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Lineage tracing of *dlx1a/2a* and *dlx5a/6a* expressing cells in the developing zebrafish brain

Cynthia M. Solek[a,b], Shengrui Feng[a,c], Sofia Perin[a], Hellen Weinschutz Mendes[a], Marc Ekker[a,*]

[a] CAREG, Department of Biology, University of Ottawa, 20 Marie-Curie Private, Ottawa, ON, Canada K1N 6N5
[b] Montreal Neurological Institute, McGill University, 3801 University Street, Montreal, QC, Canada H3A 2B4
[c] Department of Medical Biophysics, University of Toronto, 610 University Avenue, Toronto, ON, Canada M5G 2M9

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ABSTRACT

Lineage tracing of specific populations of progenitor cells provides crucial information about developmental programs. Four members of the *Dlx* homeobox gene family, *Dlx1,2, 5* and *6*, are involved in the specification of γ-aminobutyric acid (GABA)ergic neurons in the vertebrate forebrain. Orthologous genes in mammals and teleost show similarities in expression patterns and transcriptional regulation mechanisms. We have used lineage tracing to permanently label *dlx*-expressing cells in the zebrafish and have characterized the progeny of these cells in the larva and in the juvenile and adult brain. We have found that *dlx1a/2a* and *dlx5a/6a* expressing progenitors give rise, for the most part, to small populations of cells which constitute only a small proportion of GABAergic cells in the adult brain tissue. Moreover, some of the cells do not acquire a neuronal phenotype suggesting that, regardless of the time a cell expresses *dlx* genes in the brain, it can potentially give rise to cells other than neurons. In some instances, labeling larval *dlx5a/6a*-expressing cells, but not *dlx1a/2a*-expressing cells, results in massively expanding, widespread clonal expansion throughout the adult brain. Our data provide a detailed lineage analysis of the *dlx1a/2a* and *dlx5a/6a* expressing progenitors in the zebrafish brain and lays the foundation for further characterization of the role of these transcription factors beyond the specification of GABAergic neurons.

1. Introduction

Proper formation of neuronal circuits in embryogenesis requires carefully orchestrated molecular mechanisms, including tightly regulated expression of many transcription factors. Members of the *Dlx* family of homeobox transcription factors are involved in the development of forebrain interneurons and projection neurons (Anderson et al., 1997a, 1997b; Andrews et al., 2003; Li et al., 2011; MacDonald et al., 2010; Potter et al., 2009; Stühmer et al., 2002a, 2002b; Yu et al., 2011). This vastly heterogeneous population of inhibitory neurons is essential to maintain excitatory balance and proper brain function (Marín, 2012).

A large and well-characterized GABAergic precursor population in the developing mammalian telencephalon emerges from the medial (MGE), lateral (LGE) and caudal ganglionic eminences (CGE), as well as the septum and the preoptic region. Immature interneurons undergo tangential migration to the cortex and olfactory bulb where they mature into local circuit neurons (reviewed in Kelsom and Lu, 2013). A number of the molecular mechanisms involved in the proper development of these neurons have been characterized. Four members of the *Dlx* family of transcription factors are expressed in the ganglionic eminences during mouse development (Liu et al., 1997; Simeone et al., 1994). Loss of *Dlx* function results in a decrease of GABAergic neurons in the cortex (Anderson et al., 1997a, 1997b; Pleasure et al., 2000) and behaviour pathologies such as epilepsy (Cobos et al., 2005). *Dlx* mutants also have defects in the GABAergic nuclei such as the striatum,

Abbreviations: ATN, anterior tuberal nucleus; CeP, cerebellar plate; CP, central posterior thalamic nucleus; D, dorsal telencephalic area; DIL, diffuse nucleus of the inferior lobe; DT, dorsal thalamus; E, epiphysis; EG, eminentia granularis; EmT, eminentia thalami; ENd, entopeduncular nucleus, dorsal part; Ha, habenula; Hc, caudal hypothalamus; Hd, dorsal zone of periventricular hypothalamus; Hi, intermediate hypothalamus; Hr, rostral hypothalamus; Hv, ventral zone of periventricular hypothalamus; M1, migrated pretectal area; M2, migrated posterior tubercular area; M3, migrated area of EmT; M4, telencephalic migrated area; MO, medulla oblongata; NIII, oculomotor nerve nucleus; OB, olfactory bulb; P, pallium; PGZ, periventricular gray zone; Pit, pituitary; Po, preoptic region; PPa, parvocellular preoptic nucleus, anterior part; Pr, pretectum; PT, posterior tuberculum; PTN, posterior tuberal nucleus; PTv, ventral part of posterior tuberculum; S, subpallium; Sd, dorsal division of S; Sv, ventral division of S; T, midbrain tegmentum; TeO, optic tectum; TL, torus longitudinalis; TPp, periventricular nucleus of posterior tuberculum; V, ventral telencephalic area; Vc, central nucleus of V; Vd, dorsal nucleus of V; Vl, lateral nucleus of V; Vp, postcommissural nucleus of V; Vs, supracommissural nucleus of V; Vv, ventral nucleus of V

* Corresponding author.
E-mail address: mekker@uottawa.ca (M. Ekker).

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septum, globus pallidus, central nucleus of the amygdala, arcuate nucleus and reticular nucleus of the thalamus. In contrast, ectopic *dlx* expression results in anomalous expression of Glutamic Acid Decarboxylase enzymes (Gad65/67) required for synthesis of GABA from glutamate (Stühmer et al., 2002a).

The four mammalian *Dlx* genes involved in GABAergic neurons development in the forebrain are organized in bigene clusters (McGuinness et al., 1996; Simeone et al., 1994). A model has been proposed for the sequential expression of the mouse *Dlx* genes in the forebrain based on expression patterns and on the phenotype of mutants (Liu et al., 1997; Panganiban and Rubenstein, 2002). Highly conserved enhancer elements are located in the intergenic region of the *Dlx1/2* and *Dlx5/6* loci, which are in large part responsible for the expression of these genes (Ghanem et al., 2003; Zerucha et al., 2000). Another regulatory element, located upstream of the *Dlx1* coding region (URE2), is also able to target reporter transgene expression in a pattern that overlaps the expression of *Dlx1* and *Dlx2* (Ghanem et al., 2007).

Several elements of the developmental programs that give rise to GABAergic neurons are common to zebrafish and mouse (Wullimann, 2009). In the zebrafish forebrain, *gad* expressing cells are seen to extend away from the ventricular domains in the dorsal subpallium (the equivalent of the MGE and LGE) towards the pallium (Mueller et al., 2008), suggestive of a tangential migration of these cells into the postmitotic zones of the zebrafish pallium. Precise migratory patterns of GABAergic neurons in the developing zebrafish is however relatively less well understood. Genetic regulatory elements, including several transcription factors, display at least partially conserved functions in the development of GABAergic neurons throughout vertebrate evolution (Macdonald et al., 2013; Wullimann and Mueller, 2004). Four of the zebrafish *dlx* family members, *dlx1a/2a* and *dlx5a/6a,* are expressed in the developing forebrain and are involved in the specification of GABAergic neurons (Macdonald et al., 2013; MacDonald et al., 2010; Yu et al., 2011). Like in the mouse, the genes are arranged in convergently transcribed bigene clusters (Zerucha and Ekker, 2000) and their expression is regulated in part by the highly conserved intergenic enhancers (Ghanem et al., 2003; Poitras et al., 2007; Yu et al., 2011; Zerucha et al., 2000).

Although the *Dlx* genes are expressed in overlapping patterns in the forebrain of developing mouse (Liu et al., 1997; Simeone et al., 1994) and zebrafish (Ellies et al., 1997; MacDonald et al., 2010; Quint et al., 2000) embryos, they may be performing unique functions. Evidence from *Dlx* mutant mice reveals different phenotypical defects in interneuron development, whether genes are inactivated singly or in pairs (reviewed in Nord et al., 2015). Deletion of a single *Dlx* gene gives rise to subtle phenotypes, suggesting partially redundant roles for the members of bigene clusters (Cobos et al., 2005; Long et al., 2003; Qiu et al., 1997, 1995; Wang et al., 2011). However, deletion of the *Dlx1/2* gene pair results in a severe block in forebrain differentiation and neuronal migration, as well as an increase in oligodendrogenesis (Anderson et al., 1997a, 1997b; Lazarini et al., 2012; Long et al., 2007, 2009a, 2009b; Petryniak et al., 2007; Pleasure et al., 2000; Yun et al., 2002), while inactivation of the *Dlx5/6* gene pair results in exencephaly and defects in a subset of interneurons (Wang et al., 2010). Moreover, despite a vast reduction in the number of cells expressing *Dlx5* and *Dlx6* in the *Dlx1/2* null mice (a finding consistent with sequential activation model mentioned above (Panganiban and Rubenstein, 2002)), a significant population of cells remains capable of expressing the *Dlx5/6* transcription factors independently of *Dlx1/2* activity (Anderson et al., 1997b). In zebrafish, knockdown of *dlx1a/2a* or *dlx5a/6a* with antisense morpholino oligonucleotides results in different disruption of *gad1b* expression in the developing embryo (Macdonald et al., 2013). These data suggest unique roles for the members of the *Dlx* transcription factor family in the development of GABAergic interneurons in the teleost forebrain.

There is strong evidence that links defects in specification and migration of GABAergic neurons with the etiology of a number of neurodevelopmental disorders, including schizophrenia, epilepsy and autism spectrum disorder (Chattopadhyaya and Di Cristo, 2012; Marín and Rubenstein, 2001; Rossignol, 2011). The myriad ways in which these diseases manifest in individuals suggest that small changes in circuit assembly can lead to different neurological symptoms. Studies in model animals have made headway in uncovering the molecular mechanisms that direct specification and migration of GABAergic neurons (for recent reviews see Achim et al., 2014; Faux et al., 2012; Kelsom and Lu, 2013). The complexity of the developmental programs is such, however, that their characterization is still ongoing.

Little is known about the fate of the *dlx* expressing cells in the developing zebrafish. Using a GFP reporter construct driven by the *dlx5a/dlx6a* intergenic enhancer (Ghanem et al., 2003), Mione et al. (2008) characterized the migration patterns and fate determination of a subset of these cells in early developmental and later stages of the life of the zebrafish. We have used a lineage tracing method to label the *dlx1a/2a* and the *dlx5a/6a* expressing cells in the zebrafish embryo. We use the intergenic enhancers to drive expression of CreER^{T2} recombinase in combination with a ubiquitously expressed *loxP*-dependent reporter construct [*Tg(Ubi:Switch*), (Mosimann et al., 2011)]. This method has advantages over simple reporters, which depend on promoter or enhancer activity for transgene expression and hence result in loss of labeling once transcriptional activity wanes. In this system, switched cells are permanently labeled and may no longer express nor need the function of *dlx* genes at the time they are stained for mCherry expression. We show that *dlx1a/2a* and *dlx5a/6a* expressing cells labeled in the first 5 days post fertilization (dpf) give rise in part to GABAergic neurons. However, a significant proportion of these cells do not express *gad65/67a* transcripts or HuC/D proteins. Aside from a small population of *dlx5a/6a* expressing cells in the 3 dpf or older larva, the labeled cells do not undergo extensive divisions to generate large clonal populations in the adult zebrafish brain.

2. Results

2.1. Lineage tracing of dlx-expressing cells in zebrafish embryos and early larvae

To compare the populations of cells derived from the *dlx1a/2a* and the *dlx5a/6a* expressing progenitors, we generated transgenic lines expressing the CreER^{T2} recombinase under the previously characterized regulatory elements of these bigene clusters (Fig. 1A). We verified that CreER^{T2} expression recapitulated *dlx* expression in the forebrain by *in situ* hybridization (Suppl. Fig. 1). We crossed the *Tg(dlx1a/2a:CreER^{T2})* and *Tg(dlx5a/6a:CreER^{T2})* lines with the *Tg(Ubi:Switch)* reporter line (Fig. 1B; Mosimann et al., 2011) to obtain double transgenic animals (Fig. 1C, henceforth referred to as *Tg(dlx1/2Cre;Switch)* and *Tg(dlx5/6Cre;Switch)*, respectively). We administered 4-hydroxytamoxifen (4-OHT) at several time points during early development to induce CreER^{T2} activity and promote recombination of the Switch reporter transgene resulting in expression of mCherry in the cells that harbor the recombinase. Mock induced animals (with ethanol vehicle) did not display mCherry labeled cells (Suppl Fig. 2 and data not shown), suggesting the absence of leakiness of the transgenes. We traced the labeled cells by live confocal imaging over the days following induction until 10–14 dpf (Fig. 1C). We compared the population of labeled cells following induction at 24 h post fertilization (hpf) (Fig. 2A-F, henceforth designated as *Tg(dlx1/2Cre;Switch)^{24h}* and *Tg(dlx5/6Cre;Switch)^{24h}*), 48 hpf (Fig. 3A-C, G-I), 3 dpf (Fig. 3D-F, J-L) and 5 dpf (Fig. 2G-M). We also performed an earlier induction at 13 hpf for the *Tg(dlx1/2Cre;Switch)* line (Suppl. Fig. 3A-C), and at 18 hpf for the *Tg(dlx5/6Cre;Switch)* line (Suppl. Fig. 3D-F), the approximate onset of expression of each gene pair (Akimenko et al., 1994; Ellies et al., 1997). We did not observe significant differences in resulting mCherry positive cells following induction at the onset of