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***Dlx5/6*-Enhancer Directed Expression of Cre Recombinase in the Pharyngeal Arches and Brain**

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Summary

Dlx5 and *Dlx6*, two members of the *Distalless* gene family, are required for development of numerous tissues during embryogenesis, including facial and limb development. This gene pair is expressed in tandem, transcribed toward each other and separated by a short intergenic region containing multiple putative enhancers. Targeted inactivation of *Dlx5* and *Dlx6* in mice results in multiple developmental defects in craniofacial and limb structures, suggesting that these genes are crucial for aspects of both neural crest and nonneural crest development. To further investigate potential developmental roles of *Dlx5* and *Dlx6*, we used one of the *Dlx5/6* intergenic enhancers to drive *Cre* recombinase expression in transgenic mice. Crossing *Dlx5/6-Cre* transgenic mice with mice from the *R26R* strain results in β -galactosidase staining in the apical ectodermal ridge, brain, and neural crest-derived mesenchyme of the pharyngeal arches, with staining in term embryos observed in the facial skeleton and specific brain structures. However, in contrast to endogenous expression patterns of *Dlx5* and *Dlx6*, *Cre* expression within the pharyngeal arches occurs during a very narrow window in early development. Our studies suggest that *Dlx5/6-Cre* mice may prove useful both in further understanding the function and regulation of *Distalless* genes during development and in studies of gene function in conditional knockout mice.

Keywords

Distal-less; homeobox gene; Cre recombinase; neural crest; craniofacial development; cell fate analysis; transgenic mouse

Signaling cascades initiated by cell–cell or tissue–tissue interactions during development are often conserved between various tissue types. This is especially true for craniofacial, limb, and brain development (Bulfone *et al.*, 1993; Depew *et al.*, 1999). *Dlx5* and *Dlx6*, two of the six mammalian orthologs of the *Drosophila Distalless (Dll)* gene, are intimately involved in development of all three tissues (for reviews, see Merlo *et al.*, 2000; Panganiban and Rubenstein, 2002; Zerucha and Ekker, 2000). These genes are found in a tail–tail tandem in mammals (Simeone *et al.*, 1994) and are expressed in a variety of tissues during embryogenesis, including the developing pharyngeal arches, brain and apical ectodermal ridge (AER) of the limbs. (Acampora *et al.*, 1999; Depew *et al.*, 1999; Qiu *et al.*, 1997), *Dlx5*^{−/−} mice die shortly

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after birth, with defects in the facial skeleton and middle ear ossicles (Acampora *et al.*, 1999; Depew *et al.*, 1999). More extensive developmental defects are observed following targeted inactivation of both *Dlx5* and *Dlx6*, including severe facial and limb defects similar to human Split Hand/Foot Malformation Type 1 (Beverdam *et al.*, 2002; Depew *et al.*, 2002; Robledo *et al.*, 2002). As *Dlx5/6*^{-/-} embryos have an apparent homeotic transformation of the lower jaw into a maxilla-like structure, *Dlx5* and *Dlx6* likely play a crucial role in conferring mandibular identity to cells in the mandibular pharyngeal arch (Beverdam *et al.*, 2002; Depew *et al.*, 2002; Robledo *et al.*, 2002).

The signaling cascades that include *Dlx5* and *Dlx6* in facial, limb, and brain development appear quite complex. For example, expression of *Dlx5* and *Dlx6* in the pharyngeal arches is at least partially induced through endothelin-A (ET_A) receptor signaling (Charité *et al.*, 2001; Yanagisawa *et al.*, 2003), with both required for expression of the gene encoding the bHLH transcription factor dHAND (Charité *et al.*, 2001; Depew *et al.*, 2002). In the limb bud, BMP4 is crucial for initiating *Dlx5* transcription, as blocking BMP4 signaling disrupts *Dlx5* expression (Merlo *et al.*, 2002). Further, *Dlx1*, *Dlx2*, and *Dlx5* can induce expression of a *Dlx5/6-lacZ* construct in isolated slices from an embryonic mouse forebrain (Stuhmer *et al.*, 2002a).

To further characterize the potential roles of *Dlx5* and *Dlx6* in embryogenesis, we have undertaken a fate lineage analysis of *Dlx5* and *Dlx6* daughter cells. We created transgenic mice in which an enhancer from the mouse *Dlx5/6* intergenic region, termed mI56i, was used to direct *Cre* recombinase expression to *Dlx5/6* expression domains (Fig. 1A). Two highly conserved enhancers, mI56i (~440 bp) and mI56ii (~310bp), lie in the intergenic region between *Dlx5* and *Dlx6* (Ghanem *et al.*, 2003). The mI56i enhancer is closest to *Dlx6*, ~1.5 kb from the 3' end of exon 3 and is identical to the human ortholog (hI56i), excluding a 3 bp insertion in the human gene (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003). Further, this enhancer is 81–99% identical in pairwise comparisons between mouse, human, zebrafish, *Sphoeroides nephelus*, and *Takifuga rubripes* (Ghanem *et al.*, 2003). When used in transgenic constructs, the mI56i enhancer directs transgene expression to the pharyngeal arches and brain in mice in a *Dlx5/6*-specific pattern (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003). In contrast, the mI56ii enhancer directs variable expression to the brain and very little expression to the arches (Ghanem *et al.*, 2003). By crossing *Dlx5/6-Cre* transgenic mice with mice from the *R26R* strain, in which *Cre* expression permanently activates *lacZ* expression, we have been able to follow the fate of the *Dlx5/6* cell lineage by staining for β-galactosidase (β-gal) activity.

Microinjection of the transgenic construct in one-cell eggs resulted in the generation of 14 founder transgenic mice. Four male founders with transgene copy number between 10–20 copies per cell were test-bred with *R26R* female mice, with embryos collected at E (embryonic day) 10.5 and stained for β-gal activity. While similar staining patterns were obtained for all four lines, embryos from the 6-1 and 6-2 lines showed the strongest staining in the pharyngeal arches, brain, and limbs and were thus used in our subsequent analysis. In general, expression of the *Dlx5/6-Cre* transgene in the 6-1 line was stronger than in the 6-2 line in all areas other than the brain.

We first examined the extent of *Cre* expression in *Dlx5/6-Cre;R26R* embryos through analysis of β-gal staining. To better determine whether the observed staining was due to active expression of the mI56i enhancer, we also examined *Cre* expression through in situ hybridization analysis. We also compared β-gal staining with *Dlx5* and *Dlx6* expression to understand the extent to which the mI56i enhancer recapitulated endogenous *Dlx5/6* expression. β-Gal staining was first obvious at E9.5 within the first and second pharyngeal arches (Fig. 1B), a pattern mirrored by the expression patterns of *Cre* (Fig. 1E), *Dlx5* (Fig. 1H), and *Dlx6* (Fig. 1K). These cells are presumably neural crest-derived, although expression in

resident non-neural crest mesenchyme cannot be excluded. While *DLX5* expression has been found along the border of the chick neural plate prior to crest cell migration (McLarren *et al.*, 2003), we did not detect β -gal staining or *Cre* expression within the hindbrain or in streams extending from the hindbrain towards the pharyngeal arches. This supports previous studies in which neither *Dlx5* nor *Dlx6* expression has been observed in premigratory neural crest cells (Acampora *et al.*, 1999; Depew *et al.*, 1999; Robledo *et al.*, 2002; Zerucha *et al.*, 2000).

By E10.5, β -gal staining within arches 1 and 2 was more intense than observed at E9.5, with staining also observed in the diencephalon, telencephalon, olfactory placode, and apical ectodermal ridge (Fig. 1C). In contrast, *Cre* expression was found only near the first pharyngeal cleft and in the caudal portion of arch 2 (Fig. 1F), two places of strong *Dlx5* expression (arrows in Fig. 1I). *Cre* expression was detected in other *Dlx5* (Fig. 1I) and *Dlx6* (Fig. 1L) expression domains, including the diencephalon, telencephalon, and AER. By E11.5, β -gal staining in *Dlx5/6-Cre;R26R* embryos (Fig. 1D) resembled the expression patterns of *Dlx5* (Fig. 1J) and *Dlx6* (Fig. 1M) in the pharyngeal arches, where *Cre* expression was not observed (Fig. 1G). The *Dlx5/6* enhancer did not direct *Cre* expression in the *Dlx5/6* expression domain within the otic placode, an aspect previously noted for this enhancer (Zerucha *et al.*, 2000). While our findings suggest that most staining within the pharyngeal arches and arch-derived structures of *Dlx5/6-Cre;R26R* embryos after E9.5 represents a true fate of *Dlx5/6* daughter cells, previous reports have shown that the mI56i enhancer directs *lacZ* expression in the mandibular arch of transgenic embryos at E10.5, as judged by β -gal staining (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003). However, without actual in situ hybridization analysis of *lacZ* expression, it is not possible to determine whether the β -gal staining in these other mI56i transgenic embryos was due to active expression of the *lacZ* transgene or remaining β -gal enzyme, the latter having a half-life of at least 20–30 h (Bachmair *et al.*, 1986).

While the staining patterns in *Dlx5/6-Cre;R26R* embryos closely matched the endogenous expression patterns of *Dlx5* and *Dlx6* (Acampora *et al.*, 1999; Depew *et al.*, 1999; Robledo *et al.*, 2002; Zerucha *et al.*, 2000) and the β -gal staining in *Dlx5/6-lacZ* transgenic mouse embryos staining (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003), the overall intensity of staining in most structures excluding the mandible appears to be less. This suggests that the enhancer unit required for *Dlx5/6* expression in other sites may only be partially encompassed by the mI56i enhancer, resulting in only marginal expression in these sites. It is also likely that this enhancer requires other noncontiguous sequences to drive endogenous expression in specific tissues. Ghanem *et al.* (2003) recently illustrated that the mI56i enhancer drove more robust expression of a GFP construct in the forebrain of transgenic zebrafish when attached to the *dlx6* promoter and a portion of the 5' UTR rather than to the β -globin promoter.

By E13.5, labeled cells in the lower jaw were observed in the primary condensation of the mandibular bone and in the dental mesenchyme surrounding the tooth buds of the lower molars (Fig. 2A) and incisors (data not shown). Labeled cells were also present in the chondrocytes and surrounding perichondrium of Meckel's cartilage (Fig. 2A) the mesenchyme surrounding the future glandular epithelium of the submandibular gland and tongue (data not shown). Labeled cells were not observed in the maxillary bone, although a few β -gal-positive cells were observed in the olfactory epithelium and in oral cavity epithelium overlying the maxillary incisors (data not shown).

As observed at E10.5 and E11.5, *Cre* expression was absent in these structures (data not shown), even though *Dlx5* is expressed within these areas and structures (our unpubl. data; Acampora *et al.*, 1999; Robledo *et al.*, 2002). That *Dlx5/6* daughter cells are observed in the developing jaw in a *Dlx5/6*-specific manner even though the mI56i enhancer is downregulated by E10.5 likely indicates that the mI56i enhancer is mediating *Dlx5/6* expression during early patterning of mandibular crest cells (Depew *et al.*, 2002). Other enhancers would then be required for

later events involving *Dlx* molecules (Acampora *et al.*, 1999; Depew *et al.*, 1999; Ferrari and Kosher, 2002). These findings also illustrate that comparison of β -gal staining in Cre transgene:*R26R* crosses with endogenous gene expression patterns may not be the most accurate method to determine whether β -gal staining is due to fated or active transgene expression.

Although it is thought that tissues and structures within the lower jaw of *Dlx5/6-Cre;R26R* embryos containing labeled cells are all neural crest derivatives, we cannot exclude the possibility that some stained cells are non-neural crest-derived. This is especially true in the mesenchyme surrounding the incisors and molars, as this tissue is composed of both neural crest and non-neural crest-derived cells (Chai *et al.*, 2000). However, our overall staining pattern correlates well with that of Chai *et al.*, suggesting that, within the mandible, stained cells represent postmigratory neural crest cells.

At both E16.5 and E18.5, labeled cells were most abundant in the ossified mandible, where they were observed along the entire proximal-distal axis (Figs. 2B–D, 3A,B,D–F). In whole mount-stained E16.5 embryos, labeled cells were also observed along the midline of the snout just distal to the maxillary incisors (arrows, Fig. 3B). When these embryos were cleared to better visualize staining, labeled cells were observed in the mandibular incisors and entire mandible, including the articular, coronoid, and condylar processes, as well as in the ear pinna, olfactory epithelium, maxillary incisors, and oral epithelium distal to the incisors (Fig. 3D–F). Sections through the lower jaw illustrated the large contribution of stained cells to the mandible, while labeled cells in the mandibular incisor and molar dental papilla were extensively mixed with unlabeled cells (Fig. 2B–D). Labeled cells were also observed in the maxillary incisor dental papilla (Fig. 2E); maxillary molars did not contain labeled cells (Fig. 2C). More scattered staining was observed in the lamina propria of the tongue and Meckel's cartilage (Fig. 2B,D) and the columnar (Fig. 2F) and stratified (Fig. 2E) epithelium of the nasal cavity. Within the middle ear and throat, neural crest-derived structures, including the malleus, incus, stapes, hyoid, and thyroid cartilage, all contained labeled cells, although their contribution to these structures was limited (data not shown).

Labeled cells were also found in areas of the developing brain. In E13.5 *Dlx5/6-Cre;R26R* embryos, labeled cells were restricted in the diencephalon in areas that will eventually form the thalamus and hypothalamus and in the ganglionic eminences and future caudate nucleus of the telencephalon (Fig. 4A). This pattern of staining reflected the endogenous expression patterns of *Dlx5* and *Dlx6* (Acampora *et al.*, 1999; Eisenstat *et al.*, 1999; Liu *et al.*, 1997; Simeone *et al.*, 1994; Stuhmer *et al.*, 2002a; Zerucha *et al.*, 2000). In the brain of E18.5 *Dlx5/6-Cre;R26R* embryos, labeled cells were observed in telencephalon-derived structures, including the caudate nucleus (cn), basal layer of the cortex (bc), and medial nucleus of the amygdala (am) (Fig. 4B–D). In the thalamus, labeled cells were restricted to specific areas that included the bed nucleus (bn), ventral nucleus (vn), and posterior nucleus (pn) (Fig. 4C,D). In the hypothalamus, labeled cells were observed in the lateral (data not shown) and medial preoptic (po) nuclei and premamillary nucleus (pmn) (Fig. 4C,D). Scattered labeled cells were also observed in the olfactory bulb and cerebral cortex (data not shown). These areas represent areas in the mouse brain in which *Dlx5* and *Dlx6* are expressed (Long *et al.*, 2003) and where *zfDlx4/6-LacZ* (Stuhmer *et al.*, 2002b) and *Dlx5/6-Cre* (Stenman *et al.*, 2003) transgenes have been previously reported to be expressed. This active expression prevents any conclusions from being drawn about the specific fate of *Dlx5/6* daughter cells within the CNS. However, our results do illustrate that *Dlx5/6-Cre* expression may be useful in inactivating conditionally targeted alleles within the CNS.

Labeled cells were also observed along the AER and its remnants of the limbs at both E13.5 (data not shown) and E16.5 (Fig. 3C), with β -gal staining in the latter composing the tips of

the limb digits (data not shown). Other sites of β -gal labeling at E18.5 included scattered cells in the neural layer of the eye, kidney, and intestine (data not shown). β -Gal staining was not observed in the membranous bone of the ribs, limbs, and digits, all of which are affected in *Dlx5/Dlx6*^{-/-} embryos. This further illustrates that other enhancers in the *Dlx5/6* locus work in combination with the enhancer used in this study to direct proper temporospatial expression of *Dlx5* and *Dlx6*. Intermittent staining was observed along the smooth muscle of the outflow tract of the heart (data not shown). This was considered to be due to ectopic transgene expression, as the occurrence, extent, and intensity of β -gal staining differed between E18.5 embryos from the 6-1 line and was not observed in the 6-2 line. Further, when the *lacZ* gene is knocked into the *Dlx5/6* locus, β -gal staining is not observed in the outflow tract (Robledo *et al.*, 2002).

In conclusion, we have shown, using a *Dlx5/6-Cre* transgene, that *Dlx5/6* daughter cells are prominently observed throughout the bone and teeth of the lower jaw and in the maxillary incisors of near-term embryos. Fewer numbers of labeled cells are also observed within specific regions of the developing brain and in the AER of the developing limbs. These sites correspond to areas in which *Dlx5* and *Dlx6* are known or hypothesized to have functions during embryonic development. While a previous study used a *Dlx5/6-Cre* transgene to examine *Dlx5/6* daughter cells within the developing brain (Stenman *et al.*, 2003), this is the first report using this enhancer to fate-map a subset of presumably cephalic neural crest cells during craniofacial development. These *Dlx5/6-Cre* transgenic mice should be useful both in better understanding the roles of *Dlx5* and *Dlx6* during development of the mandible, dentition, and the brain and as a valuable tool for creating tissue-specific gene deletions in mice containing conditionally targeted alleles. This is especially true in the mandible, as our mI56i enhancer appears to be downregulated in the mandibular arch by E10.5, allowing a tight temporal inactivation of genes within that tissue.

MATERIALS AND METHODS

Generation of the *Dlx5/6-Cre* Transgenic Construct

The plasmid *pCre* contains a β -globin minimal promoter (Yee and Rigby, 1993) driving the expression of a *Cre* cDNA containing a nuclear localization signal. To construct the *Dlx5/6-Cre* transgene, a mouse genomic *Xho*I fragment containing the mouse id6/id5 enhancer 1 (mI56i; Zerucha *et al.*, 2000) was subcloned into the *Xho*I site of *pCre*, 3' of the SV40 poly A cassette (both the enhancer and *pCre* were kind gifts from M. Ekker). This 440 bp enhancer is one of two highly conserved enhancers in the intergenic region between *Dlx5* and *Dlx6* and lies closer to *Dlx6* than the second enhancer, mI56ii (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003). The resulting plasmid, *pDlx5/6-Cre*, was then restricted with *Kpn*I and *Sac*I. This digest liberated the complete 1.8 kb *Dlx5/6-Cre* transgene, which was separated on a 1% agarose gel, purified by perchlorate elution, and quantified.

Production of Transgenic Mice

To construct *Dlx5/6-Cre* transgenic mice, the purified *Dlx5/6-Cre* construct was injected at 3 ng/ μ l into C57BL6/J $\times$ SJL F₂ fertilized one-cell eggs and two-cell embryos were transferred to pseudopregnant females as previously described (Clouthier *et al.*, 1997). Fourteen founder transgenic mice were identified by dot blot hybridization, with transgene copy number ranging from 2–40 copies per cell. Four of these founders were bred to R26R mice (strain 129/Sv-*Gt*roa26^{tm1Sor}, The Jackson Laboratory, Bar Harbor, ME) (Soriano, 1999). Embryos were collected at E10.5 and stained for β -gal activity to evaluate *Cre* expression. Two lines (6-1 and 6-2) that produced compound hemizygous pups with β -gal activity in a *Dlx5/6*-specific pattern were used for this study.

Genotyping of *Dlx5/6-Cre* Transgenic Mice and Embryos

PCR genotyping was performed using genomic DNA isolated from tail biopsy or embryonic yolk sac. Detection of the *Dlx5/6-Cre* transgene was determined using the primers 5'-AGGGCAGAGCCATCTATTGC and 5'-GCATAACCAGTGAAACAGCATTGCTG. The presence of the *R26R* allele was determined using primers 5'-GCGAAGAGTTTGTCTCAACC-3' (R1295) and 5'-AAAGTCGCTCTGAGTTGTTAT-3' (R26RF) as previously described (Soriano, 1999). Bands were visualized on 1% agarose gels.

β -Gal Staining and Histological Analysis

β -Gal staining was performed as previously described (Ruest *et al.*, 2003). In short, E9.5–11.5 embryos and E16.5 embryos were fixed in 4% paraformaldehyde, rinsed in *lacZ* buffer, and stained overnight at room temperature. Embryos were analyzed and photographed on an Olympus SZX12 stereomicroscope fitted with a DP11 digital camera. After analysis and photography, some E16.5 embryos were dehydrated in methanol before clearing in 2:1 benzyl benzoate: benzyl alcohol to allow better visualization of staining patterns. For staining at E13.5 and E18.5, embryos were embedded in OCT (TissueTek) and sectioned at 14 μ . Sections were fixed in 0.5% glutaraldehyde in PBS, rinsed, and stained at 37°C as described above. Slides were counterstained either with eosin or nuclear fast red, coverslipped with DPX mounting media (BDH), and analyzed with a Nikon E600 microscope and attached SPOT RT digital camera.

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FIG. 1.

Early *Dlx5/6-Cre* expression in *Dlx5/6-Cre;R26R* mouse embryos. **A:** The *Dlx5/6-Cre* transgene consisted of a minimal β -globin promoter fused to a *Cre* cDNA containing a nuclear localization signal. The mouse *id6/id5* enhancer 1 (mI56i) fragment provided tissue-specific expression. **B–M:** Lateral views of E9.5 (**B,E,H,K**), E10.5 (**C,F,I,L**), and E11.5 (**D,G,J,M**) *Dlx5/6-Cre; R26R* embryos stained in whole mount for β -galactosidase (β -gal) activity (**B–D**) or processed for whole mount in situ hybridization using cRNA probes against *Cre* (**E–G**), *Dlx5* (**H–J**), or *Dlx6* (**K–M**). **B–D:** Between E9.5 and E11.5, β -gal-labeled cells (blue) are observed in the mandibular (1) and second (2) pharyngeal arches (red arrows), with staining area and intensity increasing with age. Labeled cells are also observed in the diencephalon (di)

telencephalon (te), and olfactory placode (op). Insets in **C** and **D** illustrate staining in the apical ectodermal ridge (aer). **E–G:** At E9.5, *Cre* expression is observed in a similar pattern to β -gal activity. However, at E10.5, most expression is absent, although small areas near the first pharyngeal cleft and caudal second arch still show *Cre* expression (arrows in **F**). Labeled cells are also present in the diencephalon and telencephalon. By E11.5, *Cre* expression is absent in the arches. **H–J:** *Dlx5* expression mirrors that of β -gal staining between E9.5 and E11.5. Staining at E10.5 is strongest near the first arch cleft and caudal arch 2 (arrows in **I**), correlating to areas in which *Cre* expression is still observed. Note expression in the otic placode (otp), a site in which the mI56 enhancer does not direct transgene expression. **K–M:** *Dlx6* expression appears similar to that of β -gal and *Dlx5* expression between E9.5 and E11.5.

FIG. 2.

Craniofacial contribution of *Dlx5/6* lineage cells in *Dlx5/6-Cre;R26R* embryos. **A–F:** Transverse (**A**) and frontal (**B–F**) sections of E13.5 (**A**) and E18.5 (**B–F**) embryos through the mandible and nasal cavity of *Dlx5/6-Cre;R26R* embryos. After β -gal labeling, sections were counterstained with nuclear fast red. **A:** At E13.5, β -gal-labeled cells are observed in the mandibular bone (md) and dental mesenchyme (arrows) surrounding the mandibular molars (*), but not the maxillary molars (**). Faint staining is also observed in Meckel's cartilage (mc) and surrounding perichondrium. **B–E:** By E18.5, labeled cells are observed in the mandibular bone and are mixed with unlabeled cells in the dental papilla (dp) of the mandibular molars (**B,C**) and mandibular (**D**) and maxillary (**E**) incisors (i). The oral epithelium (oep)

remains unlabeled (**B–D**) except for a few labeled cells underlying the upper incisors (**E**). **F**: In the nasal cavity, labeled cells are observed in the columnar epithelial cells, although contribution is patchy. ns, nasal septum.

FIG. 3.

Contribution of *Dlx5/6* daughter cells to mandibular and maxillary structures. β -gal staining in E16.5 *Dlx5/6-Cre; R26R* embryos. Lateral (**A,C-F**) and ventral (**B**) views of an E16.5 *Dlx5/6-Cre; R26R* embryo stained in whole mount and either photographed (**A-C**) or cleared in benzyl benzoate:benzyl alcohol before photographing. **A,B**: Strong staining is observed throughout the mandible (md), ear pinna (ep), nasal pit (black arrow in **B**), and medial maxilla epithelium (yellow arrows in **B**; see also inset). **C**: An enlarged view of the forelimb (lb) shows β -gal cells along the tips of the digits (arrows). **D-F**: Embryo in **A** cleared, illustrating a contribution of labeled cells throughout the mandible, including the articular (ar), condylar (cp), and coronoid (crp) processes (**F**) and mandibular incisors (i^* in **E**). Staining in the upper jaw is observed in the olfactory epithelium, maxillary incisors (i^{**}) and oral epithelium just distal to the maxillary incisors (arrow in **E**).

FIG. 4.

Analysis of β -gal staining in the brain of *Dlx5/6-Cre;R26R* embryos. Transverse (A), sagittal (B) and frontal (C,D) sections of E13.5 (A) and E18.5 (B–D) *Dlx5/6-Cre;R26R* embryos.

A,B: β -gal-labeled cells are observed in parts of the diencephalon (di) and ganglionic eminences of the telencephalon (te) at E13.5 (A) and in the thalamus (th), hypothalamus (ht) and caudate nucleus (cn) at E18.5 (B). **C,D:** In frontal views showing the right half of brain sections from an E18.5 embryo, labeled cells are observed in areas derived from the telencephalon, including the caudate nucleus (cn), globus pallidus (gp), and basal cortex (bc) (C). In areas derived from the diencephalon, β -gal-labeled cells are observed in the bed nucleus (bn) and preoptic nuclei (po), and are scattered in the middle forebrain bundle (mfb). Labeled

cells are observed in the medial nucleus of the amygdala (am) and in parts derived from the diencephalon, including the reticularis nucleus (rn), ventral nucleus (vn) and posterior nucleus (pn) of the thalamus, and zona incerta (zi) and premamillary nucleus (pmn) of the hypothalamus (**D**). oc, otic capsule; sl, lateral nucleus of the septum; tm, medial nucleus of the thalamus.