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Characterization of a distinct subpopulation of striatal projection neurons expressing the Dlx genes in the basal ganglia through the activity of the I56ii enhancer

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Regulation of region-specific neuronal differentiation and migration in the embryonic forebrain is a complex mechanism that involves a variety of transcription factors such as the Dlx genes. At least four cis-acting regulatory elements (CREs) are responsible for the Dlx transcriptional regulation in the subcortical telencephalon and the rostral diencephalon. These include I12b and URE2 in the Dlx1/2 bigene cluster, and, I56i and I56ii in the Dlx5/6 cluster. We previously reported that URE2, I12b, and I56i mark different progenitor cell populations in the ganglionic eminences as well as different subtypes of adult cortical interneurons. Here, we carried out a detailed spatial and temporal analysis of the I56ii CRE activity in the developing telencephalon between E10.5 and E15.5, and compared its activity with the other three Dlx CREs using lacZ reporter genes in transgenic mice. We show that I56ii marks distinct group(s) of neurons located in the superficial mantle of the LGE and MGE between E11.5 and E13.5. The I56ii-positive cells are Dlx- and GABA-immunoreactive. However, unlike the other CREs, I56ii does not label interneuron progenitors in the basal ganglia, nor tangentially migrating cells to the cortex at E13.5. Instead, I56ii-positive cells mark a subpopulation(s) of post-mitotic projection neurons that tangentially migrate from the LGE to the deep mantle of the MGE and reside between the subventricular zone and the globus pallidus during midgestation. The majority of these neurons express the striatal markers Meis2 and Isl1. Moreover, both Meis2 and Isl1 activate transcription of a reporter gene containing the I56ii sequence in co-transfection assays, indicating that these transcriptional factors may be potential upstream modulators of the Dlx genes in vivo.

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Introduction

Over the past fifteen years, genetic studies have focused on elucidating the mechanisms underlying the development of the telencephalon, one of the most complex and diverse regions in the central nervous system (CNS). Several models of telencephalic organization and development have emerged implicating a diverse array of signaling molecules and transcription factors that control cell fate specification and proliferation as well as differentiation and migration (for reviews; Guillemot, 2007; Puelles and Rubenstein, 2003; Schuurmans and Guillemot, 2002; Zaki et al., 2003). Hence, programs of regional identity as defined by the expression of distinct transcription factors in different progenitor regions control most aspects of histogenesis within the developing telencephalon. As a result, the embryonic telencephalon is divided into the pallium (primordium of neocortex, hippocampus, piriform cortex and amygdala) and the subpallium (primordium of basal ganglia), which are, in turn, subdivided into distinct progenitor domains (Marin et al., 2003b; Puelles et al., 2000, 1999). Post-mitotic (immature) neurons are generally derived from progenitor cells located nearby in the ventricular zone (VZ) of the neuroepithelium before they translocate to adjacent locations in the mantle through radial migration like during the generation of cortical glutamatergic neurons (Rakic and Lombruso, 1998). However, in some cases, cell types generated in specific progenitor zone(s) migrate orthogonally to the radial axis of the neural tube and intermingle with other neurons in a common final destination, where that cell type apparently is not made, at least in large numbers. This process, known as tangential migration, is widely spread in the developing CNS and contributes to the complexity of the neuronal network found in the vertebrate brain (Corbin et al., 2001; Marin and Rubenstein, 2001b). For instance, the majority of cortical GABAergic interneurons (Anderson et al., 1997a, 2001; Cobos et al., 2001; de Carlos et al., 1996; Gorski et al., 2002; He et al., 2001; Lavdas et al., 1999; Nery et al., 2002; Tamamaki et al., 1997; Wichterle et al., 2001) and cortical oligodendrocytes (Tekki-Kessaris et al., 2001; Thomas et al., 2000) in mouse and chick are born to ventral progenitors located in the subpallium and reach their final destination by tangential migration.

Several genes including Lhx6, Arx and the Dlx genes are involved in the regulation of neuronal migration in the ventral telencephalon.
The Dlx genes are transcription factors that are required for the proper differentiation and migration of most ventrally-derived neurons including striatal projection neurons and cortical interneurons. Thus, Dlx1/Dlx2 null mice display a major block of cell differentiation in the lateral and medial ganglionic eminences (LGE and MGE), and lack both radial and tangential migration of several types of GABAergic, dopaminergic and cholinergic interneurons derived from the subpallium (Anderson et al., 1997a,b, 2001; Bulfone et al., 1998; Long et al., 2007; Marin et al., 2000; Marin and Rubenstein, 2001b; Pleasure et al., 2000; Yun et al., 2002). Four Dlx genes, Dlx1, Dlx2, Dlx5 and Dlx6, are expressed in the forebrain and show highly overlapping expression patterns but with subtle spatio-temporal differences in the telencephalon and diencephalon, suggesting the presence of both redundant and unique functions among these genes (Panganiban and Rubenstein, 2002). Towards elucidating the biochemical mechanisms that control development of ventral progenitors in the telencephalon, we have investigated the regulatory elements and mechanisms controlling Dlx gene expression. We previously showed that shared regulatory mechanisms may underlie the functional redundancy among Dlx paralogs (Ghanem et al., 2003; Zerucha et al., 2000). Hence, we identified and characterized four conserved regulatory elements (CREs) acting as forebrain-specific enhancers for the Dlx genes in vertebrates; I56i and I56ii in the Dlk3/Dlx6 bigene cluster (Zerucha et al., 2000), and, URE2 and 112b in the Dlx1/Dlx2 cluster (Ghanem et al., 2003, 2007). Furthermore, we reported that URE2, 112b and I56i, mark different progenitor cell populations in the ganglionic eminences and different subtypes of adult cortical interneurons (Ghanem et al., 2007). These findings suggest that distinct Dlx functions could be mediated by different regulatory elements and/or mechanisms.

Here we expand our previous findings by demonstrating the existence of a distinct regulatory role played by I56ii when compared with the three Dlx CREs described earlier. We carried out a detailed spatial and temporal analysis of the lacZ reporter transgene expression driven by the I56ii CRE between E10.5 and E15.5 and compared its activities with that of three previously described Dlx CREs. We report that, unlike the other CREs, I56ii is not active in GABAergic interneuron progenitors in the basl ganglia, nor in tangentially migrating cells to the cortex. Instead, it targets lacZ expression specifically to subpopulation(s) of post-mitotic projection neurons that are probably derived from LGE progenitors and have tangentially migrated to the deep mantle of the LGE and MGE between E11.5 and E13.5. In addition, we also identified that the I56ii-positive neurons express two striatal markers, Meis2 and Islet1, during midgestation, both of which can activate transcription via I56ii in co-transfection assays in vitro, suggesting these transcriptional factors may be potential upstream regulators of Dlx genes in vivo. Together, our data reflect a complex and dynamic regulation of Dlx gene expression during the early stages of embryonic development through several regulatory elements with overlapping and distinct function(s).

Materials and methods

Transgenic animals

For transgenic mice, sequences containing the four mouse enhancers (URE2, 112b, I56i and I56ii) were subcloned separately into the p1229/p1230 vectors (Yee and Rigby, 1993) that contain a human β-globin minimal promoter and the lacZ reporter gene. Subclonings were done using a PCR-based approach or using convenient restriction sites and followed by sequencing to verify the integrity of each insert. The p1230-based 112b-alkaline phosphatase (112b-AP) construct was generated as described earlier (Ghanem et al., 2007). At least two independent transgenic lines that show lacZ or AP staining were generated with each construct. Staining results on whole mount embryos and on coronal sections of the forebrain were replicated from independent lines. Transgenic animals were produced and analyzed as previously described (Zerucha et al., 2000).

Histology

E10 to E12.5 mouse embryos were fixed for 45 min to 2 h in 4% cold paraformaldehyde (PFA) in 1× PBS at 4 °C, then, washed and stained with β-galactosidase activity overnight (O/N) at 28 °C in a solution of 1 mg/ml X-gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, and 0.02% NP-40 in PBS. The stained brains were dissected and equilibrated in 20% sucrose solution O/N at 4 °C prior to sectioning. Brains were embedded in Tissue-Tek media the following day, cryoprotected and processed for frozen sectioning at 20–50 μm using a cryostat (Leica CM3050 S). Sections were mounted with Aquatex (EM Science, VWR). E13.5–P0 mouse brains were fixed for 2 h to O/N in 4% cold PFA in PBS at 4 °C, dissected and sectioned prior to staining. lacZ staining and mounting was performed as described above.

Double immunohistochemistry

Frozen sections of E10.5–E15.5 mouse brain were dried for 1 h at room temperature, then, washed for 3 × 5 min each in 0.1 M phosphate buffer (PB) to eliminate residues from tissue protection medium. Immunostaining was performed as described earlier (Ghanem et al., 2007). The following antibodies have been applied in this study: guinea pig anti-β-gal (1:1000, a generous gift from Dr. Thomas Sargent), rabbit pan-Dll antibody (anti-Dlx; 1:100, a kind gift from Drs. Grace Panganiban and JLL.R.R.), rabbit anti-human placental alkaline phosphatase (PLAP, 1:100, Serotec), mouse anti-PCNA (1:300; Vector Laboratories), mouse anti-MAP2 (1:300, Sigma), rabbit anti-active caspase 3 (1:500, BD Biosciences), rabbit anti-GABA (1:4000, Sigma), rabbit anti-calbindin (1:1000, Chemicon), rat anti-somatostatin (1:100, Chemicon), rabbit anti-NNP (1:4000, Immunostar), rabbit anti-TH (1:350, Chemicon), mouse anti-Islet1 (1:100, Developmental Studies Hybridoma Bank), and goat anti-Meis2 (1:100, Santa Cruz Biotechnology). Secondary antibodies were all purchased from Invitrogen: goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 488, goat anti-rat Alexa Fluor 488, donkey anti-goat Alexa Fluor 488, and goat anti-guinea pig Alexa Fluor 594. For PCNA detection, sodium citrate antigen retrieval, pH 6.0, was performed on sections before incubation with PCNA antibodies.

In situ RNA hybridization

In situ hybridization on frozen tissue sections and digoxigenin RNA probe labeling were performed according to the procedures described in (Wallace and Raff, 1999). Hybridized probes were detected with an AP-conjugated anti-digoxigenin Fab fragment antibody (1:2000, Roche) and visualized with the NBT/BCIP substrate system. Antisense riboprobes for Dlx5 and Dlx6 were prepared as previously described (Cobos et al., 2006; Long et al., 2007; Quint et al., 2000).

Co-transfection and chloramphenicol acetyltransferase (CAT) assays

The mouse I56ii CRE was amplified by PCR and inserted upstream of the thymidine kinase (tk) minimal promoter in a pBLCAT2 vector, we called it mI56ii-pBLCAT2. We also created, using the overlapping-PCR method, a modified mI56ii-pBLCAT2 plasmid (mI56ii-mutant-pBLCAT2) containing the mutated Meis2 consensus binding site (TGTC to TCTA) in mI56ii sequence (position nt. 262 to nt. 265, Supplementary Fig. S1). The mouse Islet1 cDNA encompassing the full-length coding sequence (kindly provided by Dr. Steve M Sperber, National Institute of Child Health and Human Development) was subcloned into the EcoRI site of a pC52+ expression vector, designated
Fig. 1. Comparative activities of URE2, I12b, I56i, and I56ii CREs in E10.5 transgenic mouse embryos (A–D) and coronal sections (E–H), as shown by the expression of lacZ reporter gene. A, E: URE2-lacZ; B, F: I12b-lacZ; C, G: I56i-lacZ; D, H: I56ii-lacZ. Lateral views of the mouse embryos (A–D) show that all four CREs are able to target lacZ gene expression in two main domains in the forebrain, the diencephalon (I) and most of the subpallial telencephalon (II). In URE2-lacZ, I12b-lacZ, and I56i-lacZ transgenic lines, lacZ positive cells are largely absent from the VZ except for scattered cells in the URE2 and I56i lines (E, G; arrowheads), and are concentrated as a thick subpial layer (E–G). In contrast, I56ii-lacZ transgenics produce only a few lacZ positive cells present in the subpial region of the LGE at this age (H, arrows). The red arrow in panel E indicates the boundary between the LGE and MGE. 3, third branchial arch; H, hyoid arch (second arch); Hy, hypothalamus; L, limbs; LV, lateral ventricle; Md, mandible; OP, optic eminences; PT, prethalamus (ventral thalamus); ST, subpallial telencephalon; St, somites. Scale bar: (A–D), 1 mm; (E–H), 12.5 μm.

Fig. 2. Different enhancer activities of URE2, I12b, I56i, and I56ii in the subpallial telencephalon of transgenic mice. Coronal hemisections showing lacZ expression under the control of each enhancer at E11.5 (A–D) and E13.5 (E–H) in medial levels. A, E: URE2-lacZ; B, F: I12b-lacZ; C, G: I56i-lacZ; D, H: I56ii-lacZ. At both E11.5 and E13.5, URE2-lacZ, I12b-lacZ, and I56i-lacZ positive cells display a nearly homogeneous pattern of expression in most areas of the subpallium (A–C, E–G). In contrast, expression of the I56ii-lacZ transgene is mainly restricted to a subset of cells in the superficial mantle of the vLGE, MGE, AEP and POA (D, H, arrows), the regions where the other three CRE lines produce weak or no lacZ expression (E–G, arrows). Unlike in the URE-lacZ, I12b-lacZ, and I56i-lacZ lines, I56ii-lacZ does not label cells migrating tangentially to the cortex at E13.5 (H versus E–G, arrowheads). DP, dorsal telencephalon; LV, lateral ventricle; AEP, anterior entopeduncular area; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MZ, mantle zone; POA, pre-optic area; SVZ, subventricular zone; VZ, ventricular zone. Scale bar: (A–H), 250 μm.
as Islet1-pCS2+. The Meis2-pCS2+ expression vector was previously generated in our laboratory (Zerucha et al., 2000). The pSV-β-Gal reporter plasmid encoding β-galactosidase was purchased from Promega and used as an internal control.

Co-transfection assays were performed as follows: briefly, p19 murine embryonic carcinoma cells were seeded at a density of $3 \times 10^5$ in 60-mm-diameter tissue culture plates for 24–36 h prior to transfection. Cells were subsequently transfected with 2 μg of mI56ii-pBLCAT2, mI56ii-mutant-pBLCAT2, or enhancer-less pBLCAT2, 2 μg of each expression vector (Meis2-pCS2+, Islet1-pCS2+, or empty pCS2+), and 2 μg of pSV-β-Gal using the lipofectamine 2000 reagent (Invitrogen). The CAT activity in cell extracts was analyzed 48 h post-transfection on TCL plates by applying the chloroform:methanol solvent system and was quantified by a PhosphorImager as described earlier (Zerucha et al., 2000). After normalizing to the β-galactosidase activity, the relative CAT activity for each expression vector was measured and compared to the basal level of activity seen in the control. All experiments were repeated at least two times in triplicate.

Results

Spatio-temporal comparisons of lacZ expression in four Dlx CRE lines during the development of the telencephalon

Divergence in the sequences of the four Dlx forebrain CREs namely, URE2, I12b, I56i and I56ii, suggests that each of them is involved in distinct aspects of Dlx regulation either spatially and/or temporally. Previously, we showed that URE2, I12b and I56i, have some distinct and overlapping regulatory properties in the developing telencephalon despite highly overlapping activities (Ghanem et al., 2007). To test whether I56ii promotes expression with a distinct pattern, we conducted a detailed spatial and temporal analysis of lacZ reporter gene expression driven by this CRE in the telencephalon. We then compared the lacZ reporter expression of I56ii-lacZ, and, I56i-lacZ, URE2-lacZ and I12b-lacZ on coronal sections between E10.5 and E15.5. All results were confirmed in at least two independent transgenic lines for each CRE.

E10–E10.5

The onset of lacZ expression conferred by all four CREs is detected around E10 in a small cluster of cells located in the region of the primordia of the prethalamus (previously known as the ventral thalamus) (data not shown). This time point is comparable to the onset of endogenous Dlx1 and Dlx2 expression in the prethalamus at E9.0 and E9.5, respectively, as determined by in situ RNA hybridization (Bulfone et al., 1993a; Price et al., 1991) and by RNA blot analysis (McGuinness et al., 1996). This is also consistent with the onset of Dlx5 and Dlx6 expression which occurs around E9.5 (Simeone et al., 1994).

Shortly after their onset, lacZ expression in all four CRE lines becomes visible in the two domains where endogenous Dlx genes are expressed in whole mount embryos. Domain I (diencephalon) includes expression in the prethalamus and a Dlx+ longitudinal domain in the hypothalamus. Domain II encompasses most of the subpallial telencephalon (Figs. 1A–D). In URE2-lacZ, I12b-lacZ, and I56i-lacZ transgensics, β-galactosidase-positive cells are largely absent.

Fig. 3. The I56ii CRE is active in a number of Dlx5- and Dlx6-expressing cells in the telencephalon. Distribution of Dlx5 (A, B) and Dlx6 (C, D) transcripts was compared to the expression of the I56ii-lacZ transgene (E, F) in the telencephalon at E13.5. Note that a stripe of cells, where the I56ii CRE is particularly active, express relatively high levels of Dlx6 transcripts (C, E; arrowheads). Scale bar: (A, C, E), 200 μm; (B, D, F), 100 μm.
from the ventricular zone (VZ) at E10.5 on coronal sections except for scattered cells in the URE2 and I56i lines (Figs. 1E, G; arrowheads), and are concentrated as a thick subpial layer (Figs. 1E–G). At this age, the subpial tissue consists of the emerging subventricular zone (SVZ) and mantle zone (MZ) (Yun et al., 2002). Unlike expression from the other three CREs, I56ii-lacZ transgenics produce only a few lacZ positive cells that are present in the subpial region of the LGE but not the MGE at this age (Fig. 1H, arrows).

E11.5

Transgenic animals produced with all four reporter constructs show stronger lacZ expression in the subpallial telencephalon at E11.5 compared to E10.5. Transgene expression in the forebrain is restricted to regions where endogenous Dlx genes are expressed. Expression patterns of the four transgenes overlap extensively, even though discrete and reproducible differences in enhancer activity are observed, especially between the I56ii-lacZ transgene and the other CREs (Figs. 2A–D and Supplementary Fig. S2). Therefore, URE2-lacZ, I12b-lacZ, and I56i-lacZ are characterized by a nearly homogeneous expression in the SVZ and MZ in most areas of the subpallium (Figs. 2A–C and Supplementary Figs. S2A–C, E–G) with the exception of the VZ in I12b-lacZ and I56i-lacZ lines (Figs. 2B, C, and Supplementary Figs. S2B, C, F, G), and, the ventral MGE (vMGE) in URE2-lacZ lines (Fig. 2A and Supplementary Figs. S2A, E). In contrast, expression of the I56ii-lacZ transgene is only detected in a subset of cells in the MZ that are largely limited to parts of the ventral LGE (vLGE), dorsal and medial MGE (dmMGE and mMGE), pre-optic area (POA) and caudal ganglionic eminence (CGE) (Fig. 2D and Supplementary Figs. S2D, H). Expression of lacZ from all four transgenes is sparse in the septum at this age.

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**Fig. 4.** Characterization of the I56ii CRE activity in the LGE and MGE at E13.5. (A–D) Double immunohistochemistry showing that I56ii-lacZ-expressing cells are Dlx-positive by co-labeling using a pan-Dll antibody and β-galactosidase specific antibody; (E–H) I56ii-lacZ positive cells do not overlap with I12b-AP-expressing cells, indicating that I56ii and I12b/I56i are active in distinct subpopulation(s) of cells at this time point; (I–L) at E13.5, most I56ii-lacZ positive cells have already exited the cell cycle because they do not express the PCNA, which marks all the proliferating cells; (M–P) I56ii-lacZ positive cells are post-mitotic cells since they are co-localized with the molecular marker MAP2; (Q–T) these cells are not apoptotic as they do not co-express the cell death marker, active-caspase 3. Cells expressing I56ii-lacZ are shown in red; while cells expressing Dlx, AP, PCNA, MAP2 or active-caspase 3 are shown in green, respectively. Panels D, H, L, P, T are high-magnification pictures of boxes shown in panels C, G, K, O, S, GP, globus pallidus. Scale bars: (A–C, E–G, I–K, M–O, Q–S), 12.5 μm; (D, H, L, P, T), 8.7 μm.
Regional differences in the activity of I56ii and the other three CREs become more pronounced between E12.5 and E13.5, particularly in the mantle of the ventral telencephalon. Consequently, I56ii-lacZ expression is still restricted to a subgroup of cells located in the deep mantle of the LGE and septum at rostral level (Supplementary Figs. S3D and S4D). It is also found along the superficial mantle of the vLGE, MGE, anterior entopeduncular area (AEP) and POA at medial level (Fig. 2H, arrows, and Supplementary Fig. S3H), and in the deep M2 of the CGE more caudally (Supplementary Figs. S3L and S4H, arrow). In contrast, all of the above regions display weak or no lacZ expression in the other CRE lines at E12.5 (Supplementary Figs. S3E-G, I-K) and E13.5 (Figs. 2E-G and Supplementary Figs. S4E-G, arrows), suggesting that I56ii may be labeling a distinct group(s) of cells at these stages.

I56ii-lacZ expression is still restricted to a subgroup of cells located in the deep mantle of the LGE and septum at rostral level (Supplementary Figs. S3D and S4D). It is also found along the superficial mantle of the vLGE, MGE, anterior entopeduncular area (AEP) and POA at medial level (Fig. 2H, arrows, and Supplementary Fig. S3H), and in the deep M2 of the CGE more caudally (Supplementary Figs. S3L and S4H, arrow). In contrast, all of the above regions display weak or no lacZ expression in the other CRE lines at E12.5 (Supplementary Figs. S3E-G, I-K) and E13.5 (Figs. 2E-G and Supplementary Figs. S4E-G, arrows), suggesting that I56ii may be labeling a distinct group(s) of cells at these stages.

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Furthermore, unlike in the URE2-lacZ, I12b-lacZ, and I56i-lacZ lines, I56ii-lacZ does not label tangentially migrating cells to the cortex at E13.5 (compare Fig. 2H, Supplementary Figs. S4D, H with Figs. 2E-G, Supplementary Figs. S4A-C, E-G, respectively; arrowheads) and E15.5 (compare Supplementary Figs. S5D, H, and L with Supplementary Figs. S5A-C, E-G, and I-K, respectively; arrowheads). Starting at E14.5, I56ii activity is greatly reduced in the ventral telencephalon (data not shown) and is only maintained in a group of hypothalamic cells after E15.5 (Supplementary Figs. S5D, H, L). In contrast, the expression of URE2-lacZ and I56i-lacZ is still strong in the ganglionic eminences at this time (Supplementary Figs. S5A, C, E, G, I, K and data not shown). I12b-lacZ expression remains also visible in most subdivisions of the basal ganglia but with weaker intensity (Supplementary Figs. S5B, F, J).

In summary, our data suggest that I56ii CRE displays a different activity compared with URE2, I12b and I56i, that is confined to group(s) of cells lining the mantle of the ganglionic eminences between E11.5 and E13.5.

I56ii marks a subgroup of striatal projection neurons expressing Meis2 and Islet1

We sought to characterize the identity of the I56ii-lacZ positive cells. First, we checked that I56ii-lacZ-expressing cells are Dlx-positive between E11.5 and E13.5 comparing sections stained with X-gal with consecutive sections hybridized to Dlx5 or Dlx6 cRNA probes (Fig. 3 and data not shown). We also applied double labeling with a pan-Dll antibody and the β-galactosidase antibody (Figs. 4A-D and data not shown). Interestingly, we found that a stripe of cells that extends from the ventral MGE towards the LGE and express the reporter transgene also express relatively high levels of Dlx6 transcripts (Figs. 3C, E; arrowheads).

Then, in order to test whether I56ii and I12b/I56i are active in the same or distinct group(s) of cells, we crossed the I56ii-lacZ line with an alkaline phosphatase (AP) reporter line under the control of I12b (I12b-AP). We then performed double immunohistochemistry on brain sections from double hemizygote embryos (AP+/lacZ+) at E12.5.

![Image](https://example.com/image.png)

**Fig. 5.** I56ii-lacZ positive cells are immunoreactive for GABA (A–D) at E13.5. In contrast to the other Dlx CREs (URE2, I12b, and I56i, Ghanem et al., 2007), I56ii is not active in interneuron progenitor cells (calbindin: E–H; somatostatin: I–L; NPY: M–P) in the basal ganglia or tangentially migrating cells to the cortex at E13.5, except for a few I56ii-lacZ-expressing cells that are calbindin-positive (H, arrowheads). Cells expressing I56ii-lacZ are shown in red; whereas cells expressing GABA, calbindin, somatostatin or NPY are shown in green, respectively. Panels D, H, L, P are high-magnification pictures of boxes shown in panels C, G, K, O. Scale bars: (A–C, E–G, I–K, M–O) 12.5 μm; (D, H, L, P) 8.7 μm.
and E13.5 using β-gal and AP antibodies. As a result, we found that I56ii-lacZ positive cells almost never overlap with cells expressing the I12b-AP transgene at all levels and ages examined (Figs. 4E–H and data not shown).

Next, we investigated whether I56ii-lacZ positive cells proliferate and whether they undergo apoptosis after E13.5. We found that between E11.5 and E13.5, most I56ii-lacZ positive cells have already exited the cell cycle since they do not express the proliferating cell nuclear antigen (PCNA), which marks all proliferating cells (Figs. 4I–L and data not shown). In contrast, the vast majority of these cells express microtubule associated protein-2 (MAP2), a marker of post-mitotic neurons (Figs. 4M–P and data not shown). Furthermore, the I56ii-lacZ-expressing cells do not appear to be in the process of undergoing apoptosis at E13.5 as they did not co-express the cell death marker, active-caspase 3 (Figs. 4Q–T).

Since Dlx genes are expressed in striatal projection neurons as well as GABAergic interneurons both of which are derived from subpallial progenitors, we tested whether I56ii labels neurons belonging to either of these two groups. First, we found that I56ii-lacZ-expressing cells express GABA between E11.5 and E13.5 (Figs. 5A–D and data not shown). However, they do not express any of the interneuron subtype-specific molecular markers expressed at E13.5 including somatostatin and neuropeptide Y (NPY) or the dopaminergic marker — tyrosine hydroxylase (TH), suggesting that they are not immature interneurons (Figs. 5E–P and data not shown). This is true except for a few I56ii-lacZ-expressing cells that are calbindin-positive cells at E11.5 and E13.5 (Fig. 5H, arrowheads and data not shown). As comparisons, the other three CREs label one or more of these interneuron subtypes at E13.5 (Ghanem et al., 2007).

Some I56ii-lacZ positive cells express calbindin, a marker of both striatal projection neurons and cortical interneurons; thus we investigated whether I56ii-positive cells are striatal projection neurons. We co-labeled these cells with two markers of striatal projection neurons, Meis2 and Islet1, whose expression largely resembles that of I56ii-lacZ in the MZ of the ganglionic eminences. The majority of I56ii-lacZ cells are located in a deep mantle layer that is intercalated between the SVZ and more superficial parts of the mantle of the ventral LGE (striatum) and the MGE (globus pallidus) (note: superficial, as in the cortex, refers to being close to pia). This region also expresses the Islet1 (Figs. 6A–D) and Meis2 (Figs. 6I–L) transcription factors at E13.5. In contrast, we did not detect I56ii-lacZ- nor I12b-lacZ-expressing cells that were co-labeled with anti-Islet1 or anti-Meis2 antibodies at this age (Figs. 6E–H, M–P, and Supplementary Figs. S6E–H, M–P). Only a few weakly labeled URE2-lacZ positive cells express either of the two transcription factors in the mantle of the MGE (Supplementary Figs. S6C, D, K, L). Note that, many cells that strongly express URE2-lacZ also express Meis2 or Islet1, but these are located in the mantle of the dorsal LGE where I56ii is not active (Supplementary Figs. S6C, D, K, L).

Taken together, our results lead to the conclusion that I56ii-lacZ-expressing cells in the subpallial telencephalon are a subpopulation(s) of post-mitotic projection neurons that may be derived from ventral LGE progenitor cells, as suggested by their expression of two striatal

Fig. 6. The I56ii CRE is active in a subset of cells expressing Islet1 and Meis2. The vast majority of I56ii-lacZ-expressing cells located in a deep mantle layer that is intercalated between the SVZ and more superficial parts of the mantle of the ventral LGE (striatum) and the MGE (globus pallidus) express two markers of striatal projection neurons, Islet1 (A–D) and Meis2 (I–L) at E13.5. No such co-localization is observed for the I56ii-lacZ positive cells at this stage (Islet1: E–H; Meis2: M–P). Cell expressing I56ii-lacZ or I56i-lacZ are shown in red; Islet1- or Meis2-positive cells are shown in green. Panels D, H, I, P: high magnifications of boxes shown in panels C, G, K, O, respectively. Scale bars: (A–C, E–G, I–K, M–O), 12.5 μm; (D, H, L, P), 8.7 μm.
markers, Meis2 and Islet1. They constitute a distinct population of Dlx-expressing cells compared to those where the other CREs, URE2, I12b, and I56i, are active.

**Meis2 and Islet1 proteins can bind and activate reporter gene transcription via the I56ii enhancer sequence**

To further clarify the existence of a potential regulation of the Dlx genes by Meis2 and Islet1 proteins, we searched the I56ii sequence using the genomatix software and identified two putative binding sites (TGTC) for Meis homeodomain proteins as well as several potential core binding sites (ATTA) for Islet proteins (Supplementary Fig. S1). It is noteworthy that these ATTA-core motifs are also shared by a large number of other homeodomain proteins, such as Hox and Oct proteins (Gehring et al., 1994; Pankratova and Polanovsky, 1998). To explore whether Meis2 or Islet1 binds and activates transcription via these binding sites, we conducted transient co-transfection reporter assays. As noted above, p19 embryonic carcinoma cells were transfected with the mI56ii-pBLCAT reporter construct, together with Meis2-pCS2+ or Islet1-pCS2+ expression vectors (see material and methods for experimental details). We then quantified the relative CAT activity in the presence of Meis2 or Islet1, and found that it was significantly increased by about 4.50- and 3.53-fold, respectively, when compared to controls and after normalization with the β-galactosidase expression (Fig. 7). Co-transfection of a modified mI56ii-pBLCAT vector containing the mutant Meis2 binding site (mI56ii-mutant-pBLCAT2) significantly decreased the transcriptional activation of the reporter gene in the same assay and virtually no activation by Meis2 was obtained with an enhancer-less vector (empty pBLCAT2) (Fig. 7). These data suggest that both Meis2 and Islet1 proteins are potential upstream regulators of Dlx gene expression in vivo and this regulation could be mediated by the I56ii CRE.

**Discussion**

In the present report, we analyzed the regulatory role of the I56ii CRE in the mouse telencephalon using lacZ reporter transgenes. We report that the I56ii CRE shows a strikingly different activity during midgestation when compared with the other three Dlx CREs that are also active in the forebrain: I56i from the Dlx5/6 bigene cluster and, URE2 and I12b, from the Dlx1/2 cluster. Thus, the I56ii-lacZ-expressing cells display the following properties: 1) they express the transgene more transiently, between E11.5 and E13.5. Their expression is restricted to a deep mantle layer that is intercalated between the subpallial SVZ and more superficial parts of the mantle of the ventral LGE (striatum) and the MGE (globus pallidus). The other three Dlx CREs are weakly or not active in these cells (Fig. 2, arrows); 2) unlike the other three Dlx CREs, the I56ii-lacZ-expressing cells do not express markers of interneurons including somatostatin and NPY (Fig. 5 and data not shown) and do not tangentially migrate to the cortex. Rather, the I56ii-lacZ-expressing cells are a subpopulation of post-mitotic GABAergic projection neurons expressing two striatal markers, Meis2 and Islet1 (Figs. 4–6); and, 3) both Meis2 and Islet1 proteins can induce transcription in vitro via one or more putative binding sites found in the I56ii sequence (Fig. 7).

**Comparison of the lacZ reporter gene expression driven by the four Dlx CREs with endogenous Dlx gene expression**

The onset of expression of the lacZ transgene in all four CRE lines is detected in a group of prethalamic cells around E10 and, therefore, slightly contrasts with the onset of the endogenous Dlx gene expression which occurs between E9 and E9.5. This difference could be due to low levels of the β-galactosidase protein that are undetectable before E10 and/or to the existence of other regulatory sequence(s) that are necessary to refine the temporal expression of the Dlx genes. In all four transgenic lines, the β-galactosidase-positive cells are Dlx-positive as suggested by their co-labeling with a pan-Dil antibody (Figs. 4A–D and data not shown; Ghanem et al., 2007). In addition, the patterns of enhancer activity between E10.5 and E15.5 overlap with the endogenous patterns of Dlx expression (Fig. 3 and data not shown). However, the transgene expression in all lines examined lacks one feature of telencephalic Dlx expression at E10.5: previous studies showed that the dorsal LGE (dLGE) has a high concentration of Dlx2+ cells in the VZ (Eisenstat et al., 1999; Yun et al., 2002). As none of the transgenic lines studied here shows this property, it suggests that this expression is conferred by an unknown enhancer element. Furthermore, the β-galactosidase-positive neurons are still found in the mouse neocortex at birth as well as one month after birth in all CRE lines (Ghanem et al., 2007) except in the I56ii lines where lacZ expression is maintained only in a group of hypothalamic cells after E14.5 (Supplementary Figs. S5D, H, L and data not shown).

**I56ii CRE displays a differential activity in comparison with the I56i, I12b and URE2 CREs**

Using double labeling, we showed that I56ii-lacZ- and I12b-AP-expressing cells do not overlap between at E12.5 and E13.5 in the ganglionic eminences (Figs. 4E–H and data not shown). We previously showed that I12b and I56i are co-active in more than 80% of the progenitor population(s) found in the proliferative zones between E11.5 and E13.5 (Ghanem et al., 2007). Therefore, we can infer that I56ii and I56i are likely to be active in distinct group(s) of cells at these time points. Several lines of additional evidence support our conclusion: 1) we found that I56ii-positive cells are Dlx2+/GABA+. They also specifically express Meis2 and Islet1, two markers of striatal projection neurons that are primarily derived from the LGE. In contrast, none of the other three lacZ-CRE transgenes co-expresses Meis2 or Islet1 in the superficial mantle of the ganglionic eminences, except for a few cells weakly expressing URE2-lacZ (Fig. 6 and Supplementary Fig. S6); 2) we showed that URE2, I12b, and I56i are active in distinct subpopulations of immature cortical and hippocampal interneurons that tangentially migrate from subpallium, including populations expressing calbindin, somatostatin and NPY at E13.5 (Ghanem et al. 2007 and this study, data not shown). In addition, we showed that expression from the three elements continue to mark different subtypes of adult interneurons (Ghanem et al., 2007). In
contrast, the i56ii CRE is inactive in cells migrating tangentially to the cortex and its activity is not detectable past E14.5 in the basal ganglia as discussed earlier.

i56ii marks a subgroup of striatal projection neurons that are probably derived from the ventral LGE that tangentially migrate into the pallium

Based on their profile and pattern of expression as well as the orientation of their migratory processes, we suggest that the i56ii-lacZ-expressing cells are GABAergic projection neurons that may derive from progenitors found in the ventral LGE and then migrate tangentially following a dorsal-to-ventral route before they finally settle down between the SVZ and the globus pallidus in the deep mantle of the MGE (Figs. 2 and 6).

There is recent evidence of a novel dorsal-to-ventral tangential migration of neural stem cells in the perinatal forebrain (Willaime-Morawek et al., 2006). Furthermore, tangentially migrating cells can translocate across subdivisions within the subpallium itself including the LGE and MGE as well as the AEP and POA. For instance, progenitor cells found in the AEP/POA region migrate dorsally into the striatum to become cholinergic interneurons (Marin et al., 2000; Zhao et al., 2003). Also, it was shown recently that a subgroup of projections neurons, namely “corridor cells”, tangentially migrate from the LGE to the MGE around E13.5 to establish a permissive route that is essential for thalamocortical axon guidance in mouse (Lopez-Bendito et al., 2006). Furthermore, the so-called “corridor cells” are GABAergic neurons that, like the i56ii-lacZ-expressing cells described here, specifically express LGE markers such as Islet1, Ebf1 and Meis2 (Lopez-Bendito et al., 2006). It will be interesting to determine in the future whether i56ii is a marker of these “corridor cells” during midgestation.

Meis2 and Islet1 are potential upstream regulators of Dlx genes

Our data suggest that the Dlx genes may be potentially regulated by Meis2 and Islet1 to possibly maintain their activities via the i56ii sequence, at least between E11.5 to E13.5. This observation is supported by the ability of both proteins to activate the transcription (by about 3.5–4.5 fold) of a reporter gene via i56ii in transient co-transfection assays in vitro (Fig. 7). There are two potential binding sites for Meis2 in i56ii (Supplementary Fig. S1) and when one of the two sites is mutated in the above sequence, activation by Meis2 is almost totally abolished. Future experiments such as chromatin immunoprecipitation (ChIP) could determine whether this interaction also takes place in vivo. As for Islet1, it was not possible to identify a precise binding site in i56ii as the binding preferences of this transcriptional activator are poorly defined as the ATTA sequence which is found in several locations in i56ii (Supplementary Fig. S1) and which is shared by numerous homeodomain transcription factors.

In conclusion, we showed in the present study that i56ii-lacZ-expressing cells in the ventral forebrain are a subpopulation(s) of post-mitotic projection neurons that may be derived from progenitor cells located in the LGE as suggested by their expression of two striatal markers, Meis2 and Islet1. They constitute a distinct population of Dlx-expressing cells compared to those where the other CREs, URE2, 112b and i56i, are active. Therefore, the distinct activities of the four Dlx CREs, we have identified thus far, reflect a complex and dynamic spatio-temporal regulation of Dlx gene expression during the early stages of embryonic development. Moreover, it highlights that the Dlx genes may have distinct functions in distinct progenitor populations born and derived from the basal ganglia.

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Appendix A. Supplementary data


References


