dpf *dlx1a/dlx2a* morphant zebrafish brain. F,L) merged images of both CR and GAD expression 7dpf *dlx1a/dlx2a* morphant zebrafish brain. Panel A-F and G-L are two pairs of larvae which showing the most representative phenotype of the experimental group they are in, basically they representing the same planes of brain and similar phenotype. Green represents CR expression, red represents GAD expression and yellow represents overlap of CR and GAD expression. Dorsal is to the top. Scale bar= 80µm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p
PTd
PTv
J
GAD mo 7dpf
K
merged
L

Figure 4.7. Somatostatin and GAD expression in 3dpf wildtype and *dlx1a/dlx2a* morphant zebrafish brain by immunohistochemistry on zebrafish brain transverse sections (10µm) A, G) anti-somatostatin (SOM) immunohistochemistry on 3dpf wildtype zebrafish brain. B, H) anti-GAD65/67 immunohistochemistry on 3dpf wildtype zebrafish brain. C, F, I, K) merged images of both SOM and GAD expression. D, J) anti-somatostatin immunohistochemistry on 3dpf *dlx1a/dlx2a* morphant zebrafish brain. E, K) anti-GAD65/67 immunohistochemistry on 3dpf *dlx1a/dlx2a* morphant zebrafish brain. Panel A-F and G-L are two pairs of larvae which showing the most representative phenotype of the experimental group they are in, basically they representing the same planes of brain and similar phenotype. Red represents SOM expression, green represents GAD expression and yellow represents overlap of SOM and GAD expression S: subpallium. Dorsal is to the top. Scale bar= 80µm

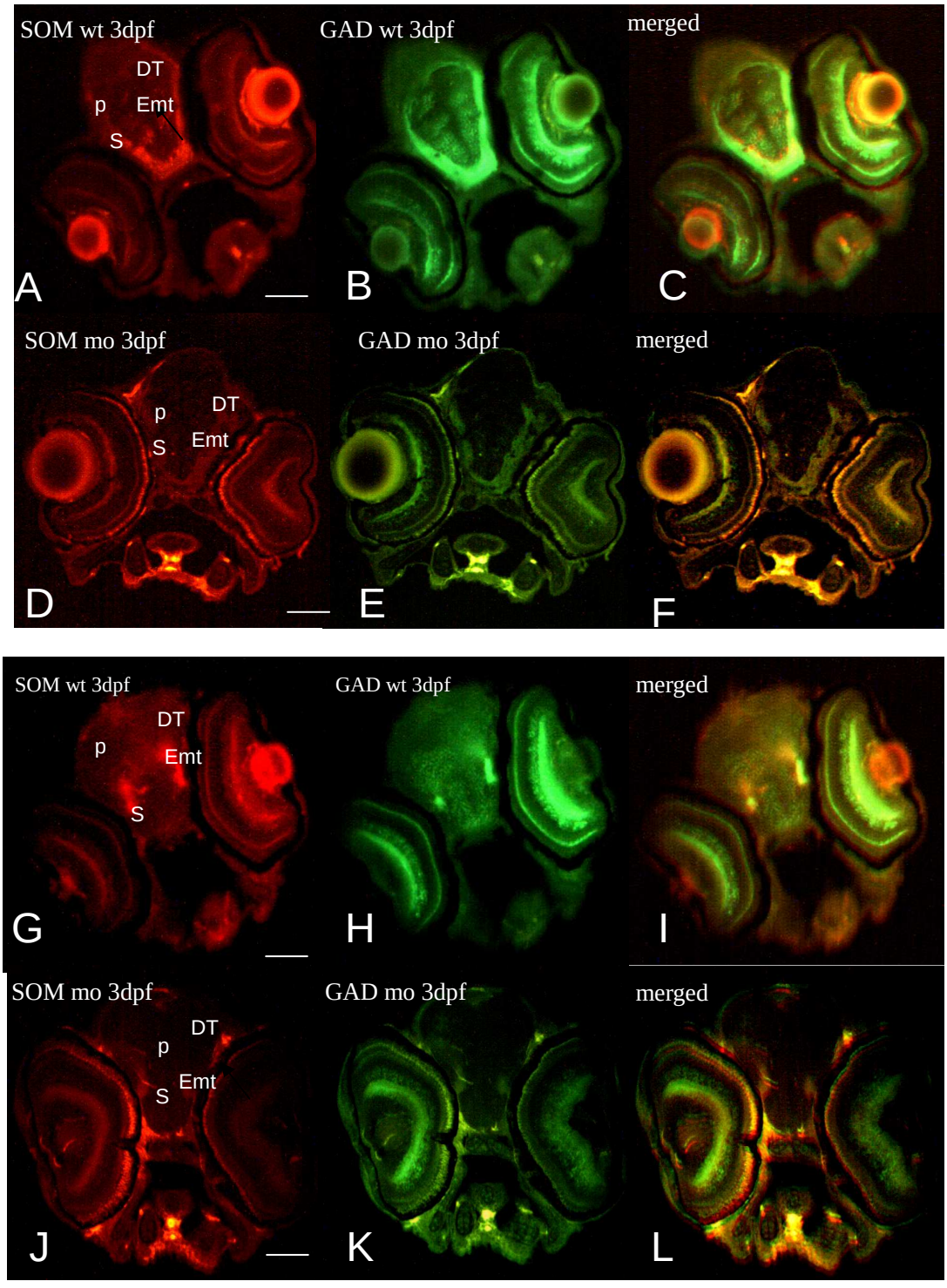
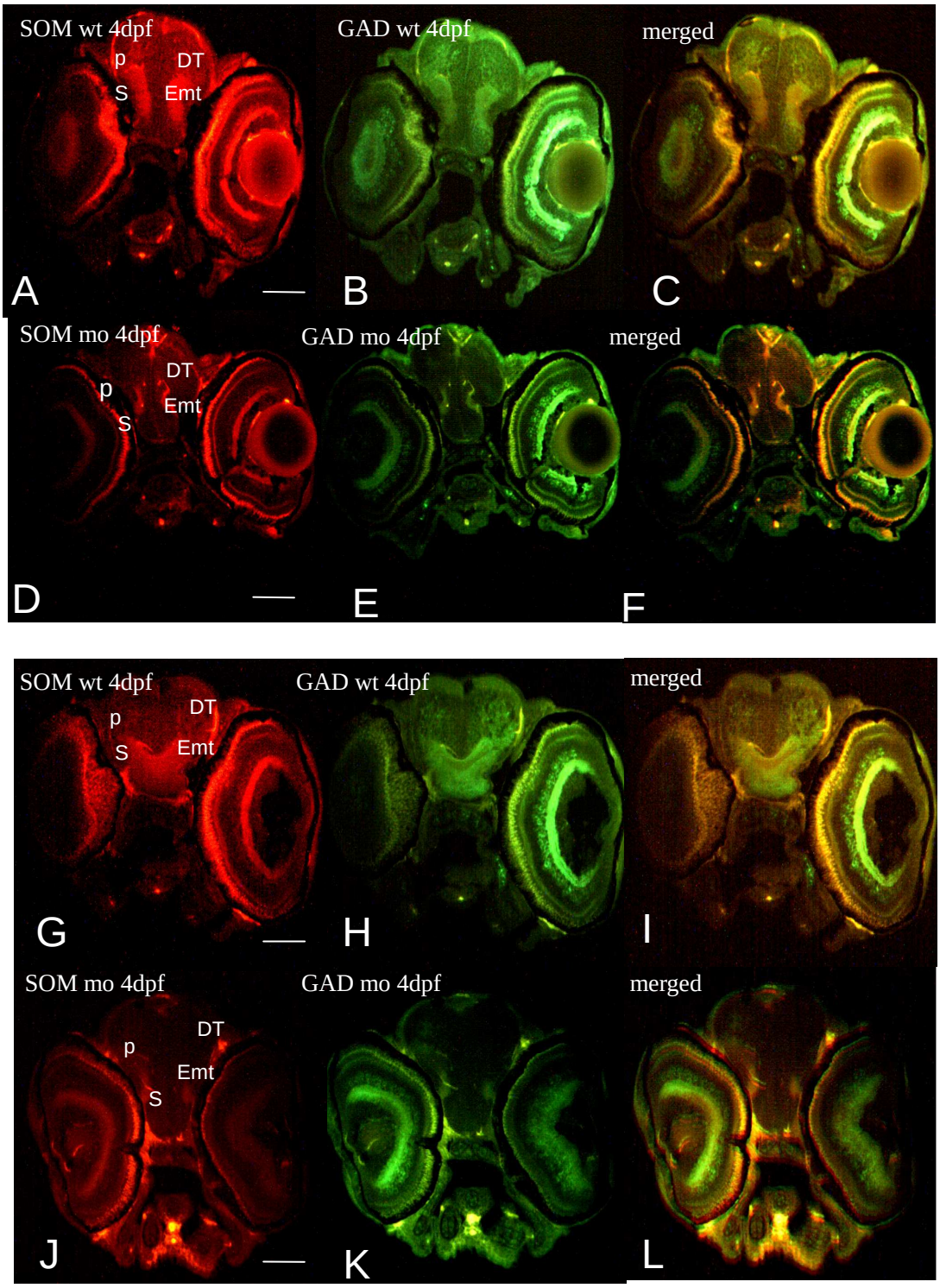

SOM wt 3dpf
DT
p
Emt
S
A
GAD wt 3dpf
B
merged
C
SOM mo 3dpf
p
DT
S
Emt
D
GAD mo 3dpf
E
merged
F
SOM wt 3dpf
DT
p
Emt
S
G
GAD wt 3dpf
H
merged
I
SOM mo 3dpf
DT
p
S
Emt
J
GAD mo 3dpf
K
merged
L

Figure 4.8. Somatostatin and GAD expression in 4dpf wildtype and *dlx1a/dlx2a* morphant zebrafish brain by immunohistochemistry on zebrafish brain transverse sections (10µm) A, G) anti-somatostatin (SOM) immunohistochemistry on 4dpf wildtype zebrafish brain. B,H) anti-GAD65/67 immunohistochemistry on 4dpf wildtype zebrafish brain. C, F, I, L) merged images of both SOM and GAD expression. D, J) anti-somatostatin immunohistochemistry on 4dpf *dlx1a/dlx2a* morphant zebrafish brain. E, K) anti-GAD65/67 immunohistochemistry on 4dpf *dlx1a/dlx2a* morphant zebrafish brain. Panel A-F and G-K are two pairs of larvae which showing the most representative phenotype of the experimental group they are in, basically they representing the same planes of brain and similar phenotype. Red represents SOM expression, green represents GAD expression and yellow represents overlap of SOM and GAD expression. Dorsal is to the top. Scale bar = 80µm


SOM wt 4dpf
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A
GAD wt 4dpf
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C
SOM mo 4dpf
DT
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Emt
S
D
GAD mo 4dpf
E
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F
SOM wt 4dpf
p
DT
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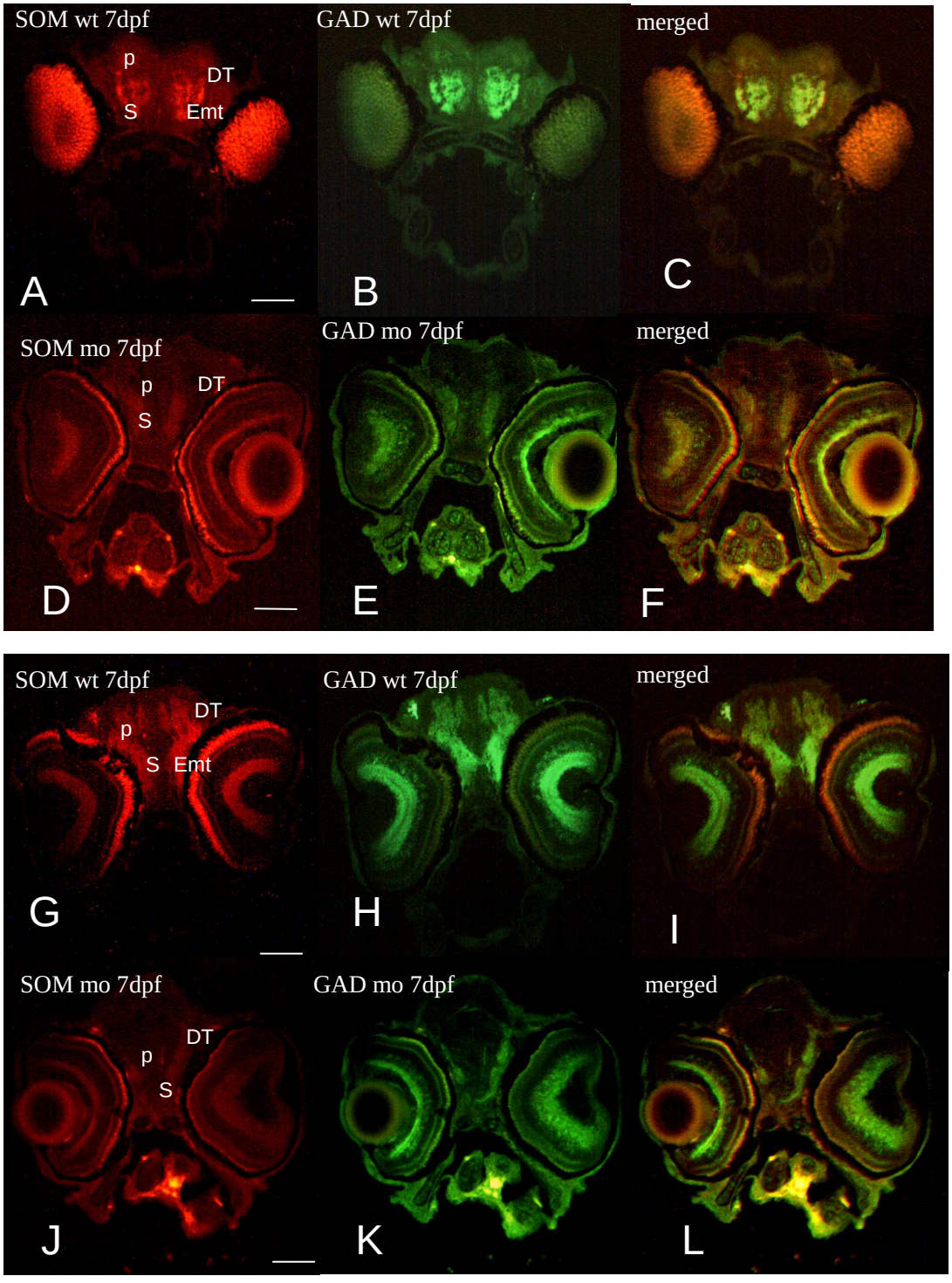
Emt
G
GAD wt 4dpf
H
merged
I
SOM mo 4dpf
DT
p
Emt
S
J
GAD mo 4dpf
K
merged
L

Figure 4.9. Somatostatin and GAD expression in 4dpf wildtype and *dlx1a/dlx2a* morphant zebrafish brain by immunohistochemistry on zebrafish brain transverse sections (10µm) A,G) anti-somatostatin (SOM) immunohistochemistry on 4dpf wildtype zebrafish brain. B,H) anti-GAD65/67 immunohistochemistry on 4dpf wildtype zebrafish brain. C, F, I, L) merged images of both SOM and GAD expression. D, J) anti-somatostatin immunohistochemistry on 4dpf *dlx1a/dlx2a* morphant zebrafish brain. E, K) anti-GAD65/67 immunohistochemistry on 4dpf *dlx1a/dlx2a* morphant zebrafish brain. Panel A-F and G-L are two pairs of larvae which showing the most representative phenotype of the experimental group they are in, basically they representing the same planes of brain and similar phenotype. Red represents SOM expression, green represents GAD expression and yellow represents overlap SOM and GAD expression. Dorsal is to the top. Scale bar= 80µm


SOM wt 7dpf
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GAD wt 7dpf
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SOM mo 7dpf
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GAD mo 7dpf
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GAD wt 7dpf
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SOM mo 7dpf
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GAD mo 7dpf
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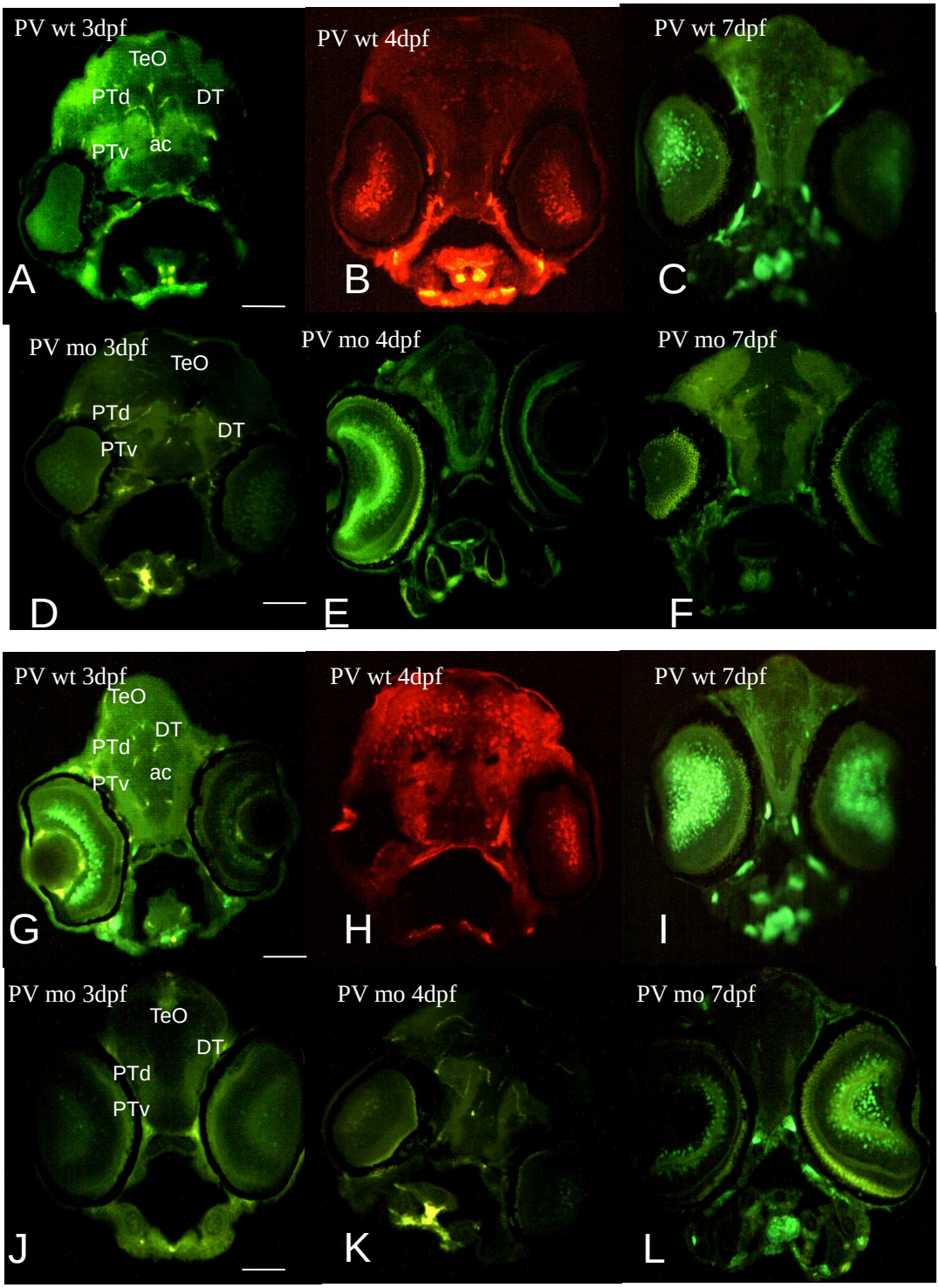
Figure 4.10. Parvalbumin expression in 3dpf, 4dpf and 7dpf wildtype and *dlx1a/dlx2a* morphant zebrafish brain via immunohistochemistry on zebrafish brain transverse sections (10μm).

A,G) anti-parvalbumin(PV) immunohistochemistry 3dpf wildtype zebrafish brain. B,H) anti-parvalbumin immunohistochemistry of 4dpf wildtype zebrafish brain. C,I) anti-parvalbumin immunohistochemistry on sections (10μm) of 7dpf wildtype zebrafish brain. D,J) anti-parvalbumin immunohistochemistry on 3dpf *dlx1a/dlx2a* morphant zebrafish brain. E,K) anti-parvalbumin immunohistochemistry on 4dpf *dlx1a/dlx2a* morphant zebrafish brain. F,L) anti-parvalbumin immunohistochemistry on 7dpf *dlx1a/dlx2a* morphant zebrafish brain. PV expression is shown in green in A, C, D, F, G-L and in red in B, E. Different colors here due to different second antibodies were used. Dorsal is to the top. Scale bar= 80μm


PV wt 3dpf
TeO
PTd
DT
PTv
ac
A
PV wt 4dpf
B
PV wt 7dpf
C
PV mo 3dpf
TeO
PTd
DT
PTv
D
PV mo 4dpf
E
PV mo 7dpf
F
PV wt 3dpf
TeO
DT
PTd
ac
PTv
G
PV wt 4dpf
H
PV wt 7dpf
I
PV mo 3dpf
TeO
DT
PTd
PTv
J
PV mo 4dpf
K
PV mo 7dpf
L

5. Discussion

5.1 *Dlx* genes are important for Calbindin positive GABAergic neuron development.

In this report, we showed that in *dlx1a/dlx2a* morphant zebrafish brain at 3dpf, 4dpf and 7dpf, the number of Calbindin- and GAD-positive cells is remarkably reduced compared with wildtype zebrafish brain at the same developmental stages. By inhibiting translation of a gene, morpholinos are used to knockdown the gene function and to learn about its normal function. The knockdown of *dlx1a/dlx2a* by morpholino could be the cause of reduction of Calbindin- and GAD-positive cells number. This is consistent with what has been shown in the mouse.

A previous study has shown that the number of neocortical GABA- and calbindin-expressing cells is greatly reduced in the *Dlx1/2* double mutant mice (Anderson et al, 1997). The expression of the synthesizing enzyme for GABA was also reduced in the neocortex of these mutants. The reduction of the expression of the interneuron markers was observed throughout the neocortex and the hippocampal CA fields (Anderson et al., 1997). It is also known that in the mouse, the overexpression of Necdin, a member of the MAGE (melanoma antigen) protein family, significantly increased the populations of cells expressing Calbindin D-28k and GAD in cultured forebrain slices. Dlx5N, a truncated form of Dlx5 that competes with Dlx2 to bind MAGE-D1, suppresses GABAergic neuron differentiation by dissociating the endogenous necdin-MAGE-D1-Dlx2 complex. Furthermore, *in vitro* experiments have indicated that necdin deficiency diminishes Dlx2-dependent GABAergic

differentiation but not neuronal differentiation in general (Kuwajima et al, 2006**)**. It could be possible that inhibition of *dlx1a* and *dlx2a* function in *dlx1a/dlx2a* morphant zebrafish impaired the necdin-MAGE-D1-Dlx2 complex which led to the suppression of GABAergic neuron differentiation and reduction of calbindin- and GAD-positive cells.

Therefore, our results suggest that *dlx1a* and *dlx2a* are involved in Calbindin positive GABAergic neuron development in zebrafish.

5.2 The regulatory function of *Dlx* genes in Calretinin-positive GABAergic neuron development is not obvious.

It is shown in this report that in the forebrain of the *dlx1a/dlx2a* morphant zebrafish at 3dpf, 4dpf and 7dpf, the expression of Calretinin and GAD was not significantly altered when compared with wildtype zebrafish forebrain at the same developmental stages.

This result contradicts with what have been shown in mouse. Xu et al showed that Calretinin interneurons are absent in *Dlx1-/- Dlx2-/-* mice, in which both MGE (medial ganglionic eminences) and CGE (caudal ganglionic eminence) are abnormal (Xu et al, 2004). The difference between the *dlx1a/dlx2a* knockdown zebrafish and *Dlx1-/- Dlx2-/-* knockout mice may be due to different developmental mechanism between the two animal models. Yet there are also other possible explanations that can not be ruled out.

In $Dlx1^{-/-}$ mice there was no significant difference in expression of Calretinin and

GAD67 at early postnatal stages (P12) compared with WT. In contrast, the number of Calretinin+ interneurons was significantly reduced in the mutant mice older than one month (Cobos et al., 2005). This suggests that expression of Calretinin is age-dependent. In this report, we only examined zebrafish at 3dpf, 4dpf and 7dpf and it is reasonable to hypothesize that these developmental stages are too early for the development of Calretinin+ cells to be affected by loss-of-function of *dlx1a* and *dlx2a*.

Furthermore, although the majority of calretinin+ interneurons express *Dlx1*, these cell subtypes are only partially lost in *Dlx1-/-* mice, suggesting the existence of molecular properties that distinguish which *Dlx1*-positive interneurons survive in the mutants (Cobos et al, 2005). It is known that there are at least two distinguishable subtypes of CR-positive cells, termed bipolar (BCR) and multipolar (MCR) CR cells. These cell types differ in most of characteristics, including firing pattern, biochemical markers, neurite arborization, and synaptic plasticity (Caputi et al, 2009). Therefore it is possible that in our experiments, knocking down of *dlx1a* and *dlx2a* in zebrafish affects only specific subtype(s) of CR+ cells but not the others, which results in minor reduction of CR expression in contrast to an obvious change in CR expression in *Dlx1/Dlx2* knockout mice.

More experiments are required to verify and further interpret the role of *dlx1a* and *dlx2a* in regulation of Calretinin-positive GABAergic neuron development in zebrafish. Until further work is done, results from this study do not support a role of *dlx1a* and *dlx2a* in regulating Calretinin-positive GABAergic neuron development

in zebrafish.

5.3 *Dlx* genes are important for somatostatin positive GABAergic neuron development.

In this study, we showed that at 3dpf, 4dpf and 7dpf the expression of somatostatin- and GAD in the forebrain of the *dlx1a/dlx2a* morphants is remarkably reduced when compared with wildtype at the same developmental stages. This is similar to what has been observed in *Dlx1* knockout mice (Cobos et al., 2005).

Previous studies have implicated *Lhx6*, the LIM-homeodomain (LIM-hd) factor, as a mediator for the development of the interneurons expressing somatostatin. *Lhx6* exerts this function by promoting the expression of CXCR4 and CXCR7 that regulate interneuron migration and the expression of transcription factors Arx to control interneuron development (Liodis et al, 2007). In the *Dlx1-/-* and *Dlx2-/-* mice, interneuron migration to the cortex is greatly diminished because of reduced *Arx* and *Lhx6* expression caused by molecular changes in the MGE (Cobos et al., 2005; Petryniak et al., 2007). It is possible that reduction of SOM-positive cells in *dlx1a/dlx2a* morphant zebrafish is the result of reduced *Lhx6* expression.

In addition, a previous study demonstrated that in mice, CXCR4, the receptor for the CXC motif chemokine stromal cell-derived factor-1 (SDF-1), contributes to the development of the morphology and connectivity of somatostatin-expressing GABAergic neurons (Stumm, 2007). CXCR4 is reduced in *Dlx1/2* mutant mice and leads to disruption of interneuron migration (Long et al., 2009a). This suggests that

knockdown of *dlx1a/dlx2a* in zebrafish might reduce CXCR4 and result in disrupted somatostatin-expressing GABAergic neuron development.

In conclusion, the results of this study suggest that in the zebrafish brain, *dlx1a* and *dlx2a*, are important for somatostatin-positive GABAergic neuron development.

5.4 *Dlx* genes are important for Parvalbumin-positive GABAergic neuron development.

Results above have demonstrated that at 3dpf, 4dpf and 7dpf, expression of Parvalbumin is remarkably reduced in the brain of *dlx1a/dlx2a* morphant zebrafish compared with wildtype at the same developmental stages. This suggests that *dlx1a* and *dlx2a* genes may be required for Parvalbumin-positive GABAergic neuron development.

Previous studies have suggested that *Lhx6* is involved in the development of the interneurons expressing parvalbumin in mice (Liodis et al, 2007). Insufficient Lhx6 in *Dlx1*/*Dlx2*$^{-/-}$ mice may disrupt the development of PV+ neurons. This might also explain the reduction of PV-positive neurons in *dlx1a/dlx2a* morphant zebrafish.

However, in *Dlx1*$^{-/-}$ mice the number of parvalbumin+ cells did not change significantly while somatostatin- and calretinin-expressing neurons were lost (Cobos et al., 2005). This may be due to functional redundancy between *Dlx1* and *Dlx2* in regulating PV+ cells development or due to different developmental mechanism of PV+ neurons in zebrafish and mice.

6. Conclusion

This study furthers the knowledge of *dlx* gene function in zebrafish GABAergic interneuron development. By injecting morpholinos targeted against the *dlx1a* and *dlx2a* mRNA, we examined the functions of these genes in the development of the zebrafish GABAergic interneuron. Using immunohistochemistry on wildtype and morphant zebrafish brain transverse sections with various GABAergic interneuron markers, we have shown that expression of Calbindin, Somatostatin and Parvalbumin are remarkably reduced in *dlx1a/dlx2a* morphant zebrafish brain while expression of Calretinin is slightly reduced compared to wildtype zebrafish.

. Previous studies have demonstrated that *Dlx1* and *Dlx2* are essential for GABAergic neuron formation throughout the forebrain in mice. Yet the role of *dlx* genes in GABAergic interneuron development in zebrafish is unknown. Our study suggests that in zebrafish, *dlx1a* and *dlx2a* genes are important for the development of certain subtypes of GABAergic interneuron (Calbindin-, Somatostatin- and Parvalbumin-positive neurons) and may have minor influence on Calretinin-positive neuron development. This also suggests that different regulatory mechanisms are involved in the development of the different subtypes of GABAergic interneurons.

7. References

Abe H, Watanabe M, Yamakuni T, Kuwano R, Tankahashi Y, Kondo H.(1992). Localization of gene expression of calbindin in the brain of adult rats. *Brain Research* 138: 211-215.

Acampora D, Merlo GR, Paleari L, Zerega B, Postiglione MP, Mantero S, Bober E, Barbieri O, Simeone A, Levi G. (1999). Craniofacial, vestibular and bone defects in mice lacking the *Distal-less*-related gene *Dlx5*. *Development.* 126:3795–3809.

Acosta MT, Pearl PL. (2003).The neurobiology of autism: new pieces of the puzzle. *Curr Neurol Neurosci Rep*. 3:149-56

Akimenko MA, Ekker M, Wegner J, Lin W, Westerfield M. (1994). Combinatorial expression of three zebrafish genes related to *Distal-Less*: Part of a Homeobox gene code for the head. *J. Neurosci.* 14: 3475-3486.

Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH. (1998). Zebrafish Hox clusters and vertebrate genome evolution. *Science* 282: 1711-1714.

Anderson SA, Eisenstat DD, Shi L, Rubenstein JLR. (1997). Interneuron migration from basal forebrain to neocortex: Dependence on *Dlx* genes. *Science* 278:17

Anderson SA, Qiu M, Bulfone A, Eisenstat DD, Meneses J, Pedersen R, Rubenstein JLR. (1997a). Mutations of the homeobox genes *Dlx-1* and *Dlx-2* disrupt the striatal subventricular zone and differentiation of late-born striatal neurons. *Neuron* 19: 27-37.

Anderson SA, Eisenstat DD, Shi L, Rubenstein JLR. (1997b). Interneuron migration from basal forebrain to neocortex: Dependence on *Dlx* genes. *Science* 278: 474-476.

Anderson SA, Marin O, Horn C, Jennings K, Rubenstein JL. (2001). Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development* 128: 353-363.

Arai R, Jacob Owitz DM, Deura S. (1994). Heterogeneity in calbindin-D28k expression in oxytocin-containing magnocellular neurons of the rat hypothalamus. *Histochemistry* 101: 9-12.

Argenton F, Zecchin E. (1999). Early appearance of pancreatic hormone-expressing cells in the zebrafish embryo. *Mech Dev* 87: 217-21

Arif SH. (2009). A Ca2+-binding protein with numerous roles and uses: parvalbumin in molecular biology and physiology. *BioEssays* 31:410–421

Baylor SM, Hollingwort S. (2003). Sarcoplasmic reticulum calcium release compared in slowtwitch and fast-twitch fibres of mouse muscle. *J Physiol* 551:125–138

Bendall AJ, Hu G, Levi G, Abate-Shen C. (2003) Dlx5 regulates chondrocyte differentiation at multiple stages. *Int. J. Dev. Biol.* 47: 335-344.

Benson MD, Bargeon JL, Xiao G, Thomas PE, Kim A, Cui Y, Franceschi RT. (2000). Identification of a homeodomain binding element in the bone sialoprotein promoter that is required for its osteoblast-selective expression. *J. Biol. Chem.* 275: 13907-13917.

Beverdam A, Merlo GR, Paleari L, Mantero S, Genova S, Barbieri O, Janvier P, Levi G. (2002). Jaw transformation with gain of symmetry after *Dlx5/Dlx6* inactivation: Mirror of the past? *Genesis* 34: 221-227.

Brazeau P, Vale W, Burgus R, Ling N, Butcher M, Rivier J, Guillemin R.(1973). Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science*. 179: 77–79.

Camp AJ, Wijesinghe R. (2009). Calretinin: Modulator of neuronal excitability. *The International Journal of Biochemistry & Cell Biology.* 41: 2118–2121

Caputi A, Rozov A, Blatow M, Monyer H. (2009). Two calretinin-positive GABAergic cell types in layer 2/3 of the mouse neocortex provide different forms of inhibition. *Cereb Cortex.*19:1345-59.

Casarosa S, Fode C, Guillemot F. (1999). *Mash1* regulates neurogenesis in the ventral telencephalon. *Development* 126: 525-534.

Castro A, Becerra M, Manso MJ, Anado'n R. (2006). Calretinin immunoreactivity in the brain of the zebrafish, *Danio rerio*: Distribution and comparison with some neuropeptides and neurotransmitter-synthesizing enzymes. II. midbrain, hindbrain, and rostral spinal cord. *The Journal of Comparative Neurology* 494:792–814

Celio MR. (1990). Calbindin D28k and parvalbumin in the rat nervous system. *Neuroscience*. 35: 375-475.

Chen X, Li X, Wang W, Lufkin T. (1996) *Dlx5* and *Dlx6*: an evolutionary conserved pair of murine homeobox genes expressed in the embryonic skeleton. *Annals New York Acad Sci.* 785: 38–47

Chung IH, Han J, Iwata J, Chai Y.(2010). Msx1 and Dlx5 function synergistically to regulate frontal bone development. *genesis* 48: 645–655.

Cobos I, Calcagnotto ME, Vilaythong AJ, Thwin MT, Noebels JL, Baraban SC, Rubenstein JL. (2005). Mice lacking *Dlx1* show subtype-specific loss of interneurons, reduced inhibition and epilepsy. *Nat. Neurosci.* 8: 1059-1068.

Cobos I, Borello U, Rubenstein JL. (2007). Dlx transcription factors promote migration through repression of axon and dendrite growth. *Neuron* 54: 873-888.

Cohen B, Cohen SM, O'hara , E MeGinnis W. (1993). Distal-less is a downstream gene of Deformed required for ventral maxillary indentity. *Development.*117:847-56

Cohen SM, Jurgens G. (1989). Proximal-Distal pattern formation in Drosophila: cell autonomous requirement for *Distal-less* gene activity in limb development. *EMBO J.* 8: 2045-2055.

Colasante G, Collombat P, Raimondi V, Bonanomi D, Ferrai C, Maira M, Yoshikawa K, Mansouri A, Valtorta F, Rubenstein JL, Broccoli V. (2008). Arx is a direct target of Dlx2 and thereby contributes to the tangential migration of GABAergic interneurons. *J. Neurosci.* 28: 10674-10686.

Condie BG, Bain G, Gottlied DI, Capecchi MR.(1997). Left palate in mice with a targeted mutation in the gamma-aminobutyric acid-producing enzyme glutamic acid decarboxylase 67. *Proc Natl Acad Sci.* 94:11451-5.

Costa M, Patel Y, Furness JB, Arimura A. (1977). Evidence that some intrinsic neurons of the intestine contain somatostatin. *Neurosci Lett*. 6:215–222.

Crackower MA, Scherer SW, Rommens JM, Hui CC, Poorkaj P, Soder S, Cobben JM, Hudgins L, Evans JP, Tsui L. (1996). Characterization of the split hand/split foot malformation locus SHFM1 at 7q21.3-q22.1 and analysis of a candidate gene for its expression during limb development. *Hum Mol Genet*. 5:571-9.

Danglot L, Triller A, Marty S. (2006). The development of hippocampal interneurons in rodents. *Hippocampus*.16:1032-60

Deacon TW, Pakzaban P, Isacson O (1994) The lateral ganglionic eminence is the origin of cells committed to striatal phenotypes: neural transplantation and developmental evidence. *Brain Res* 668: 211–219

Depew MJ, Simpson CA, Morasso M, Rubenstein JLR. (2005). Reassessing the Dlx Code: The genetic regulation of branchial arch skeletal pattern and development. *J. Anat.* 207: 501-561.

Depew MJ, Lufkin T, Rubenstein JLR. (2002). Specification of jaw subdivisions by *Dlx* genes. *Science* 298: 381-385.

Depew MJ, Liu JK, Long JE, Presley R, Meneses JJ, Pedersen RA, Rubenstein JLR. (1999). *Dlx5* regulates regional development of the branchial arches and sensory capsules. *Development.* 126: 3831-3846.

Devos N, Deflorian G. (2002). Eifferential expression of two somatostatin genes during zebrafish embryonic development. *Mech Dev* 115: 133-7

Dodig M, Kronenberg MS, Bedalov A, Kream BE, Gronowicz G, Clark SH, Mack K, Liu YH, Maxon R, Pan ZZ, Upholt WB, Rowe DW, Lichtler AC. (1996). Identification of a TAAT-containing motif required for high level expression of the COL1A1 promoter in differentiated osteoblasts of transgenic mice. *J. Biol. Chem.* 271: 16422-16429.

Eisenstat DD, Liu J, Mione M, Zhong W, Yu G, Anderson SA, Ghattas I, Puelles L, Rubenstein JLR. (1999). DLX-1, DLX-2 and DLX-5 expression define distinct stages of basal forebrain differentiation. *J. Comp. Neurol.* 414: 217-237.

Ekker M, Akimenko M, Bremiller R, Westerfield M. (1992). Regional expression of three homeobox transcripts in the inner ear of zebrafish embryos. *Neuron* 9: 27-35.

Elina Y, Hitoshi K. (2002). Stage-specific control of neuronal migration by somatostatin. *Nature* 415: 77-81

Ellies DL, Stock DW, Hatch G, Giroux G, Weiss KM, Ekker M. (1997). Relationship between the genomic organization and the overlapping embryonic expression patterns of the zebrafish *Dlx* genes. *Genomics* 45: 580-590.

Ferrari D, Kosher RA. (2002). Dlx5 is a positive regulator of chondrocyte differentiation during endochondral ossification. *Dev Biol.*252:257–270

Fode C, Ma Q, Casarosa S, Ang SL, Anderson DJ, Guillemot F. (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. *Genes Dev.* 14: 67-80.

Frantz GD, Tobin AJ. (1994). Cellular distribution of calbindin D28K mRNAs in the adult mouse brain. *Journal of Neuroscience Research.* 37: 287-302.

Freund TF, Buzsaki G. (1996). Interneurons of the hippocampus. *Hippocampus* 6: 347– 470.

Freund TF, Gulyás AI. (1997). Inhibitory control of GABAergic interneurons in the hippocampus. *Canadian Journal of Physiology and Pharmacology*. 75: 479-487,

Ghanem N, Jarinova O, Amores A, Hatch G, Park BK, Rubenstein JLR, Ekker M. (2003). Regulatory roles of conserved intergenic domains in vertebrate *Dlx* bigene clusters. *Genome Res.* 13: 533-543.

Ghanem N, Yu M, Long J, Hatch G, Rubenstein JL, Ekker M. (2007). Distinct cis-regulatory elements from the Dlx1/Dlx2 locus mark different progenitor cell populations in the ganglionic eminences and different subtypes of adult cortical interneurons. *J. Neurosci.* 27: 5012-5022.

Ghanem N, Yu M, Poitras L, Rubenstein JL, Ekker M. (2008). Characterization of a distinct subpopulation of striatal projection neurons expressing the *Dlx* genes in the basal ganglia through the activity of the I56ii enhancer. *Dev. Biol.* 322: 415-424.

Gonchar Y. (2008). Multiple distinct subtypes of GABAergic neurons in mouse visualcortex identified by triple immunostaining. *Front. Neuroanat*. 1: 11.

Gonchar Y, Burkhalter A. (1997). Three distinct families of GABAergic neurons in rat visual cortex. *Cereb Cortex* 7:347–358.

Gordon CT, Brinas IM, Rodda FA, Farlie PG. (2010).Role of *Dlx* genes in craniofacial morphogenesis: *Dlx2* influences skeletal patterning by inducing ectomesenchymal aggregation *in ovo*. *Evol Dev*. 12:459-73

Gordon CT, Rodda FA, Farlie PG. (2009).The RCAS retroviral expression system in the study of skeletal development. *Dev Dyn*. 238:797-811.

Haldeman RJ, Cooper LF, Hart TC, Phillips C, Boyd C, Lester GE, Wright JT. (2004). Increased bone density associated with *Dlx3* mutation in the tricho-dento-osseous syndrome. *Bone* 35: 988-997.

Hsu S-hC, Noamani B, Abernethy DE, Zhu H, Levi G.(2006). Dlx5- and Dlx6-mediated chondrogenesis: Differential domain requirements for a conserved function. *Mech Dev.* 123: 819–830.

Iler N, Rowitch DH, Echelard Y, McMahon AP, Abate-Shen C. (1995). A single homeodomain binding site restricts spatial expression of Wnt-1 in the developing brain. *Mech Dev*. 53: 87-96

Jinno S, Kosaka T. (2006). Cellular architecture of the mouse hippocampus: a quantitative aspect of chemically defined GABAergic neurons with stereology. *Neurosci. Res*. 56: 229-245

Kawasaki H, Nakayama S, Kretsinger RH. (1998). Classification and evolution of EF-hand proteins. *Biometals*.11: 277–295.

Keilhoff G, Becker A, Grecksch G, Wolf G, Bernstein HG. (2004). Repeated application of ketamine to rats induces changes in the hippocampal expression of Parvalbumin neuronal nitric oxide synthase nad cFos similar to those found in human schizophrenia. *Neuroscience* 126: 591–598

Kojetin DJ, Venters RA, Kordys DR, Thompson RJ, Kumar R, Cavanagh J (2006). Structure, binding interface and hydrophobic transitions of Ca2+-loaded calbindin-D(28K). *Nat. Struct. Mol. Biol.*13: 641–7

Korotkova TM, Sergeeva OA, Eriksson KS, Haas HL, Brown RE. (2003) Excitation of ventral tegmental area dopaminergic and nondopaminergic neurons by orexins/hypocretins. *J. Neurosci.* 23: 7–11

Kubota Y, Kawaguchi Y. (1994). Three classes of GABAergic interneurons in neocortex and neostriatum. *Jpn J Physiol*. 44: 145–148

Kuwajima T, Nishimura I, Yoshikawa K (2006). Necdin promotes GABAergic neuron differentiation in cooperation with Dlx homeodomain proteins. *J Neurosci* 26: 5383–5392.

Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG. (1999). The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. *J. Neurosci.* 19: 7881-7888.

Levi G, Puche AC, Manter S, Barbieri O, Trombino S, Paleari L, Egeo A, Merlo GR. (2003). The Dlx5 homeodomain gene is essential for olfactory development and connectivity in the mouse. *Mol Cell Neurosci* 22: 530–543.

Lewis DA, Konopaske GT, Sweet RA, Wu Q, Sampson A. (2006). Regional specificity of chandelier neuron axon terminal alterations in schizophrenia. *Neuroscience* 138:189-96

Lewis DA, Hashimoto T, Morris HM.(2008).Cell and receptor type-specific alterations in markers of GABA neurotransmission in the prefrontal cortex of subjects with schizophrenia. *Neurotox Res*. 14: 237-48

Lichtenstein J, Warson R, Jorgenson R, Dorst JP, Mckusick VA. (1972). The tricho-dento-osseous (TDO) syndrome. *Am J Hum Genet*. 24: 569-82

Liodis P, Denaxa M, Grigoriou M, Akufo-Addo C, Yanagawa Y, Pachnis V. (2007). Lhx6 activity is required for the normal migration and specification of cortical interneuron subtypes. *J Neurosci* 27: 3078–3089.

Liu JK, Ghattas I, Liu S, Chen S, Rubenstein JLR. (1997). *Dlx* genes encode DNA-Binding proteins that are expressed in an overlapping and sequential pattern during basal ganglia differentiation. *Dev. Dyn*. 210: 498-512.

Long JE, Garel S, Depew MJ, Tobet S, Rubenstein JL. (2003). DLX5 regulates development of peripheral and central components of the olfactory system. *J Neurosci* 23: 568–578

Long JE, Garel S, Alvarez-Dolado M, Yoshikawa K, Osumi N, Alvarez-Buylla A, Rubenstein JL. (2007). Dlx-dependent and -independent regulation of olfactory bulb interneuron differentiation. *J. Neurosci.* 27: 3230-3243.

Long JE, Cobos I, Potter GB, Rubenstein JL. (2009a). Dlx1&2 and Mash1 transcription factors control MGE and CGE patterning and differentiation through parallel and overlapping pathways. *Cereb Cortex.* 19: 96-106.

Long JE, Swan C, Liang WS, Cobos I, Potter GB, Rubenstein JL. (2009b). Dlx1&2 and Mash1 transcription factors control striatal patterning and differentiation through parallel and overlapping pathways. *J. Comp. Neurol.* 512: 556-572.

Marin O, Anderson SA, Rubenstein JL. (2000). Origin and molecular specification of striatal interneurons. *J. Neurosci.* 20: 6063-6076.

Marin O, Rubenstein JL. (2001). A long, remarkable journey: Tangential migration in the telencephalon. *Nat. Rev. Neurosci.* 2**:** 780-790

Marin O, Rubenstin JL. (2003). Cell migration in the forebrain. *Annu Rev Neurosci* 26: 441-83

McConnell JA. (1981). Identification of early neurons in the brainstem and spinal cord. II: An autoradiographic study in the mouse. *J. comp. Neurol.* 200: 273-288

Mesulam MM, Geula C, Bothwell MA, Hersh LB. (1989). Human reticular formation: cholinergic neurons of the pedunculopontine and laterodorsal tegmental nuclei and some cytochemical comparisons to forebrain cholinergic neurons. *The Journal of Comparative Neurology.* 283: 611–633

Mesulam MM, Mufson EJ, Wainer BH, Levey AI. (1983). Central cholinergic pahtways in the rat: an overview based on an alternative nomenclature (Ch1-Ch6) *Neuroscience* 10: 1185-1201.

Miettinen R, Gulyas AI, Baimeridge KG, Jacobowitz DM, Freund TF. (1992). Calretinin is present in non-pyramidal cells of the rat hippocampus- II. co-existence with other Calcium binding proteins and GABA. *Neuroscience.*48: 29-43

Miyoshi G, Butt SJ, Takebayashi H, Fishell G. (2007). Physiologically distinct temporal cohorts of cortical interneurons arise from telencephalic Olig2-expressing precursors. *J. Neurosci.* 27: 7786–7798

Mohler H, Crestani F, Rudolph U. (2002).BAB A-receptor subtypes: a new pharmacology. *Curr Opin Pharmacol*. 1:22-5

Morasso MI, Markova NG, Sargent TD. (1996). Regulation of epidermal differentiation by a Distal-less homeodomain gene. *J Cell Biol*. 135:1879-87

Mueller T., Wullimann M.F. (2003). Anatomy of neurogenesis in the early zebrafish brain. *Developmental Brain Research.* 140: 137-155

Neidert AH, Virupannavar V, Hooker GW, Langeland JA. (2001). Lamprey *Dlx* genes and early vertebrate evolution. *Proc Natl Acad Sci U S A.* 98: 1665-70

Panganiban G, Rubenstein JLR. (2002). Developmental functions of the *Distal-less/Dlx* homeobox genes. *Development* 129: 4371-4386

Park BK, Sperber S, Choudhury A, Ghanem N, Hatch G, Sharpe PT, Thomas BL, Ekker M. (2004). Intergenic enhancers with distinct activities regulate *Dlx* Gene expression in the mesenchyme of the branchial arches. *Developmental Biology*. 268:532-45

Parras CM, Schuurmans C, Scardigli R, Kim J, Anderson DJ, Guillimot F. (2002). Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. *Genes Dev* 16: 324-338.

Petryniak M, Potter G, Rowitch D, Rubenstein J. (2007). Dlx1 and *Dlx2* control neuronal versus oligodendroglial cell fate acquisition in the developing forebrain. *Neuron* 55:417–433

Pleasure SJ, Anderson S, Hevner R, Bagri A, Marin O, Lowenstein DH, Rubenstein JLR. (2000). Cell migration from the ganglionic eminences is required for the development of hippocampal GABAergic interneurons. *Neuron* 28: 727-740.

Poitras L, Ghanem N, Hatch G, Ekker M. (2007). The proneural determinant MASH1 regulates forebrain *Dlx1/2* expression through the I12b intergenic enhancer. *Development* 134:1755-1765.

Price JA, Bowden DW, Wright JT, Pettenati MJ, Hart TC. (1998). Identification of a mutation in DLX3 associated with Tricho-Dento-Osseous (TDO) syndrome. *Hum. Mol. Genet.* 7: 563-569.

Price JA, Wright JT, Walker SJ, Crawford PJ, Aldred MJ, Hart TC. (1999). Tricho-dento-osseous syndrome and amelogenesis imperfecta with taurodontism are genetically distinct conditions. *Clin. Genet.* 56: 35–40.

Puelles L, Kuwana E, Puelles E, Bulfone A, Shimamura K, Keleher J, Smiga S, Rubenstein JL. (2000). Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes *Dlx-2, Emx-1, Nkx-2.1,* *Pax-6,* and *Tbr-1. J. Comp. Neurol.* 424: 409-438.

Qiu M, Bulfone A, Martinez S, Meneses JJ, Shimamura K, Pedersen RA, Rubenstein JLR. (1995). Null mutations of *Dlx-2* results in abnormal morphogenesis of proximal first and second branchial arch derivatives and abnormal differentiation in the forebrain. *Genes Dev.* 9: 2523-2538.

Qiu M, Bulfone A, Ghattas I, Meneses JJ, Christensen L, Sharpe PT, Presley R, Pedersen RA, Rubenstein JLR. (1997). Role of the *Dlx* homeobox genes in proximodistal patterning of the branchial arches: Mutations of Dlx-1, Dlx-2, and Dlx-1 and -2 alter morphogenesis of proximal skeletal and soft tissue structures derived from the first and second arches. *Dev. Biol.* 185:165-184.

Quint E, Zerucha T, Ekker M. (2000). Differential expression of orthologous *Dlx* genes in zebrafish and mice: Implications for the evolution of the *Dlx* homeobox gene family. *J. Exp. Zool. Mol. Dev. Evol.* 288: 235-241.

Roberson MS, Meermann S, Morasso MI, Mulvaney-Musa JM, Zhang T.(2001).A role for the homeobox protein Distal-less 3 in the activation of the glycoprotein hormone alpha subunit gene in choriocarcinoma cells. *J Biol Chem* 276:10016-24

Robledo RF, Rajan L, Lufkin T. (2002). The *Dlx5* and *Dlx6* homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. *Genes Dev.* 16:1089-1101.

Rogers JH. (1987). Calretinin: a gene for a novel calcium-binding protein expressed principally in neurons. *J Cell Biol* 105:1343–1353.

Rymar VV, Sasseville R, Luk KC, Sadikot AF.(2004). Neurogenesis and stereological morphometry of calretinin-immunoreactive GABAergic interneurons of the neostriatum. *J Comp Neurol.*469:325-39

Ryoo HM, Hoffmann HM, Beumer T, Frenkel B, Towler DA, Stein GS, Stein JL, Wijnen AJ, Lian JB. (1997). Stage-specific expression of Dlx-5 during osteoblast differentiation: Involvement in regulation of osteocalcin gene expression. *Mol. Endocrinol.* 11:1681-1694.

Sanchez F, Alonso JR, Arevalo R, Carretero J, Vanzquez R, Aijon J. (1992). Calbindin D28K- and parvalbumin-reacting neurons in the hypothalamic magnocellular neurosecretory neuclei of the rat. *Brain Research Bulletin* 28: 39-46.

Schurmans S, Schiffmann SN, Gurden H, Lemaire M, Lipp HP, Schwam V. (1997) Impaired long-term potentiation induction in dentate gyrus of calretinindeficient mice. *Proc Natl Acad Sci USA*. 94:10415–20

Shimamoto T, Nakamura S, Bollekens J, Ruddle FH, Takeshita K. (1997). Inhibition of DLX-7 Homeobox gene causes decreased expression of GATA-1 and c-Myc genes and apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* 94: 3245-3249.

Shimamoto T, Ohyashiki K, Takeshita K.(2000). Overexpression of the homeobox gene DLX-7 inhibits apoptosis by induced expression of intercellular adhesion molecule-1. *Exp Hematol.*28:433-41

Shulkes A. (1994). Somatostatin: physiology and applications. *London Bailliere Tindall.* 3: 215–236

Simeone A, Acampora D, Pannese M, Desposito M, Stornaiuolo A, Gulisano M, Mallamaci A, Kastury K, Druck T, Huebner K, Boncinelli E. (1994). Cloning and characterization of two members of the vertebrate *Dlx* gene family. *Proc. Natl. Acad. Sci. USA* 91: 2250-2254.

Sperber SM, Saxena V, Hatch G, Ekker M. (2008). Zebrafish *dlx2a* contributes to hindbrain neural crest survival, is necessary for differentiation of sensory ganglia and functions with *dlx1a* in maturation of the arch cartilage elements. *Dev. Biol.* 314: 59-70.

Stock DW, Ellies DL, Zhao Z, Ekker M, Ruddle FH, Weiss KM. (1996). The evolution of the vertebrate *Dlx* gene family. *Proc. Natl. Acad. Sci. USA* 93:10858-10863.

Stuhmer T, Puelles L, Ekker M, Rubenstein JL. (2002). Expression from a *Dlx* gene enhancer marks aldult mouse cortical GABAergic neurons. *Cereb Cortex* 12: 75-85.

Stumm R, Kolodziej A, Schulz S, Kohtz JD, Volker HL. (2007). Patterns of SDF-1 mRNAs migration pathways, and phenotypes of CXCR4-expressing neurons in the Developing Rat Telencephalon. *The Journal of Comparative Neurology*. 502:382–399

Sumiyama K, Ruddle FH. (2003). Regulation of Dlx3 gene expression in visceral arches by evolutionary conserved enhancer elements. *Proc. Natl. Acad. Sci. USA* 100: 4030-4034.

Tadic T, Dodig M, Erceg I, Marijanovic I, Mina M, Kalajzic Z, Velonis D, Mark S. Kronenberg M S, Koshel R A, Ferrari D, Lichtler AC.(2002).Overexpression of *Dlx5* in chicken calvarial cells accelerates osteoblastic differentiation. *Journal of Bone and Mineral Research*.17: 1008–1014

Toresson H, Campbell K. (2001). A role for Gsh1 in the developing striatum and olfactory bulb of Gsh2 mutant mice. *Development* 128: 4769-4780.

Walsh J. (1994). Somatostatin Physiology of the gastrointestinal tract. *New York: Raven Press*. 6: 31–48.

Wichterle H, Garcia-Verdugo JM, Herrera DG, Alvarez-Buylla A. (1999). Young neurons from medial ganglionic eminence disperse in adult and embryonic brain. *Nat. Neurosci.* 2:461-466.

Wichterle H, Turnbull DH, Nery S, Fishell G, Alvarez-Buylla A. (2001). In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development* 128:3759-3771.

Wonders CP, Anderson SA. (2006). The origin and specification of cortical interneurons. *Nat Rev Neurosci.* 7:687-96

Xu Q, Anderson SA.(2003). Cortical interneuron fate determination: diverse sources for distinct subtypes. *Cerebral Cortex*.13: 670–676

Xu Q, Cobos I, De La Cruz E, Rubenstein JL, Anderson SA. (2004). Calretinin interneurons are absent in *Dlx1-/- Dlx2-/-* mice, in which both MGE and CGE are abnormal origins of cortical interneuron subtypes. *J. Neurosci*. 24: 2612–2622

Xu Q, Tam M, Anderson SA. (2008). Fate mapping Nkx2.1-lineage cells in the mouse telencephalon. *J. Comp. Neurol.* 506: 16–29

Yun K, Fischman S, Johnson J, Hrabe de Angelis M, Weinmaster G, Rubenstein JL. (2002). Modulation of the Notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. *Development* 129: 5029-5040.

Zerucha T, Ekker M. (2000). *Distal-Less*-Related homeobox genes of vertebrates: evolution, function, and regulation. *Biochem Cell Biol.* 78: 593-601

Zerucha T, Stuhmer T, Hatch G, Park BK, Long Q, Yu G, Gambarotta A, Schultz JR, Rubenstein JLR, Ekker M. (2000). A highly conserved enhancer in the *Dlx5/Dlx6* intergenic region is the site of cross-regulatory interactions between *Dlx g*enes in the embryonic forebrain. *J. Neurosci.* 20: 709-72

Zhu H, Bendall AJ. (2009). *Dlx5* Is a Cell Autonomous Regulator of chondrocyte hypertrophy in mice and functionally substitutes for *Dlx6* during endochondral ossification. *PLoS One.* 4: 8097