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Functional consequences of I56ii *Dlx* enhancer deletion in the developing mouse forebrain

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ABSTRACT

Dlx homeobox genes encode a group of transcription factors that play an essential role during developmental processes including maintaining the differentiation, proliferation and migration of GABAergic interneurons. The *Dlx1/2* and *Dlx5/6* genes are expressed in the forebrain and are arranged in convergently transcribed bigene clusters, with I12a/I12b and I56i/I56ii *cis*-regulatory elements (CREs) located in the intergenic region of each cluster respectively. We have characterized the phenotypic consequences of deleting I56ii on forebrain development and spatial patterning of corridor cells that are involved in guiding thalamocortical projections. Here we report that deletion of I56ii impairs expression of *Dlx* genes and that of potential targets including *Gad2* as well as striatal markers *Islet1, Meis2,* and *Ebf1*. In addition, I56ii deletion reduces both the binding of DLX2 in the *Dlx5/Dlx6* intergenic region and the presence of H3K9Ac at the *Dlx5/Dlx6* locus, consistent with the reduced expression of these genes. Deletion of I56ii reduces the expression of the ISLET1 and CTIP2 in the striatum and disrupts the number of parvalbumin and calretinin expressing cells in the adult somatosensory cortex of the ΔI56ii mice. These data suggest an important regulatory role for I56ii in the developing forebrain by means of a potential regulatory mechanism which may regulate the expression of *Dlx* genes, notably *Dlx6* as well as the spatial patterning of the ventral telencephalon, including possibly corridor cells.

1. Introduction

The majority of neurons that make up the cortex are of two types. The first type consists of excitatory projection neurons that release the neurotransmitter glutamate. The second type consists of inhibitory interneurons that release GABA as their primary neurotransmitter (Parnavelas, 2000; Pleasure et al., 2000; Zhu et al., 1999). These neurons are derived from progenitor cells in the ventricular zone of the telencephalon and migrate to their final destinations through the two distinct mode of migration, namely radial or tangential migration (López-Bendito and Molnár, 2003; Pleasure et al., 2000). One of the features of the developing central nervous system is the ability of neuronal cells to migrate away from their origin to their final destination (Gleeson and Walsh, 2000; Lambert de Rouvroit and Goffinet, 2001). This process is essential for the proper brain development and function (Wichterle et al., 2003).

Cortical interneurons migrate from the ventral telencephalon to the developing neocortex via tangential migration (Parnavelas, 2000). During tangential migration, also known as “neurophilic migration”, neurons move parallel to the surface of the brain along the trajectory of axons and often disobey the regional boundaries within the developing brain (Hatten, 1999). Lineage studies have reconciled the divergent migratory patterns of the cortical interneurons, demonstrating that the migration of these interneurons have distinct patterns (Mione et al., 1997). It has been shown that the interneurons that originate from the ventral telencephalon depend largely on a wide spectrum of guidance cues to navigate from the ventral telencephalon to the developing cortex (Zhu et al., 1999). For instance, SLIT1 is a secretory protein that is produced in both the lateral and medial ganglionic eminences (LGE and MGE) and has been shown to act as a chemorepellent for the tangentially migrating GABAergic interneurons (Yuan et al., 1999). Furthermore, it has been suggested that hepatocyte growth factor (HGF) could provide localized cues to interneurons and thus facilitate their migration towards the dorsal pallium (Powell et al., 2001).

Part of the mechanism that is proposed for the proper establishment of neuronal pathways involves tangentially migrating cells within

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the ventral telencephalon, also known as “corridor cells”, guiding tangentially migrating cells towards the cortex (Lopez-Bendito et al., 2006). The corridor cells are thought to create a permissive corridor between the boundaries of the diencephalon/telencephalon and pallial/subpallial regions of the developing forebrain (Lopez-Bendito et al., 2006). Corridor cells may not only function by providing positional cues, but also by forming a bridge between different regions of the telencephalon that otherwise would have been non-permissive to the tangentially migrating cells as well as the growth of the thalamocortical axons (Corbin et al., 2001). Interestingly, it has been demonstrated that unlike the tangentially migrating cells, the corridor cells migrate in the opposite direction. The corridor cells originate from the lateral ganglionic eminence (LGE) and migrate downward deep into the mantle zone (MZ) of the medial ganglionic eminence (MGE) (Corbin et al., 2001; Lopez-Bendito et al., 2006; López-Bendito and Molnár, 2003). They express a number of LGE-specific markers including *Islet1, Ebf1* and *Meis2* (Lopez-Bendito et al., 2006).

The *Dlx* gene family encode homeodomain transcription factors involved in embryonic development of the telencephalon through regulation of the differentiation and migration of GABAergic interneurons (Panganiban and Rubenstein, 2002). *Dlx* genes in mice are arranged as three bigene clusters; namely, *Dlx1/Dlx2, Dlx5/Dlx6* and *Dlx3/Dlx4* (Zerucha and Ekker, 2000). In these bigene clusters, *Dlx* genes are convergently transcribed with the *cis*-acting regulatory elements (CREs) located within the intergenic region of each cluster, namely I12a/I12b and I56i/I56ii in the *Dlx1/Dlx2* and *Dlx5/Dlx6* clusters, respectively (Zerucha and Ekker, 2000; Zerucha et al., 2000). Analysis of *Dlx* intergenic CREs using *lacZ* reporter transgenesis demonstrated overlap in their patterns of activity, yet some marked differences between the activity patterns of I12b, I56i and I56ii CREs in the developing forebrain (Ghanem et al., 2007, 2008). Spatiotemporal analysis of *lacZ* transgenes indicated little if any activity of the I56ii CRE in GABAergic interneurons or in tangentially migrating cells to the cortex. However, I56ii-driven reporter transgene expression in cells whose position deep in the mantle zone, corresponds to a group of corridor cells (Bielle et al., 2011; Ghanem et al., 2008; Lopez-Bendito et al., 2006). The I56ii-positive cells expressed the *Islet1* and *Meis2* striatal markers. This was further investigated in co-transfection assays *in vitro* where these striatal markers were able to activate transcription via the I56ii CRE (Ghanem et al., 2008).

The aim of the present study was to better understand the component of *Dlx* function that is dependent on the I56ii CRE through enhancer deletion. More specifically, we are interested to investigate the impact of the loss of *Dlx* function on the corridor cells. We have generated mice that are homozygous for a deletion of the I56ii CRE in the intergenic region of *Dlx5/Dlx6* cluster (Fig. 1). Here we report that the I56ii deletion impacts the expression of *Dlx* genes and of some of their downstream targets. Notably, the expression of the *Islet1*, *Ebf1* and *Ctip2* TFs in or near the corridor cells located in the striatum of the developing forebrain is affected. Furthermore, I56ii deletion leads to reduced DLX2 and H3K9Ac binding to the intergenic region of *Dlx5/Dlx6* locus. Unexpectedly, impaired *Dlx* expression as result of I56ii deletion also affects the number of parvalbumin- and calretinin-expressing interneurons in the adult somatosensory cortex.

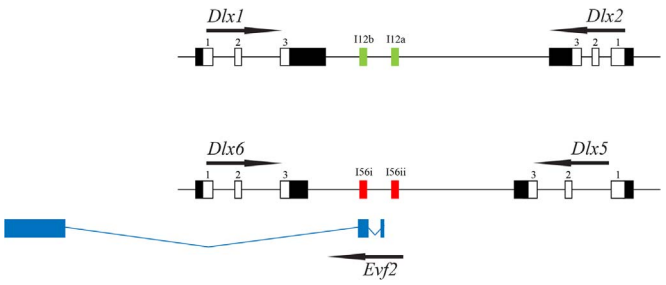

Fig. 1. Schematic representation of the genomic organization of the vertebrate *Dlx1/Dlx2* and *Dlx5/Dlx6* genes. Exons of the *Dlx* genes are numbered with black boxes representing untranslated regions and white boxes representing coding regions. *Dlx* intergenic *cis*-regulatory elements (CREs) are shown in green (*Dlx1/Dlx2*) and red (*Dlx5/Dlx6*). The long-noncoding RNA (lncRNA) *Evf2* is shown in blue on the opposite strand.

2. Materials and methods

2.1. Generation of I56ii targeted deletion mice

Mice with targeted deletion of I56ii were generated at the Transgenic Mouse Core Facility at the McGill Cancer Center. Through homologous recombination, a LoxP-flanked PGK-neomycin-resistant cassette in a pL452 vector, replaced the entire I56ii enhancer sequence on a Bacterial Artificial Chromosome (564M8 BAC; BAC/PAC resources, Children's Hospital Oakland Research Institute) harboring 2 Kb of the *Dlx5/Dlx6* locus (Liu et al., 2003). BAC clones were screened and sequenced to ensure a successful recombination. The recombined vector was electroporated into 129SV mouse embryonic stem (ES) cells. Cells were selected with gentamicin to detect cells harboring the mutant BAC. Additionally, ES cells were screened for the presence of the neomycin cassette through quantitative real-time PCR and a positive ES clone was injected into a host C57BL/6 blastocyst to generate chimeric mice. Chimeric mice were mated with C57BL/6 wild-type mice and genotyped to ensure a germ-line transmission of the mutant allele and to generate heterozygote progeny that were positive for I56ii deletion in their germ line. Once I56ii heterozygous mice were obtained, we mated two heterozygotes to produce homozygous ΔI56ii mice. The ΔI56ii mice were mated with SoxCre mice to remove the neomycin cassette from the *Dlx5/Dlx6* cluster to generate ΔI56iiΔNeo mice. This step was important to prevent the potential impact of the neomycin cassette during expression analysis.

2.2. Genomic DNA extraction and genotyping

Tissue samples were digested in a solution containing 1 mg/mL of proteinase K, 50 mM Tris-HCl pH=8.0, 100 mM EDTA, 100 mM NaCl and 1% SDS. Genomic DNA was extracted using a standard salt and ethanol precipitation protocol. A PCR-based approach was used to screen for mutant embryos by using purified genomic DNA, and primer-pairs flanking each deleted enhancer (Table 1).

2.3. Tissue preparation and RNA extraction

The ventral telencephalon of mouse embryos was dissected at E13.5 and E15.5. The ventral telencephalon (subpallium) was obtained by dissecting the forebrain from pallial-subpallial boundary to remove pallium from ventral telencephalon. Using the whole ventral telencephalon, rather than the individual ganglionic eminences, may mask some of the regional differences in the expression levels of various targets. To avoid this, we have also conducted in situ hybridization to investigate any possible spatial changes in the expression patterns of target genes.

Total RNA was extracted from the ventral telencephalon (subpallium) of wild type and homozygote embryos at various stages using QIAGEN RNeasy Plus® Kit following the manufacturer's protocol. First

Table 1
List of gene specific primers used for the quantitative real-time PCR analyses.

Primer name	Primer sequence (5′–3′)
ΔI56ii.for	GAGGGAAGAAAGACGGGAGT
ΔI56ii.rev	GTCAGAGCCCAAACCTTGAA
I56ii.for	GGATCCCTCAGCAACCCATTTGCAGT
I56ii.rev	GGATCCCAGAGGCTCTGTCTCTATATT