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# Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression

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## SUMMARY

**The expression of the Dlx homeobox genes is closely associated with neurons that express γ-aminobutyric acid (GABA) in the embryonic rostral forebrain. To test whether the Dlx genes are sufficient to induce some aspects of the phenotype of GABAergic neurons, we adapted the electroporation method to ectopically express DLX proteins in slice cultures of the mouse embryonic cerebral cortex. This approach showed that ectopic expression of *Dlx2* and *Dlx5* induced the expression of glutamic acid decarboxylases (GADs), the enzymes that synthesize GABA. We also used this method to show cross-regulation between different Dlx family members. We find that *Dlx2* can induce *Dlx5* expression, and that *Dlx1*, *Dlx2* and *Dlx5* can induce expression from a *Dlx5/6-lacZ* enhancer/reporter construct.**

Key words: Dlx, Electroporation, Forebrain, GABA, Glutamic acid decarboxylase, Mouse

## INTRODUCTION

Recent evidence suggests that neurotransmitter subtype specification is linked to dorsoventral patterning (Marín et al., 2000; Wilson and Rubenstein, 2000). For example, in the ventral spinal cord, longitudinal progenitor domains, which are arrayed as distinct dorsoventral tiers, give rise to distinct types of neurons: cholinergic motoneurons arise from more ventral positions than most GABAergic interneurons (Briscoe et al., 2000; Sander et al., 2000). A similar organization appears to be found in the embryonic telencephalon (Marín et al., 2000). In both of these tissues, Nkx genes regulate specification of cholinergic neurons (Sussel et al., 1999; Sander et al., 2000; Briscoe et al., 2000). Transcription factors that are expressed in progenitor domains for GABAergic neuronal zones are candidates for regulating GABAergic specification. In the telencephalon, members of the Dlx homeobox gene family are expressed in, and regulate the development of, the primordia of the basal ganglia (Anderson et al., 1997a; Marín et al., 2000). These large nuclei consist of GABAergic projection neurons. In addition, the Dlx genes are expressed in, and regulate the development of, neurons that tangentially migrate from the basal telencephalon into the cerebral cortex (Anderson et al., 1997b; Anderson et al., 1999; Anderson et al., 2001; Pleasure et al., 2000). These neurons give rise to GABAergic interneurons of the cerebral cortex, hippocampus and olfactory bulb (Anderson et al., 1997a; Anderson et al., 1997b; Bulfone et al., 1998; Lavdas et al., 1999; Pleasure et al., 2000; Sussel et al., 1999; Wichterle et al., 1999). These data support a model that cortical GABAergic and glutamatergic neurons arise from distinct telencephalic progenitor zones and are under distinct genetic controls (Anderson et al., 1997b; Anderson et al., 1999; Anderson et al., 2001; Bulfone et al., 1998; Parnavelas, 2000; Hevner et al., 2001).

The molecular mechanisms through which the Dlx genes regulate the development of forebrain GABAergic neurons remain to be elucidated. To complement Dlx loss-of-function analyses, we have implemented a novel electroporation gain-of-function assay in slice cultures of the embryonic mouse forebrain. Using this approach, we demonstrate that *Dlx2* and *Dlx5*, but not *Dlx1*, can robustly induce cortical cells to express the glutamic acid decarboxylase (Gad) genes. Furthermore, this assay provides evidence for a cross-regulatory cascade of the Dlx genes.

## MATERIALS AND METHODS

### Expression vectors

Coding regions of mouse *Dlx1*, *Dlx2* and *Dlx5* genes (Eisenstat et al., 1999; Zerucha et al., 2000) were cloned into pCAGGS, a chicken β-actin-promoter driven expression vector (Niwa et al., 1991). The original vector was modified to include a greater number of restriction sites 3′ of the promoter (plasmids pCAGGS/ES and pCAGGS/SE). The genes for *Dlx2* and *Dlx5* consisted of their complete and unmodified coding sequences, whereas that for *Dlx1* lacked coding sequence for the two C-terminal amino acids and, potentially, for 5 amino acids (MTMTT) at the predicted N terminus, which contains three closely spaced methionines. Untranslated gene sequences were kept as short as feasible, ranging from 0-7 bases at the 5′ end to maximally 8 bases at the 3′ end.

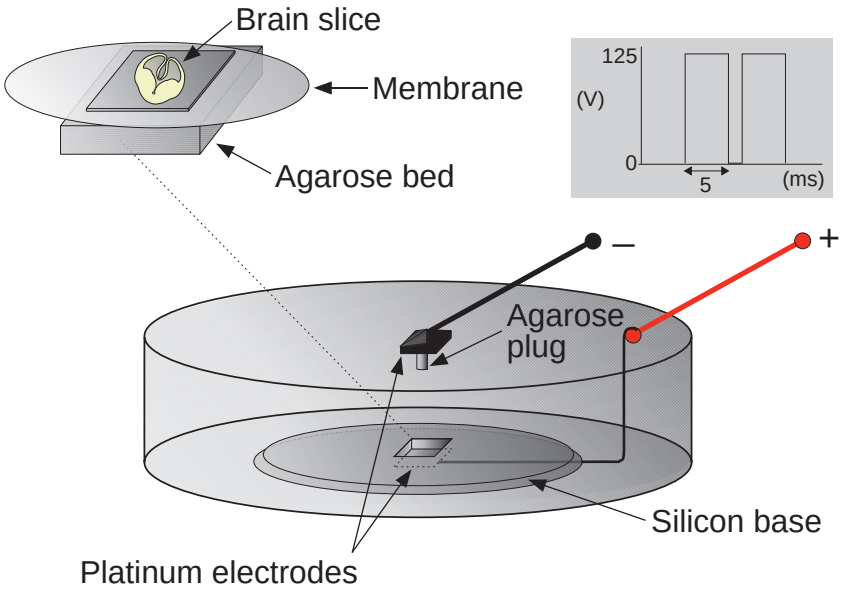


**Fig. 1.** Electroporation apparatus. A pair of horizontally oriented platinum electrodes and a tiny agarose column are used to achieve the focal electroporation of the brain tissue. The lower electrode is fixed with a silicon base at the bottom of a glass petri dish; the silicon base has a central square well. A vibratome-cut brain slice, together with its supporting filter membrane, is placed on an agarose bed, which is inserted into the square well. An agarose column, punched with a hypodermic needle, is attached to the upper electrode. A droplet (~1 μl) of plasmid solution is pipetted onto the lower end of this agarose column, and the electrode is lowered until the solution touches the chosen spot on the tissue. Two square wave pulses of 125 volts and 5 ms duration were used for the electroporations (top right). Although we have not explored whether the polarization has any effect on the success rate, we have always used the lower electrode as the anode.

### Telencephalic slices

Preparation and maintenance of slice tissue cultures followed the methods of Anderson et al. (Anderson et al., 1997a).

### Electroporation

A tissue vibratome slice (in 4% low melting point agarose in 1× Krebs), with its supporting membrane (Nucleopore Track-Etch membrane, Whatman), was placed onto a 1% agarose block (in 1× Krebs buffer) within a setup of two horizontally oriented platinum electrodes (System CUY-700-1 and CUY-700-2; Protech International, San Antonio, TX) (see Fig. 1 for a drawing of the apparatus). A tiny agarose column, punched with a clipped hypodermic needle (gauge 19 or 21) from a 1% agarose gel in 1× Krebs, was attached to the mobile upper electrode. The agarose blocks and columns were made form a gel that was cast between two glass plates (the layer of agarose should not be thinner than 1.5 mm). A small amount of plasmid solution was applied to the lower end of the agarose column, and then the electrode was lowered to let the solution contact the tissue. The system was powered by a T820 Electro Square Porator (BTX). In empirical trials, we determined that plasmid concentrations of about 1 mg/ml at charging voltages of >100 V (with two pulses of 5 ms each) yielded acceptable numbers of electroporated cells.

### Fixation and re-sectioning of slices

After incubation for up to 48 hours, slices were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS), washed in PBS and either embedded in Tissue-Tek or 5% low gelling temperature agarose/PBS for further sectioning on a cryostat (10 μm) or a vibrating blade microtome (50 μm), respectively. This material was subjected to standard in situ hybridization and/or immunohistochemistry procedures.

### In situ hybridization

In situ hybridization was performed along the lines detailed in Porteus et al. (Porteus et al., 1992).

### Immunohistochemistry

Immunohistochemistry was performed on 50 μm free-floating sections. Following pre-incubation for 2 hours in a solution of 2% normal goat serum in PBST (PBS with 0.1% Triton X-100) and 0.1% $NaN_3$, the liquid was changed for a fresh aliquot containing the primary antibody, and the tissue incubated for up to 48 hours at 4°C. Sections were subsequently washed 3× in PBST and the secondary antibody (1:200 goat anti-rabbit IgG, conjugated to either the red fluorescent dye Alexa594 (Molecular Probes), or biotin (Vector)) applied, and incubated for 4 hours at room temperature. The biotinylated antibody was visualized with the ABC kit (Vector), according to the manufacturers instructions. We used the following primary antibodies: anti-distal-less (rabbit polyclonal, 1:400; a kind gift from Dr G. Panganiban, University of Wisconsin-Madison); anti-GAD65 (rabbit polyclonal, 1:2000; Chemicon AB5082); and anti-β-galactosidase (rabbit polyclonal, 1:2000; 5prime-3prime).

## RESULTS

### The Dlx genes are expressed in all GABAergic regions of the embryonic forebrain

In the embryonic forebrain, the expression pattern of the DLX family of transcription factors is nearly identical to that of the glutamic acid decarboxylases (GAD65 and GAD67), enzymes that synthesize the neurotransmitter γ-aminobutyric acid (GABA). For example, at early stages of embryonic telencephalon development, the subpallium expresses *Gad65* (*Gad2* – Mouse Genome Informatics) and *Gad67* (*Gad1* – Mouse Genome Informatics), and *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* (Fig. 2) (Eisenstat et al., 1999; Fode et al., 2000; Liu et al., 1997; Zerucha et al., 2000; Katarova et al., 2000), whereas the pallium does not. Once the subpallial-to-pallial tangential migration begins, bringing GABAergic cells into cortical regions, GAD65 and DLX expression show similar distributions within the cerebral cortex (Fig. 2). Coincident expression of GAD65 and DLX is also notable in subdivisions of the rostral diencephalon (Fig. 2c,d,h,i). Expression of GAD posterior to the zona limitans, is not associated with DLX expression, as central nervous system expression of the Dlx genes appears to be restricted to the anterior forebrain (Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991; Eisenstat et al., 1999). The spatial and temporal coincidence between DLX and GAD expression suggests that the Dlx genes may have an important role in regulating GAD expression. To test this, we used a gain-of-function assay.

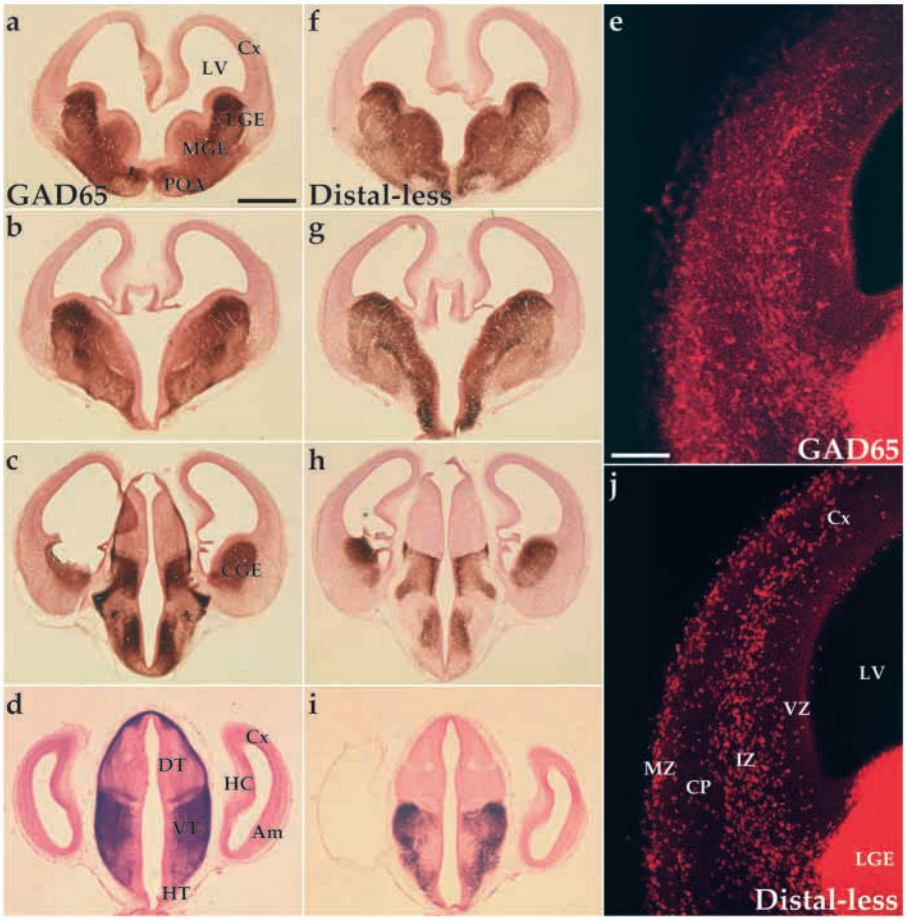


**Fig. 2.** Comparison of GAD65 and DLX protein expression in the forebrain of an E13.5 mouse. Coronal sections (a,f are most rostral) were histochemically stained (DAB reaction) with either an antibody to GAD65 (a-d) or Distal-less (f-i) (which crossreacts with Dlx1, Dlx2 and Dlx5; see Fig. 3). In regions rostral to the dorsal thalamus (DT), the coincidence of GAD65 and DLX expression is striking, except for axon tracts in the hypothalamus that are only $GAD65^+$ (c,d). Immunofluorescent labeling of $GAD65^+$ (e) and $DLX^+$ (j) cells shows the tangential migration from the basal telencephalon in two principal pathways into the cortex. Abbreviations: Am, amygdala; CGE, caudal ganglionic eminence; CP, cortical plate; Cx, cortex; DT, dorsal thalamus; HC, hippocampus; HT, hypothalamus; IZ, intermediate zone; LGE, lateral ganglionic eminence; LV, lateral ventricle; MGE, medial ganglionic eminence; MZ, marginal zone; POA, preoptic area; VT, ventral thalamus; VZ, ventricular zone. Scale bars: in a, 590 μm for a-d,f-i; in e, 140 μm for e,j.

### Use of electroporation of brain slices for ectopic expression of the DLX proteins in the cortex

We adapted an electroporation method (Funahashi et al., 1999) to transfect Dlx expression vectors into coronal slices of the embryonic brain grown in vitro (Anderson et al., 1997a; Anderson et al., 1997b) (Fig. 1). To test whether the DLX proteins could induce GADs, we electroporated the expression vectors into the cerebral cortex from E12.5 mouse embryos. At this age, the cortex is essentially free of GAD- or DLX-positive cells, and selecting the caudal-most sections of the telencephalon for the experiments (Fig. 2d,i) assured that they were severed from the subpallial sources of tangentially migrating $GAD^+/DLX^+$ neurons (Anderson et al., 1997a; Anderson et al., 2001). The caudal-most sections were also consistently more efficiently electroporated.

An expression vector encoding green fluorescent protein (GFP) was co-electroporated with the Dlx expression vectors to identify the regions that expressed the transfected plasmids (Fig. 3a,c,e,g), and to assess the efficiency of each electroporation. Electroporation of *Dlx1*, *Dlx2* and *Dlx5* expression vectors led to the appearance of ectopic DLX immunoreactivity within 3 hours (not shown); by 7 hours, strong expression (comparable with endogenous levels) was detectable (Fig. 5b). Expression from the *Dlx1*, *Dlx2* and *Dlx5* vectors produced roughly equivalent levels of DLX expression, as judged by immunofluorescence with an anti-distal-less antibody (Fig. 3b,d,f). The patterns of GFP and DLX expression were virtually identical (Fig. 3a-f), suggesting extensive co-transfection of the electroporated plasmids. Counting of GFP- and DLX-positive cells showed that >95% of electroporated cells expressed both plasmids.

### Ectopic expression of Dlx genes induces GAD expression

Electroporation of either the *Dlx2* or *Dlx5* expression plasmid readily induced GAD65 immunoreactivity (27/30 experiments for *Dlx2*; 12/20 for *Dlx5*) (Fig. 4b,f; Fig. 5). In the most effective *Dlx2* electroporations, more than 85% of the GFP-positive cells expressed GAD65 (Fig. 4a,b). *Dlx5* was less efficient at inducing GAD65 (Fig. 4e,f), with the most effective electroporations inducing GAD65 in ~50% of the GFP-positive cells. *Dlx1* was inefficient at inducing GAD65 (data not shown); only in four out of 12 electroporations were any GAD65-positive cells detected, and these constituted less than 1% of the GFP-positive cells.

Electroporations of binary combinations of the *Dlx2* and *Dlx5* expression vectors did not appear to increase the amount of GAD65 expression compared with levels seen with the *Dlx2* plasmid alone ($n$=3). Electroporation of the GFP expression plasmid alone never induced expression of GAD65 ($n$>50) (Fig. 4c,d,g,h).

The kinetics of *Dlx2*-mediated GAD65 induction was assessed by analyzing electroporated slices after 7, 19 and 35 hours in culture (Fig. 5). Whereas strong expression of DLX2 could be detected after 7 hours, ectopic expression of GAD65

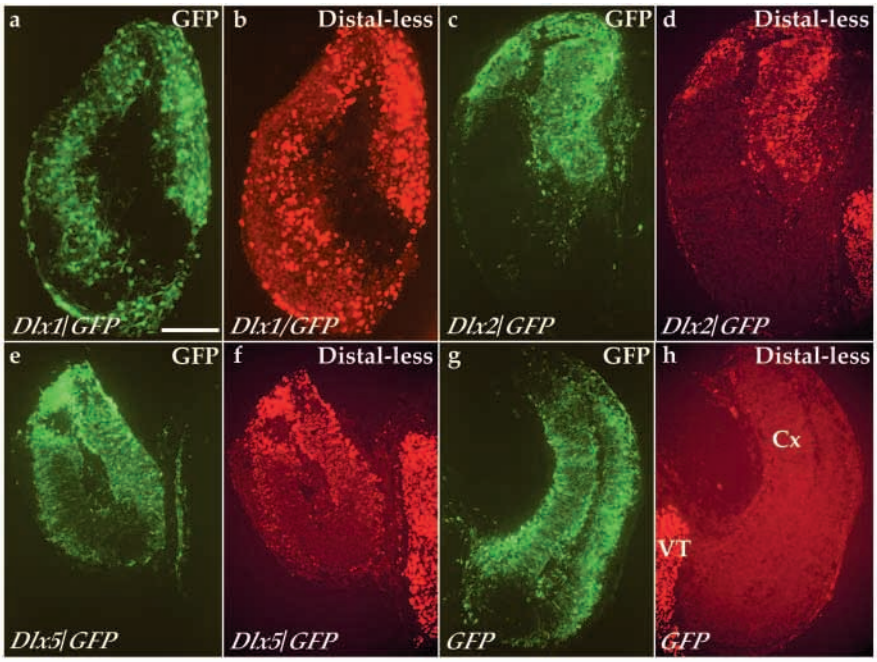


**Fig. 3.** Electroporation of Dlx and GFP expression vectors leads to robust protein expression in coronal slices from caudal regions of the E12.5 mouse telencephalon. Shown are pair-wise depictions of the same section in green and red fluorescence. The bottom left corner indicates which expression vectors were electroporated. GFP and DLX expression are studied in the same slice. The top right corner of each panel indicates which protein was being analyzed: GFP expression was detected by its intrinsic green fluorescence (a,c,e,g); DLX protein expression was detected by immunofluorescent labeling with the anti-distal-less antibody (b,d,f,h). Electroporation of *Dlx1, Dlx2* and *Dlx5* all induced strong expression of the respective DLX protein in a pattern that closely resembles that of the co-electroporated GFP. Electroporation of GFP alone did not induce DLX expression (g,h) (note the endogenous DLX expression in the VT). Abbreviations: Cx, cortex; VT, ventral thalamus. Scale bar: 260 μm in a.

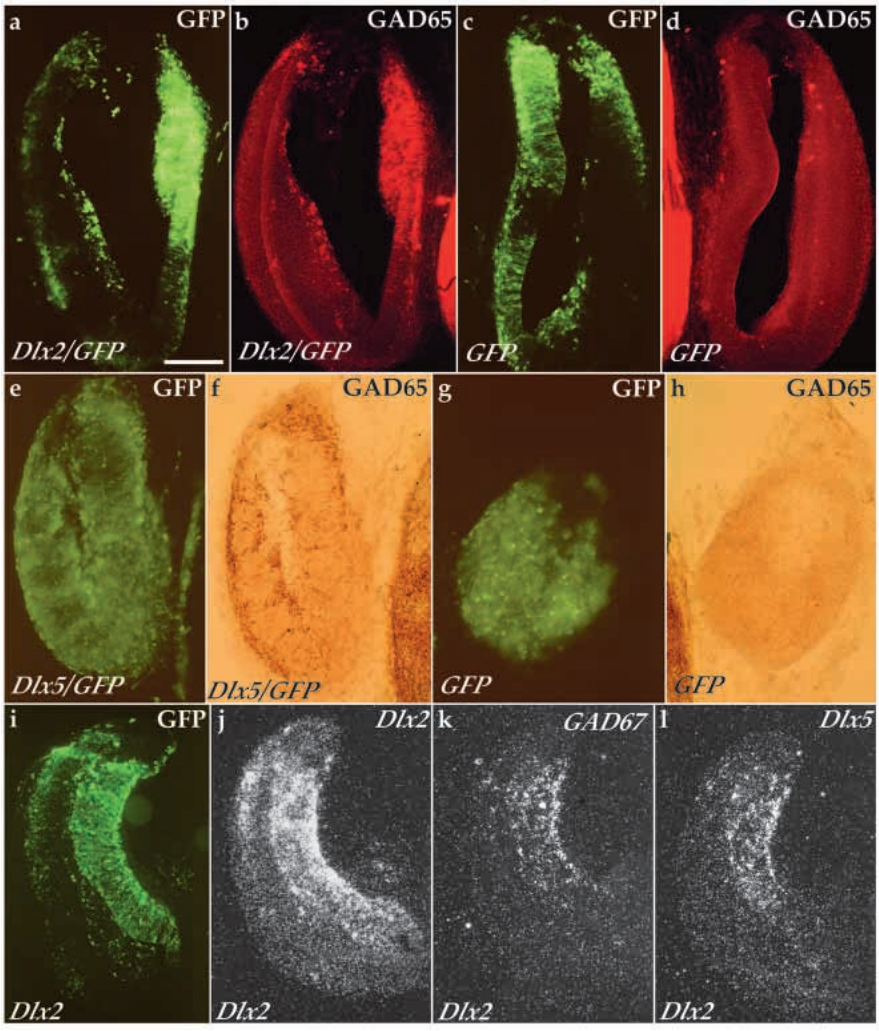


**Fig. 4.** Electroporation of *Dlx2* and *Dlx5* expression vectors induces the expression of glutamic acid decarboxylases (GADs) in caudal regions of the cerebral cortex of the E12.5 mouse telencephalon.
(a-h) Immunohistochemical detection of GAD65 protein in 50 μm free-floating sections following electroporation of *Dlx2* (a,b) or *Dlx5* (e,f). Shown are pair-wise depictions of the same section in green and red fluorescence. The control panels (c,d,g,h) represent the opposite side of the same slice, which was electroporated with the GFP plasmid alone. The bottom left-hand corners indicate which expression vectors were electroporated. The top right-hand corners indicate which proteins were being analyzed. GFP expression was detected by its intrinsic green fluorescence (a,c,e,g); GAD65 was visualized either by immunofluorescence (b,d) or with the DAB reaction (f,h). GAD65 is robustly expressed in the majority of cells electroporated with the Dlx expression vectors (b,f). (i-l) Parallel sections (10 μm) of a slice that was electroporated with the *Dlx2* and GFP expression vectors. The top right-hand corner indicates which protein or RNA was being analyzed. In situ hybridization with a probe for *Dlx2* (j) confirms the abundance of RNA from the introduced plasmid in the electroporated cells (compare the pattern for *Dlx2* with that for GFP in i). In the same region ectopic signals for *Gad67* (k) and *Dlx5* (l) are clearly detected. A probe for *Dlx6* did not produce any signal (not shown). Scale bar: 260 μm.

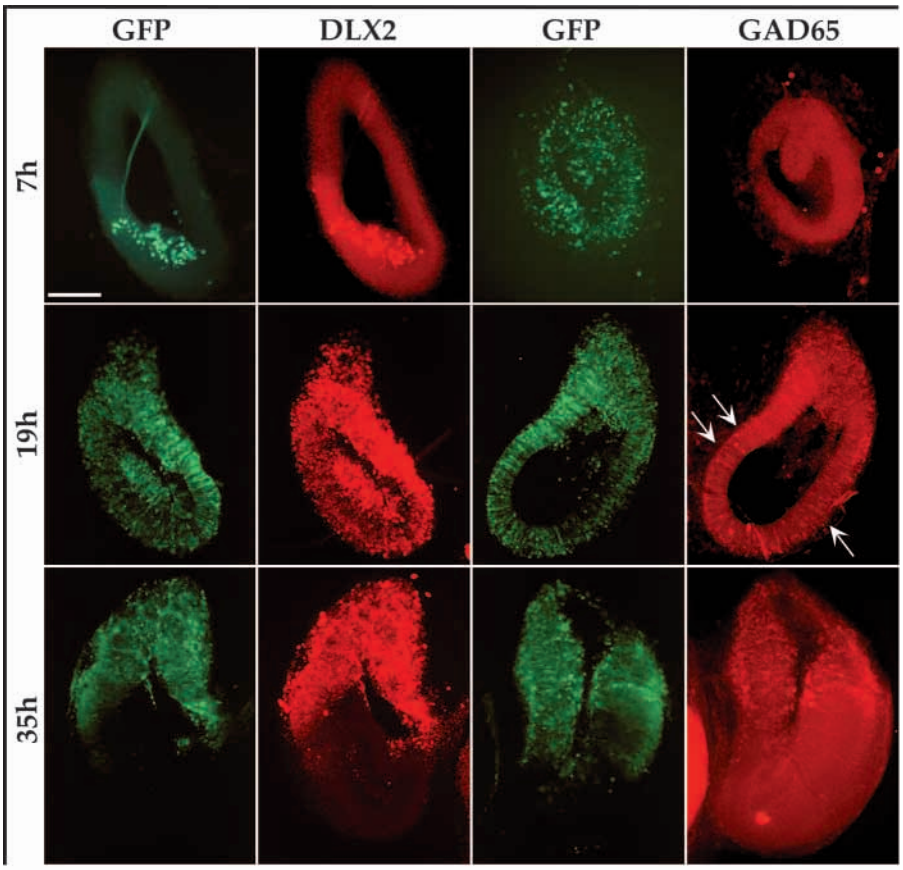


**Fig. 5.** Time course of GFP, DLX and GAD65 induction following electroporation of the *Dlx2* and GFP expression vectors. Time after electroporation is shown on the left column. Electroporated slices were re-sectioned at 50 μm; distinct sections were subjected to immunohistochemistry for Distal-less protein and GAD65. Shown are pair-wise depictions of the same section in green and red fluorescence. The second column shows DLX2 immunoreactivity and the fourth column GAD65 immunoreactivity (note that owing to an experimental misfortune the two 50 μm sections for the 7 hour time point originate from different slices). Whereas DLX2 expression is already high after 7 hours, no immunoreactivity for GAD65 is detectable at this time. GAD65 is clearly expressed 19 hours post-electroporation, and is even more abundant after 35 hours. Arrows in the panel for GAD65 at 19 hours indicate cells with low levels of GAD65. Scale bar: 250 μm.

was not (*n*=3). GAD65 expression was detectable by 19 hours, and was increased by 35 hours (Fig. 5).

Electroporation of the *Dlx2* vector also induced the expression of *Gad67*. As no antibody specific to GAD67 was available, we studied this induction using in situ RNA hybridization (Fig. 4i-k). Although only 5/28 experiments showed a clear induction of *Gad67* by *Dlx2*, we suggest that this is an underestimate, as most of the negative experiments were performed during early stages of the project, when we were establishing the protocol and often encountered low levels of electroporation. The last three experiments show *Gad67* induction by *Dlx2*. Attempts to compare the efficiencies of GAD65 and GAD67 induction have been hampered by our inability to use the same method to study both of their expression (in situ hybridization for *Gad65* has not worked, and we have no antibody specific for GAD67).

Thus, these experiments indicate that *Dlx2* and *Dlx5* are efficient inducers of a fundamental element of the GABAergic phenotype in cortical cells. However, *Dlx2* and *Dlx5* failed to clearly induce other markers that are characteristic of basal telencephalic neurons or cortical interneurons such as NPY, nNOS, substance P, enkephalin, OCT6, calretinin or calbindin, although none of these shows as close a temporal or spatial correlation during development to Dlx expression as do the Gad genes. Furthermore, ectopic expression of the DLX proteins did not seem to affect expression of TBR1, a transcription factor that marks most early cortical plate neurons (Hevner et al., 2001) (data not shown).

### Dlx genes are regulated by DLX proteins

Previous loss of function studies provided evidence that *Dlx1* and *Dlx2* together regulate *Dlx5* and *Dlx6* in most of the forebrain (Anderson et al., 1997a; Zerucha et al., 2000). In addition, an intergenic enhancer for mouse *Dlx5* and *Dlx6* and zebrafish *dlx4* and *dlx6* has been shown to be regulated by *Dlx1* and *Dlx2* in transgenic mice and in tissue culture cells (Zerucha et al., 2000). Here, we used the gain-of-function assay to test whether ectopic expression of *Dlx1*, *Dlx2* and/or *Dlx5* is sufficient to induce expression from endogenous Dlx genes and from a co-electroporated *Dlx5/6* enhancer/reporter plasmid (*mI5/6i-lacZ*) (Zerucha et al., 2000).

Electroporation of *Dlx2* in the cortex induced *Dlx5* RNA, as judged by in situ hybridization (Fig. 4i,j,l). Although the efficiency of this induction was not robust (occurring in only 4/23 cases), we suggest this is an underestimate for the same reasons discussed above regarding the induction of *Gad67*. Electroporation of *Dlx1* did not induce expression of the endogenous *Dlx5* gene (data not shown). Induction of *Dlx6* expression was not detected following electroporation with either *Dlx1*, *Dlx2* or *Dlx5* (data not shown), nor with binary combinations of these expression vectors (*n*=28).

Next we tested whether the Dlx expression vectors could induce expression from a co-electroporated *mI5/6i-lacZ* enhancer/reporter plasmid. We performed the electroporations at various rostrocaudal and dorsoventral positions of the E12.5 brain, and assessed the pattern of β-galactosidase through immunohistochemistry. Control experiments showed that electroporation of the *mI5/6i-lacZ* plasmid alone resulted in β-

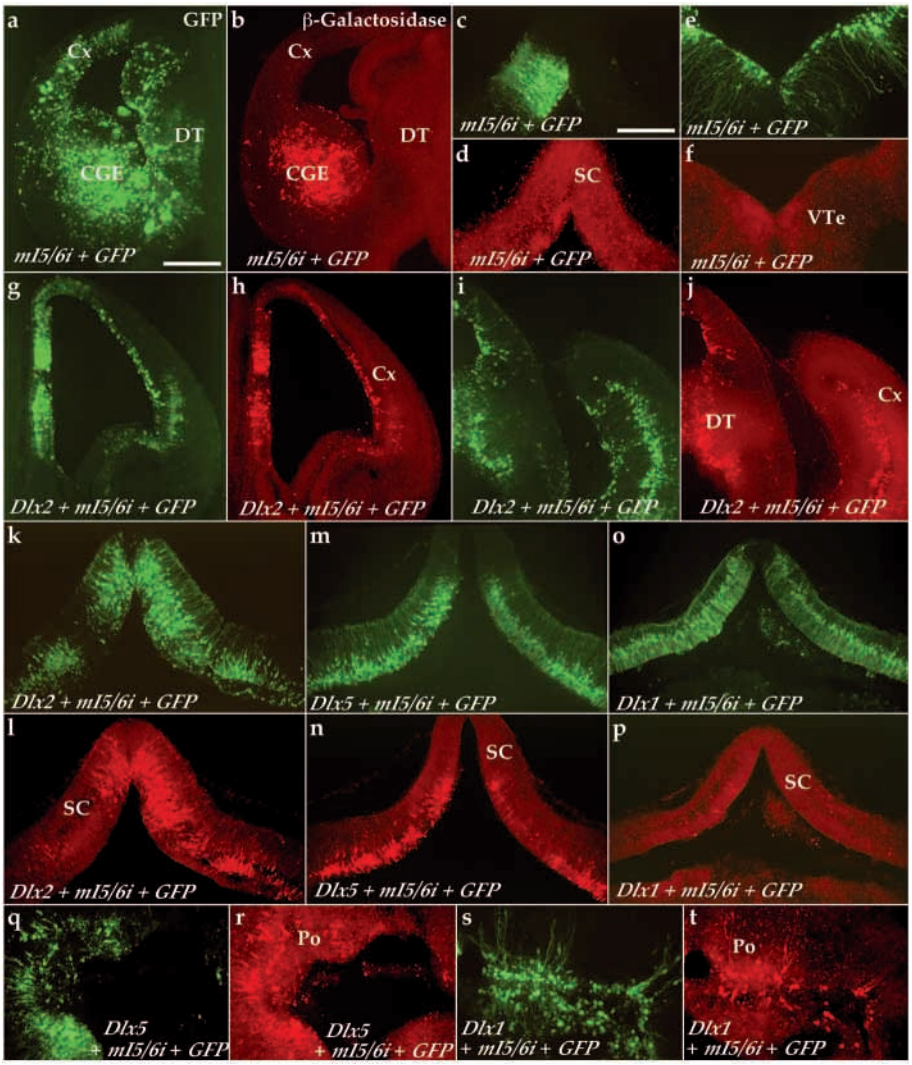


**Fig. 6.** Dlx expression vectors induce ectopic expression of a *Dlx5/6* enhancer/reporter construct. Shown are pairwise depictions of the same 50 μm free-floating section in green and red fluorescence. The bottom left-hand corner indicates which expression vectors were electroporated. Red fluorescence marks the expression of the reporter protein β-galactosidase. (a-f) A plasmid encoding an intergenic mouse *Dlx5/6* enhancer (*mI5/6I*) (Zerucha et al., 2000) upstream of the reporter gene *lacZ* is expressed only in regions that endogenously express Dlx genes (e.g. the CGE but not the cortex (a,b); compare GFP and β-galactosidase expression in the telencephalon). *mI5/6i-lacZ* is also not expressed in all regions caudal to the ventral thalamus, such as the superior colliculus (c,d) or tegmentum (e,f). Adding the *Dlx2* expression vector to the electroporation, leads to strong expression of β-galactosidase in the cortex (g-j), dorsal thalamus (i,j) and in all mid- and hindbrain regions, e.g. the superior colliculus (k,l). The *Dlx5* expression vector also leads to expression of the reporter protein in the superior colliculus (m,n) and the pons (q,r). The *Dlx1* expression vector leads to expression of the reporter protein in the pons (s,t), but not in the superior colliculus (o,p). Note that q and r are rotated 90° counterclockwise, so that dorsal is towards the right. Abbreviations: CGE, caudal ganglionic eminence; Cx, cortex; DT, dorsal thalamus; Po, pons; SC, superior colliculus; VTe, ventral tegmental area. Scale bars: 260 μm in a,c.

galactosidase expression that was restricted to areas that endogenously express the Dlx genes, such as the ganglionic eminences, septum and ventral thalamus (Fig. 6a-f). Thus, *Dlx5/6* enhancer-mediated expression appears to critically depend on the transcription factors that are present in the $Dlx^+$ regions.

To test whether *Dlx2* is sufficient to induce *mI5/6i-lacZ* expression, we co-electroporated the *Dlx2* expression vector with the *mI5/6i-lacZ* and the GFP expression vectors into the cerebral cortex, midbrain and hindbrain. The *Dlx2* expression vector induced robust β-galactosidase expression in the cerebral cortex (Fig. 6h,j), the dorsal thalamus (Fig. 6j), the tectum (Fig. 6l) and the hindbrain ($n$=15). Co-expression of GFP and β-galactosidase regularly exceeded 90% in these experiments (Fig. 6), implying that *Dlx2* induction of expression from *mI5/6i-lacZ* was very efficient. This result also suggests that mixtures of three different plasmids are efficiently electroporated into the same cells.

While the *Dlx5* expression vector was also a potent inducer of β-galactosidase expression throughout the brain (Fig. 6n,r; $n$=15), the *Dlx1* expression vector produced a very different result from the *Dlx2* and *Dlx5* vectors. *Dlx1* induced very little β-galactosidase expression in the cerebral cortex (not shown) and the tectum (Fig. 6p). However, the *Dlx1* vector was capable of inducing β-galactosidase in the dorsal thalamus, the tegmentum (ventral midbrain) and in the hindbrain (Fig. 6t) ($n$=8).

## DISCUSSION

We demonstrate that Dlx and Gad expression in the embryonic forebrain is nearly identical. We also show that ectopic expression of a subset of Dlx genes in the developing cerebral cortex is sufficient to induce the Gad genes. This study establishes that the Dlx genes are sufficient to induce the fundamental phenotype of GABAergic neurons. The results support the hypothesis that the Dlx genes play a significant role in the specification of telencephalic GABAergic neurons (e.g. the projection neurons of the basal ganglia and interneurons of the cortex). Furthermore, these findings may also prove useful for engineering GABAergic neurons from appropriate progenitor cells.

This gain-of-function study complements the analysis of the *Dlx1* and *Dlx2* loss-of-function double mutants, which show differentiation and migration defects in the development of

most telencephalic GABAergic neurons (Anderson et al., 1997a; Anderson et al., 1997b; Anderson et al., 1999; Anderson et al., 2001; Bulfone et al., 1998; Pleasure et al., 2000). Despite the severe block in GABAergic neuron development in the *Dlx1* and *Dlx2* mutants, which includes decreased expression of *Dlx5* and *Dlx6*, GAD expression persists, showing that other transcription factors are also capable of regulating GAD expression in the forebrain. Candidates for these genes include *Mash1* (Ascl1 – Mouse Genome Informatics), and *Gsh1* and *Gsh2*. These transcription factors continue to be expressed in the *Dlx1* and *Dlx2* mutants (Yun and J. L. R. R., unpublished observations), and gain-of-function evidence suggests that *Mash1* is able to induce GABAergic neurons (Fode et al., 2000).

This analysis has begun to dissect the specific roles for the Dlx genes in the development of GABAergic neurons. While they can induce both *Gad65* and *Gad67* within 20 hours, we did not find induction of other markers of GABAergic projection neurons (e.g. enkephalin and substance P) or of GABAergic interneurons (e.g. calbindin and nNOS) (data not shown). Perhaps additional time is needed to see expression of these genes. Alternatively, cortical cells may not be fully competent to express all genes found in GABAergic neurons, as other transcription factors may be needed in parallel, or in conjunction with, the DLX proteins.

While *Dlx2* and *Dlx5* were robust inducers of GADs, *Dlx1* was not. Based on their amino acid sequences, the Dlx genes fall into two major homology groups: Type A (*Dlx2*, *Dlx3* and *Dlx5*) and Type B (*Dlx1*, *Dlx6* and *Dlx7*) (Stock et al., 1996; Liu et al., 1997). These are the first results that suggest biochemical differences in the functions of A and B subtypes. Furthermore, while *Dlx2* and *Dlx5* efficiently transactivated expression from a co-electroporated *Dlx5/6* enhancer/reporter plasmid in every CNS region tested (Fig. 6h,j,l,n,r), *Dlx1* appeared more restricted in its ability to activate the *Dlx5/6* enhancer. For example, while *Dlx1* activated *Dlx5/6* enhancer expression in the ventral midbrain (tegmentum) and in the ventral hindbrain (Fig. 6t), we did not observe any effect of *Dlx1* on the *Dlx5/6* enhancer in the dorsal midbrain (superior colliculus) (Fig. 6p). We also noted that whereas *Dlx2* could induce both endogenous *Dlx5* and the *Dlx5/6* enhancer, it did not effectively induce endogenous *Dlx6* expression. This may be explained by the observation that expression from the *Dlx5/6* enhancer more closely resembles endogenous *Dlx5* than *Dlx6* expression. These results suggest that there is a separate *Dlx6* enhancer, which may be less sensitive to activation by *Dlx2*.

Therefore, the results of this and our previous studies suggest both redundant and distinct functions for different members of the Dlx gene family. *Dlx1* and *Dlx2* are redundant for the control of late-born neurons of the basal telencephalon to efficiently migrate away from the subventricular zone and to express markers of more differentiated neurons (such as DARPP32) (Anderson et al., 1997; Marín et al., 2000) (Yun and J. L. R. R., unpublished). Neither single mutant shows this phenotype. However, we provide evidence that *Dlx1* and *Dlx2* show different abilities to ectopically induce GAD expression, and to regulate the *Dlx5/6* enhancer. Thus, perhaps both Type A (*Dlx2* and *Dlx5*) and Type B (*Dlx1* and *Dlx6*) Dlx genes have redundant functions in regulating aspects of differentiation related to migration, but Type A and Type B Dlx genes may have distinct functions with respect to cross-regulation of Dlx genes and GAD expression. The hypothesis that Type A and B Dlx genes have different functions in vivo, is consistent with the observation that *Dlx1* and *Dlx2* mutants have distinct maxillary dysmorphologies, despite their similar expression patterns in the first branchial arch (Qiu et al., 1997).

The electroporation method used in these experiments is a novel adaptation of the approach devised for in ovo electroporation of chicken embryos (Funahashi et al., 1999). Slice culture electroporation is spatially more precise and it can be readily applied to diverse species. Given the large number of mouse mutants now available, this opens the possibility of rescuing mutant phenotypes. Furthermore, many developmental and physiological processes are largely unperturbed in slice cultures (e.g. neuronal migrations) (Anderson et al., 1997b; Anderson et al., 2001); thus electroporation that does not restrict the size of the transfected plasmid (unlike most viral vectors) will be an effective method with which to study rapidly the effects of genes on specific processes. Finally, because one can efficiently co-electroporate two or more plasmids into single cells, this approach promises to be an important method to study multi-component processes, such as transcriptional regulation in physiologically relevant cells. This proved to be the case for establishing that *Dlx2* and *Dlx5* are effective inducers of the *Dlx5/6* enhancer.

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