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Evolution of Developmental Control Mechanisms

The *ascl1a* and *dlx* genes have a regulatory role in the development of GABAergic interneurons in the zebrafish diencephalon

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During development of the mouse forebrain interneurons, the *Dlx* genes play a key role in a gene regulatory network (GRN) that leads to the GABAergic phenotype. Here, we have examined the regulatory relationships between the *ascl1a*, *dlx*, and *gad1b* genes in the zebrafish forebrain. Expression of *ascl1a* overlaps with *dlx1a* in the telencephalon and diencephalon during early forebrain development. The loss of Ascl1a function results in a loss of *dlx* expression, and subsequent losses of *dlx5a* and *gad1b* expression in the diencephalic prethalamus and hypothalamus. Loss of Dlx1a and Dlx2a function, and, to a lesser extent, of Dlx5a and Dlx6a, impairs *gad1b* expression in the prethalamus and hypothalamus. We conclude that *dlx1a/2a* act downstream of *ascl1a* but upstream of *dlx5a/dlx6a* and *gad1b* to activate GABAergic specification. This pathway is conserved in the diencephalon, but has diverged between mammals and teleosts in the telencephalon.

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Introduction

Gene regulatory networks (GRNs) are made up of dynamic interactions between transcription factors and *cis*-regulatory elements (CREs) found within the genome (for reviews see: Levine and Davidson (2005) and Davidson and Levine (2008)). CREs are classically thought to be non-coding regulatory sequences, comprised of clustered transcription factor binding sites; the binding of these transcription factors are able to affect the transcription of specific genes (for reviews see: Kulkarni and Arnosti (2003), Kadonaga (2004) and Panne (2008)). The overall levels and timing of gene expression are conferred by the cumulative contributions of multiple transcription factors on a myriad of regulatory regions. The genes regulated by this process during development often encode transcription factors that will play a role in the regulation of other transcription factor genes located downstream in the GRN, eventually resulting in the expression of terminal differentiation genes leading to a specified cell type.

Ascl1 (*Mash1*) is one of the basic helix–loop–helix (bHLH) transcription factors thought to play important roles in GRNs controlling neurogenesis (for reviews see: Bertrand et al. (2002) and Allan and Thor (2003)). *Ascl1* is expressed in proliferating neural precursors in the subpallial telencephalon and prethalamus of the mouse (Lo et al., 1991; Guillemot and Joyner, 1993; Porteus et al., 1994; Yun et al., 2002; Andrews et al., 2003). *Ascl1* mutants have defects in neural specification and in the timing of differentiation in the ventral forebrain, including altered telencephalic expression of the *Dlx* genes and *Gad1* (*Gad67*), which encodes glutamic acid decarboxylase, the enzyme responsible for the production of γ-amino butyric acid (GABA) (Casarosa et al., 1999; Horton et al., 1999: Yun et al., 2002; Long et al., 2009a). Ectopic expression of *Ascl1* leads to *Gad1* expression in the mouse dorsal telencephalon, further supporting a role for *Ascl1* in GABAergic interneuron development (Fode et al., 2000). In zebrafish there are two *Ascl1* orthologs, *ascl1a* and *ascl1b*. These genes are expressed in the embryonic forebrain, including the subpallial telencephalon and prethalamus, reminiscent of *Ascl1* expression in the mouse (Allende and Weinberg, 1994; Wullimann and Mueller, 2002).

In the mouse forebrain, expression of *Ascl1* and *Dlx* genes overlap suggesting these genes may genetically interact during mouse forebrain development (Porteus et al., 1994; Yun et al., 2002; Andrews et al., 2003). Consistent with this hypothesis,

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Ascl1$^{-/-}$ mutant mice have mis-expression of *Dlx* in the ganglionic eminences (Casarosa et al., 1999; Horton et al., 1999; Yun et al., 2002; Long et al., 2009a) and ASCL1 proteins have been shown to activate and directly bind to a *Dlx1/Dlx2* regulatory element (Poitras et al., 2007).

The *Dlx* genes encode homeodomain transcription factors expressed in the ganglionic eminences of the telencephalon and diencephalon in the mouse. More specifically, four *Dlx* genes are expressed in the forebrain of the mouse: *Dlx1*, *Dlx2*, *Dlx5*, and *Dlx6* (Liu et al., 1997; Yang et al., 1998; Anderson et al., 1997a; Eisenstat et al., 1999), while five orthologous *dlx* genes are expressed in the forebrain of the zebrafish: *dlx1a*, *dlx2a*, *dlx5a*, *dlx6a*, and *dlx2b* (Akimenko et al., 1994; Ellies et al., 1997; Hauptmann and Gerster, 2000). The *Dlx* genes are expressed in highly overlapping but also distinct domains within the forebrain of mice and zebrafish, often correlating with neuronal differentiation and *Gad* expression (Liu et al., 1997; Eisenstat et al., 1999; MacDonald et al., 2010a; Stühmer et al., 2002a,b; Yun et al., 2002). Functional studies have shown that the *Dlx* genes are required for the differentiation and migration of most GABAergic neurons in the telencephalon and diencephalon (Anderson et al., 1997a,b; Stühmer et al., 2002a,b; Long et al., 2007; Long et al., 2009a,b; Wang et al., 2012). Additionally, DLX1 and DLX2 are involved in the suppression of neurite growth and branching, thus enabling the proper tangential migration of GABAergic neurons (Cobos et al., 2007).

The zebrafish *dlx* genes are involved in branchial arch and sensory placode development (Solomon and Fritz, 2002; Kaji and Artinger, 2004; Walker et al., 2006; Jackman and Stock, 2006; Sperber et al., 2008; Talbot et al., 2010), as are the mouse *Dlx* genes (Qiu et al., 1995; Depew et al., 2002; Jeong et al., 2008). However, despite their common use as forebrain markers, there has been little functional analysis of the *dlx* genes in the zebrafish brain. To characterize the role of *ascl1a* and *dlx* in the GRN(s) controlling GABAergic interneuron differentiation in the zebrafish forebrain, we have knocked down their function and assayed the effects on downstream targets. Our results show that the *ascl1a* gene regulates *dlx* genes necessary for proper *gad1b* expression in the diencephalon of the zebrafish. Thus, these genes are key part of a GRN involved in early forebrain development that is conserved among bony vertebrates.

Materials and methods

Zebrafish strains and staging

Embryos were obtained and housed using standard procedures described in Westerfield (2000). The following transgenic zebrafish lines were used in this study: *Tg*(*dlx1a/2aIG:GFP*) (MacDonald et al., 2010a), *Tg*(*dlx1URE2:GFP*) (MacDonald et al., 2010b), and *Tg*(*dlx5a/6a:GFP*) (Ghanem et al., 2003). All developmental stages are reported as hours post-fertilization (hpf) All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and approved by institutional animal care committees.

Morpholino and mRNA injections

Morpholino oligonucleotides (MO) were injected into one-cell stage wild type or transgenic zebrafish embryos at concentrations ranging from 2 to 4 ng/μl. The following translation blocking morpholinos were used: *dlx1a* (Sperber et al., 2008), *dlx2a* (Sperber et al., 2008), *dlx2b* (Jackman and Stock, 2006), *dlx5a* (Walker et al., 2006), *dlx6a* (5′TGGTCATCAT–CAAATTTTCTGCTTT3′). The *ascl1a*$^{5'UTR}$ MO (Cau and Wilson, 2003) was kindly provided by Dr. S. Wilson. Splice blocking MOs for *dlx5a* (Talbot et al., 2010) were kindly provided by Dr. C. B. Kimmel, and were used to confirm the translation blocking MO phenotypes. The *dlx6a* splice-blocking morpholino binds to the end of the second exon and inhibits the splicing of the second intron (5′AAATGAGTTCACATCTCACCTGCGT3′).

In situ hybridization and imaging

Whole mount mRNA *in situ* hybridization was carried out as described in Thisse and Thisse (1998). The antisense mRNA probes were labeled with digoxygenin-11-UTP (Roche, 11277073910) and synthesized from the following cDNA clones: *dlx1a* (Ellies et al., 1997), *dlx2a* (Akimenko et al., 1994), *dlx5a* (Akimenko et al., 1994), *dlx6a* (Ellies et al., 1997), *dlx2b* (Ellies et al., 1997), *gad2* (Martin et al., 1998), *gad1b* (Mueller et al., 2008), *ascl1a* (Cau et al., 2000), *nkx2.1a* (Rohr and Concha, 2000), *emx2* (Morita et al., 1995), *lhx5a* (Toyama et al., 1995), *gfp* (Dorsky et al., 2002). After the procedure, embryos were post fixed in 4% PFA and cleared overnight in glycerol.

Fluorescent RNA *in situ* hybridization was carried out with a protocol modified from those described previously (Jowett and Yan, 1996; Welten et al., 2006; Talbot et al., 2010). The DNP-labeled probe was revealed with tyr-Cy5, whereas dig-labeled probes were revealed using tyr-Cy3. Fluorescein-labeled probes were revealed with tyr-fluorescein (available from Perkin-Elmer). The full tissue labeling protocols can be found online: http://wiki.zfin.org/display/prot/Triple+Fluorescent+In+Situ.

For confocal imaging, embryos were placed in mounting media on glass slides and positioned under coverslips. Confocal z-stacks were obtained by using a Zeiss LSM5 PASCAL (Carl Zeiss, Germany) with an excitation laser at 488 (Fluorescein), 543 nm (Cy3), and 633 nm (Cy5).

Rescue experiments and morphant phenotype scoring

For exogenous expression of *dlx* genes, capped full-length mRNA was synthesized *in vitro* using linearized PCS2+ plasmids (mMessageMachine; Ambion) and purified. The following plasmids as templates: mut*dlx2a* (mutagenized at MO binding site) and mut*dlx5a* (Supplementary Table 1). A solution containing 40 ng/μl of mRNA, along with MO, was co-injected into single cell embryos. Individuals were classified and scored in two groups: either as having reduced or normal prethalamic expression. Embryos from each treatment were scored in a double-blind manner and plotted with standard error from three individual experiments. One way ANOVA was used to compare data.

Results

The ascl1a, dlx, and gad1b genes are co-expressed in the forebrain

We utilized triple fluorescent *in situ* hybridizations to determine if the zebrafish *ascl1a*, *dlx* and *gad* genes show overlapping expression in the forebrain as they do in the mouse. The expression of *ascl1a* begins in the prospective forebrain at 10 hpf and lasts until at least 72 hpf (Allende and Weinberg, 1994). The *dlx1a* and *dlx2a* (hereafter called *dlx1a/2a*) genes are expressed starting at 13 hpf in the prospective forebrain (Akimenko et al., 1994; Ellies et al., 1997). At 24 hpf, *dlx1a* is expressed in the telencephalon and two domains of the diencephalon, the prethalamus (or ventral thalamus) and the hypothalamus (Fig. 1A). The *dlx2a* expression domains are identical to *dlx1a* (MacDonald et al., 2010a), so we consider *dlx1a* expression as representative of the two genes. At 24 hpf, expression of *ascl1a* is detected in the telencephalon and prethalamus, and partially overlaps with the *dlx1a* expression

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