Cloning and Cortical Expression of Rat *Emx2* and Adenovirus-mediated Overexpression to Assess its Regulation of Area-specific Targeting of Thalamocortical Axons

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A goal of this study was to use recombinant adenovirus (AdV) to ectopically express *Emx2* in the embryonic neocortex as a gain-of-function approach to study its role in the area-specific targeting of thalamocortical axons (TCAs), using the rat as a model. First, we cloned the cDNA for the full-length coding region of rat *Emx2*, a homologue of *Drosophila empty spiracles*. We also used this sequence to define the full-length coding region of mouse *Emx2* from genomic DNA. Our analysis of *Emx2* expression shows that in rat, as reported in mouse, *Emx2* is expressed in high caudal to low rostral, and high medial to low lateral, gradients across the cortex throughout cortical neurogenesis, and expression is primarily restricted to progenitors in the neuroepithelium. We also carried out an analysis of the distribution of cells infected with a replication defective recombinant type 5 adenovirus (AdV) containing a *CAG/LacZ* expression construct, following an injection into the lateral ventricle of the cerebral hemisphere at different stages of embryonic cortical neurogenesis. AdV-infected cells are broadly distributed tangentially, but their laminar distribution is differentially restricted and reflects the temporal sequence of generation of cortical neurons. This finding indicates that the AdV predominantly infects progenitors in the ventricular zone, which leads to a preferential labeling of their immediate progeny, and infects cells that have recently become postmitotic and have yet to move far from the ventricular surface. We then show that AdV-mediated ectopic *Emx2* expression results in aberrant intracortical pathfinding and areal targeting of TCAs from the dorsal lateral geniculate nucleus. These findings indicate that EMX2 imparts positional information normally associated with caudal cortical areas, such as the primary visual area, that influences the area-specific targeting of TCAs. These results are consistent with a role for EMX2 in areal specification of the neocortex as suggested by recent analyses of *Emx2* null mutants.

Introduction

The mammalian neocortex is parceled along its tangential extent into functionally specialized areas that are distinguished, both radially and tangentially, by unique distributions of neuronal phenotypes, as well as characteristic efferent and afferent axonal projections. Layer-specific properties of neocortical neurons are determined genetically within the ventricular zone shortly before neurons become postmitotic (McConnell and Kaznowski, 1991; Chenn *et al*., 1997). Along the tangential dimension of the neocortex, area-specific properties appear to develop as a result of the early action of regulatory genes distributed in graded patterns across the neocortex, and extrinsic influences, such as thalamocortical axon (TCA) input, which act later within the constraints of the genetic framework (Rakic, 1988; O'Leary, 1989; Chenn *et al*., 1997; O'Leary and Nakagawa, 2002).

TCAs originate in the principal sensory nuclei of dorsal thalamus, are the sole source of modality-specific sensory information relayed to the neocortex, and project to the primary sensory areas in an area-specific manner. The functional specializations of the primary sensory areas are defined by, and dependent upon, TCA input. TCA projections exhibit area-specificity throughout their development, and the gradual developmental differentiation of cytoarchitecture and connections that distinguish cortical areas depends to a large extent upon area-specific TCA input. In addition, experimental manipulations have demonstrated that the neocortex exhibits considerable plasticity in the development of area-specific features, and have implicated TCA input as a major influence controlling this plasticity (Chenn *et al*., 1997).

Evidence for the genetic regulation of arealization has begun to emerge over the past few years (Monuki and Walsh, 2001; Ragsdale and Grove, 2001; O'Leary and Nakagawa, 2002). Indirect evidence included descriptions of graded or restricted patterns of gene expression across the ventricular zone or the cortical plate that are established and maintained in the embryonic neocortex independent of TCA input, and therefore likely controlled by mechanisms intrinsic to the dorsal telencephalon (Miyashita-Lin *et al*., 1999; Nakagawa *et al*., 1999). Genes that regulate arealization presumably confer positional identities to cortical cells and regulate the expression of axon guidance molecules that control the area-specific targeting of TCAs. Two genes proposed to regulate arealization are the homeodomain transcription factor *Emx2* and the paired-box transcription factor *Pax6* (O'Leary *et al*., 1994). In mouse, *Emx2* is expressed in a low rostrolateral to high caudomedial gradient (Gulisano *et al*., 1996) and *Pax6* in a high rostrolateral to low caudomedial gradient (Stoykova and Gruss, 1994; Stoykova *et al*., 1996) across the ventricular zone of the embryonic neocortex.

Recent loss-of-function studies have provided evidence for a role for EMX2 and PAX6 in arealization by analyzing *Emx2* and *Pax6* (*small eye*; *sey*) mutant mice (Bishop *et al*., 2000, 2002; Mallamaci *et al*., 2000). Changes in marker expression and patterns of area-specific TCA projections suggested that rostral–lateral areas are expanded, whereas caudal–medial areas are reduced in *Emx2* mutants (Bishop *et al*., 2000, 2002; Mallamaci *et al*., 2000). Marker analyses of *Pax6* mutants show the opposite changes to those in *Emx2* mutants, suggesting that rostral–lateral areas are reduced and caudal–medial areas are expanded in *Pax6* mutants (Bishop *et al*., 2000, 2002). Thus, *Emx2* appears to preferentially impart caudal and medial area identities, and *Pax6* preferentially imparts rostral and lateral identities. Analyses of *Emx1* single mutants and *Emx1/Emx2* double mutants suggest that EMX1 does not regulate arealization (Bishop *et al*., 2002), despite its close similarity in sequence and

expression to EMX2 (Simeone *et al*., 1992a,b; Gulisano *et al*., 1996). To date, gain-of-function analyses of the roles of EMX2 and PAX6 in arealization have not been reported.

A goal of this study was to use a replication defective recombinant adenovirus (AdV) to ectopically express *Emx2* in the embryonic neocortex as a gain-of-function approach to study its role in the area-specific targeting of TCAs, using the rat as a model. We chose to use an AdV because it infects both mitotically active and postmitotic cells *in vivo* and transcription of the virally transferred genes begins almost immediately after infection (Moriyoshi *et al*., 1996; Tamamaki *et al*., 2001). Cells infected *in vivo* with adenoviral vectors express very high levels of the transgene, typically for 5–10 days postinfection, with a gradual decline in expression level (Verma and Somia, 1997). As a prelude to this experimental phase of our study, we cloned the cDNA for the full-length coding region of rat *Emx2*, since when this project began, coding sequence had been reported for only the homeodomain region of mammalian *Emx2* and some 3′ and 5′ flanking sequence (Simeone *et al*., 1992a,b). In addition, we examined the expression patterns of *Emx2* in rat, focusing on the cerebral cortex during the period of cortical neurogenesis, to demonstrate its patterned expression as reported in mouse [for a review, see Cecchi (Cecchi, 2002)]. We also carried out an analysis of the tangential and laminar distribution of AdV-infected cortical cells following an AdV injection into the lateral ventricle of the cerebral hemisphere at specific stages during embryonic cortical neurogenesis. Finally, we analyzed the effect of AdV-mediated ectopic *Emx2* expression on the intracortical pathfinding and areal targeting of TCAs.

Materials and Methods

Animals

Timed-pregnant Sprague–Dawley rats were obtained from Harlan. The day of implantation is considered embryonic day (E) 0. The first 24 h postnatal is postnatal day (P) 0.

Isolation and Sequencing of Emx2 cDNA Clones

Primers were designed to the conserved regions of the published human and mouse *Emx2* homeodomain sequence (5′ GCGGATCCAAAGCGG-ATTCGAAC and 3′ CCGGAATTCTGAGCCTTCTTCCTC) deposited in GenBank (Simeone *et al*., 1992b). These primers had introduced restriction enzyme sites, *Bam*HI and *Eco*RI, respectively. To obtain template for PCR reactions, RNA from embryonic day 16.5 (E16.5) whole rat brain was treated with DNase1, poly-A selected with Oligo-dT latex beads (TaKaRa), and then cDNA synthesized using Superscript II (Gibco) following the manufacturer's protocols. PCR reactions (50 µl) were prepared containing 1X PCR buffer (15 mM NH_4SO_4, 60 mM Tris pH 8.5, 2.0 mM $MgCl_2$), 0.5 mM of each primer, 0.25 mM each dNTP, and 0.8 U of Taq polymerase. The mix was heated to 90°C, then 0.5 µl of cDNA template added and cycled 35X (95°C 30 s, 54°C 30 s, 72°C 2 min), with a final 72°C extension step for 10 min before cooling to 4°C. PCR products were analyzed on 1.4% agarose gels containing ethidium bromide. The 238 bp PCR product was digested with *Bam*HI and *Eco*RI, subcloned into pBluescript (Stratagene), and sequenced from both directions using T3 and T7 primers, confirming it as *Emx2*. Preparative quantities of the insert were isolated, labeled with ^{32}P, and used to screen 1×10^6 plaques of a rat E18 whole brain cDNA library (courtesy of Prof. H. Nawa, CSH Beckman Laboratory) constructed in a λ ZapII vector (Stratagene). Nitrocellulose filters were hybridized in 5× SSPE, 5× Denhardt's solution, and 0.5% SDS at 55°C overnight. The filters were washed twice in 2× SSPE with 0.5% SDS at 55°C for 30 min and exposed to X-ray film (X-OMAT, Kodak) overnight. Eight positive clones were obtained, one of which contained the entire open reading frame. The 1.5 kb insert from this clone was ligated into the *Eco*RI and *Xho*I sites of pBluescript and sequenced entirely in both directions using the dideoxy chain termination method.

Northern Blots

Total-RNA samples were isolated from CNS tissue derived from different embryonic and postnatal ages of rats using the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). RNA (30 µg per well) was electrophoresed on a 0.8% agarose gel, blotted onto a nitrocellulose membrane overnight (Sambrook *et al*., 1989), and UV cross-linked. Blots were stained with 0.04% (w/v) methylene blue in 0.5 M sodium acetate, pH 5.2 for 45 s, followed by destaining in water for 2 min. Probes were generated by excising full-length coding regions of *Emx2* from cDNA plasmids, gel-purifying the fragments with Geneclean III (Bio 101), and labeling with ^{32}P using the Prime-a-Gene labeling kit (Promega). Pre-hybridization and hybridization were performed in a solution of 0.5 M Na_2HPO_4, 1% bovine serum albumin, 1 mM EDTA, 5% SDS and 20% formamide at 55°C. Probes were added at a concentration of 10^6 dpm/ml, hybridized overnight, and washed in decreasing concentrations of sodium succinate with 0.1% SDS (Sambrook *et al*., 1989) The blots were exposed to X-ray film (X-OMAT, Kodak) for 2.5 days. Films were scanned and figures were prepared directly from these scans using Adobe Photoshop software (Adobe).

In Situ *Hybridization and Densitometry Analysis*

Serial sections of brains were cut either in sagittal or coronal planes at 15 µm on a cryostat, and four to six adjacent series thaw-mounted on 3-aminopropyltriethoxysilane-coated slides (Sigma) and allowed to dry briefly before storing at –70°C prior to *in situ* hybridization. *Emx2* sense and antisense ^{35}S- and digoxigenin-labeled (DIG) cRNA probes were produced using a 780 bp template of the full-length coding region. *Emx1* sense and antisense digoxigenin-labeled (DIG) cRNA probes were produced using a 771 bp template of the coding region (a kind gift from J.J.A. Contos and J. Chun). Plasmids containing the cDNA templates were linearized and transcription reactions with T7 or T3 polymerase (Promega) were carried out in the presence of [^{35}S]UTP (Amersham) or digoxigenin-UTP. The template was degraded with RNase-free DNase (Promega) and extracted with phenol–chloroform. For *in situ* hybridization using radiolabeled probes, tissue sections were processed as described by Bishop *et al*. (Bishop *et al*., 2002). Sections were apposed to autoradiographic film (Hyperfilm, Amersham) and exposed for durations ranging from 6 h to 3 days. All slides for each individual probe were apposed to the same film for each exposure duration (6 h, 12 h, 1 days, 2 days, 3 days). Films were examined using a stereomicroscope and photographed with a digital camera (Kodak DCS 420) mounted on a Nikon Optiphot enlarger. Autoradiographic images were captured digitally using a Kodak DCS 420 camera and densitometric analyses performed using Image software (NIH, v1.6). Measurements of specific binding within individual cortical regions or laminae were obtained by subtracting non-specific binding, determined in near-adjacent sections incubated with sense probes, and were expressed in relative terms to reveal both temporal and spatial profiles in the levels of gene expression in each plane of section. Sections processed for *in situ* hybridization using DIG-cRNA probes were as described by Nakagawa *et al*. (Nakagawa *et al*., 1999). Sections were examined and photographed under an optical microscope (Zeiss Axiophot) and photographed with a digital camera (Kodak DCS 420).

Construction, Propagation and Injection of Recombinant Adenoviruses

We used a human adenovirus type 5 (AdV) deleted of sequences in the non-essential E3 region, and in the E1A/E1B region, impairing the ability to replicate in non-permissive cells (Miyake *et al*., 1996). Construction and propagation of the adenoviral vectors was done essentially as described in Moriyoshi *et al*. (Moriyoshi *et al*., 1996), with the exception that we used an internal ribosome entry site (IRES) from the encephalomyocarditis virus to generate dicistronic expression constructs that produce independent translation of two proteins from the single transgene transcript (Ghattas *et al*., 1991; Martinez-Salas, 1999). Expression was under the control of the strong and ubiquitous cytomegalovirus/chick β-actin hybrid promoter (CAG) (Niwa *et al*., 1991). Expression constructs were sub-cloned into the unique Swa1 site of the cosmid shuttle vector pAdex1w or pAdex1CAwt. To create recombinant adenoviruses, 8 µg of the cosmid shuttle vector carrying our expression construct together with 1 µg of adenoviral genomic DNA tagged at both

ends with a 55 kDa terminal protein were transfected into 70–80% confluent HEK 293 cells using the Lipofectamine method (Life Technologies) in serum-free medium and, 12–16 h later, 1 ml of DME supplemented with 10% FBS was added. Twenty-four hours after transfection, the cells were plated with varying dilutions of untransfected 293 cells in DMEM 5% FBS. Cells were cultured for 2–3 weeks and observed for the presence of dying cells, indicating viral propagation. Positive wells were selected, amplified, and analyzed for the presence of the LacZ reporter gene. Restriction enzyme mapping on the viral DNA confirmed that the recombinant AdV contained the appropriate expression construct. Recombinant viruses were purified over a CsCl concentration gradient, dialyzed and stored at −70°C in 10% glycerol. Recombinant adenoviruses were injected *in utero* in the lateral ventricle as described (Luskin *et al.*, 1988; Austin and Cepko, 1990).

Reporter Gene Detection and Axon Labeling

We used two reporter genes, green fluorescent protein (GFP) and LacZ, which encodes the enzyme, β-galactosidase (β-gal). GFP is a bioluminescent protein that fluoresces green when illuminated with blue light (Chalfie *et al.*, 1994). For X-gal histochemical detection of β-gal, brains were perfused in 2% PF, 0.3% gluteraldehyde and either sections or whole embryos were incubated overnight at 37°C with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside at 0.1% (XGal; Diagnostic Chemicals), 2 mM $MgCl_2$, 5 mM EGTA, 0.01% (w/v) sodium desoxycholate, 0.2% (w/v) Nonidet P-40, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6.6H_2O$. For immunohistochemical localization of β-gal, brains were fixed in 4% PF, cryoprotected in 30% sucrose and cryosectioned. Sections were blocked in PBT, 10% normal calf serum, 0.1% thimosol, 6% H_2O_2, washed in PBS, 1% GS and incubated in a β-gal polyclonal antibody (Cappel) overnight at 4°C. Sections were washed three times in PBS, 1% GS, incubated with a biotinylated goat anti-rabbit antibody for 2 h, washed in PBS and incubated in a fluorochrome or in a streptavidin-peroxidase-conjugated complex. For the latter, staining was visualized with DAB and nickel intensification.

The fluorescent, lipophilic dye, DiI (1–5% wt/vol in DMF, Molecular Probes) was used as an anterograde axon tracer to label TCAs in PF-fixed brains as described (De Carlos and O'Leary, 1992; Braisted *et al.*, 1999). For this, the brainstem was bisected at the midbrain/diencephalic junction and a crystal of DiI was targeted to the dLG or VP. Crystal placement was later verified in bisbenzimide counterstained sections through the dorsal thalamus.

Results

Sequence and Expression Analyses of Rat Emx2

Cloning and Northern Blot Analysis

Using oligonucleotides designed to the published partial *Emx2* sequence for mouse (Simeone *et al.*, 1992a,b), we generated PCR products specific for *Emx2*, and used them as probes to screen a rat E18 whole brain cDNA library to obtain cDNA for the full-length coding region (Fig. 1*A*), as well as additional 3′ and 5′ untranslated regions (data not shown). Northern blots show a single band for *Emx2* transcripts of ~2.8 kb (Fig. 2), as previously reported (Simeone *et al.*, 1992b). Based upon the rat sequence, we were able to define a predicted cDNA sequence for the coding region of mouse *Emx2* (Fig. 1*A*) through analysis of mouse genomic DNA sequence in the 'Mouse HTGS Database' (accession no. AC098733.1). Figure 1*A* shows a comparison of the cDNA sequences for the full-length coding regions of *Emx2* for rat and mouse, as well as published sequence for human (Noonan *et al.*, 2001) and chick (Bell *et al.*, 2001). Figure 1*B* shows comparisons between these species of the predicted amino acid sequences for EMX2 protein.

The predicted amino acid sequence of the homeodomain region of rat EMX2 (Fig. 1) is 100% identical to that for EMX2 of mouse (Simeone *et al.*, 1992b), human (Noonan *et al.*, 2001), chick (Bell *et al.*, 2001), zebrafish (Morita *et al.*, 1995) and dogfish (Derobert *et al.*, 2002). Comparison of predicted amino acid sequences for the full protein shows that rat and mouse EMX2 are 100% identical. Thus, the predicted amino acid sequences indicate that the EMX2, and in particular the homeodomain, is highly conserved across species.

Laminar Expression of Emx2 Compared to Emx1

We examined the laminar expression patterns of *Emx2* compared to *Emx1* in rat brain throughout cortical neurogenesis (Fig. 3). At E12, when cortical neurogenesis begins with the generation of preplate neurons (i.e. the Cajal–Retzius neurons of the marginal zone and subplate neurons), the expression of both genes appears limited to the ventricular neuroepithelium. At later stages (i.e. after E14), when cortical plate neurons are being generated and are migrating through the intermediate zone to the cortical plate, the spatial and temporal expression patterns of *Emx1* and *Emx2* become distinct. The expression of *Emx2* appears confined to the neuroepithelium throughout embryonic development [but see Mallamaci *et al.* (Mallamaci *et al.*, 1998)], *Emx1* is expressed at high levels in the transitional layers (subventricular zone and intermediate zone) and cortical plate between E15 and E18. As the neuroepithelium thins near the end of cortical neurogenesis, *Emx2* expression diminishes and becomes confined to the deeper aspect of the neuroepithelium, and later to the ependymal lining of the lateral ventricle (data not shown). At E19, *Emx1* remains expressed most highly in the neuroepithelium, but in a broader radial domain than *Emx2*, and at moderate levels in the cortical plate and deeper aspects of the subplate.

Graded Expression of Emx2 in the Embryonic Rat Neocortex

In mice, *Emx2* expression begins in the rostrolateral neural plate around E8.5 (corresponding to about E10 in rat) (Simeone *et al.*, 1992a,b; Shimamura *et al.*, 1995). However, we limited our analyses to the period of cortical neurogenesis, which in rat begins on E12 and is completed around E20 (Bayer and Altman, 1991). *Emx2* expression was examined in sagittal (Fig. 4) and coronal (Fig. 5) sections of embryonic rat brain, and found to exhibit graded expression along both rostral–caudal and medial–lateral axes of the neocortex. At E12, levels of *Emx2* were already graded, being higher in caudal cortex relative to rostral cortex; the graded expression continued throughout embryonic cortical neurogenesis. A modest high caudal to low rostral graded expression was observed at E20 (Fig. 6*B*) despite the substantial reduction in the absolute level of *Emx2* expression, and its restriction to the deepest part of the neuroepithelium (Fig. 3). *Emx2* is also expressed in a high medial to low lateral gradient in the embryonic cortex during neurogenesis (Fig. 5). At E20, near the end of cortical neurogenesis (Bayer and Altman, 1991), the M–L gradient is no longer evident (Fig. 6*C*).

We performed a densitometric analysis to quantify the relative temporal changes in expression levels of *Emx2* at successive embryonic ages, as well as its graded expression. Levels of *Emx2* expression in the neuroepithelium were highest at E12, exhibited a precipitous reduction between E13 and E14, then a gradual reduction thereafter (Fig. 6*A*). Densitometry measurements confirmed the graded expression patterns along both the rostral–caudal axis (Fig. 6*B*) and medial–lateral axis (Fig. 6*C*) of the embryonic rat neocortex. Thus, the strongest graded expression of *Emx2* is during the generation of subplate, marginal zone, and the deep layers of the cortical plate.

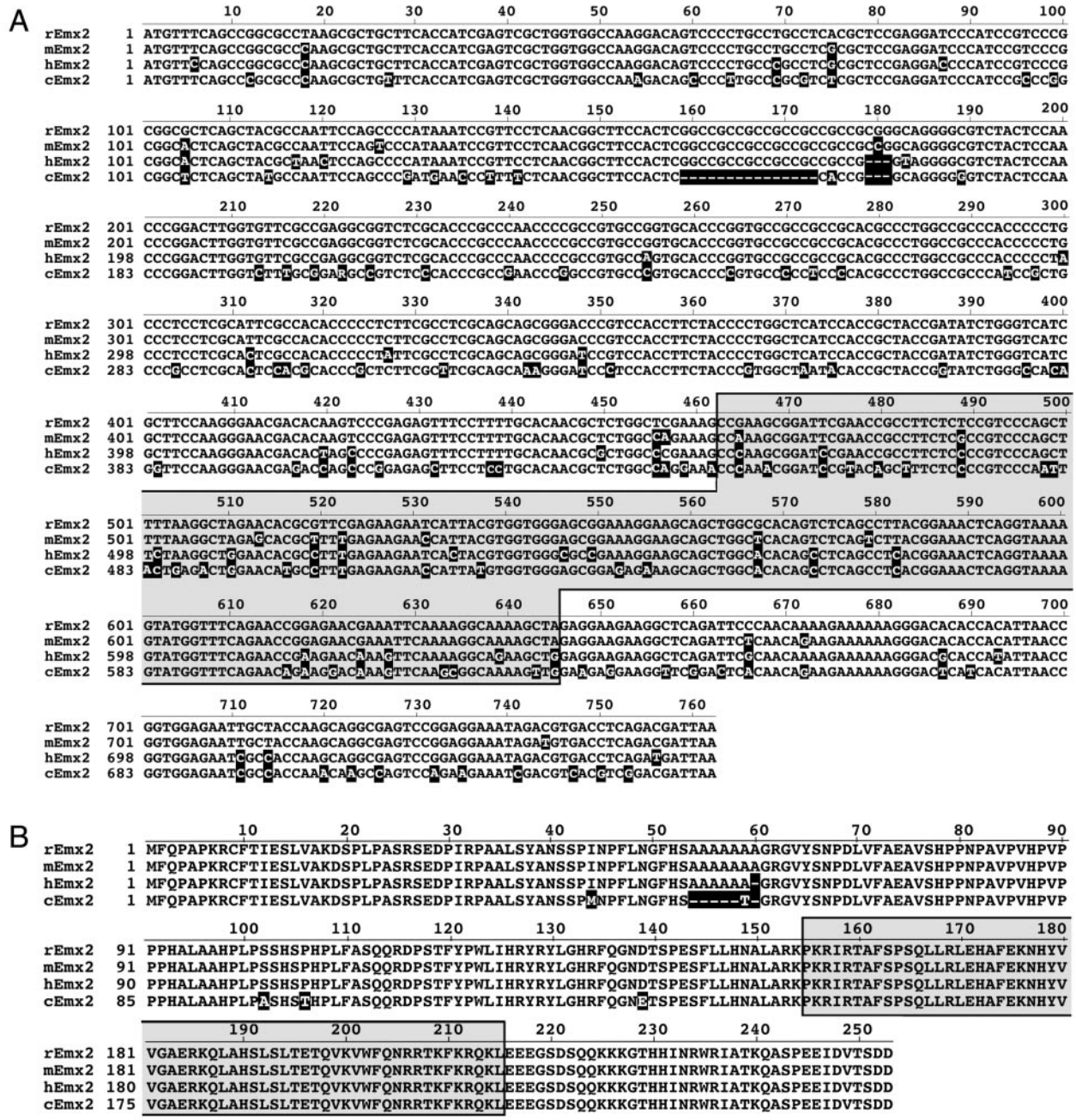

Figure 1. Comparison of cDNA and predicted protein sequences of rat, mouse, human and chick *Emx2*. (*A*) Clones containing rat *Emx2* were derived from cDNA libraries of E18 rat whole brain. Sequence analysis was performed on 1.5kb, which included some 5′ and 3′ untranslated regions. The open reading frame is shown (rEmx2), and compared with the sequence for mouse *Emx2* (mEmx2), which we derived from the database of mouse genomic DNA sequence, as well as published sequences of *Emx2* from human (hEmx2) (Noonan *et al*., 2001) and chick (cEmx2) (Bell *et al*., 2001). Numbers refer to the nucleotide sequence. (*B*) The 251 amino acids in the predicted sequence for rat and mouse EMX2 protein are shown, and compared with those for human and chick. Numbers refer to the protein sequence. In *A* and *B*, non-conserved nucleotides or amino acids, and gaps in sequence which are indicated by dashes, are highlighted in black. The homeodomain is boxed and shaded. See text for a comparison of Emx2 sequences between species.

Expression Efficiency of Dicistronic Vectors

To ectopically express *Emx2* in embryonic neocortex, we constructed a replication defective recombinant adenovirus containing an expression cassette with a CAG promoter, cDNA for the coding region of *Emx2*, an IRES sequence, and as a reporter, *LacZ*, which encodes β-galactosidase (Fig. 7*A*). Although a single RNA transcript is generated, the IRES enables the mRNA for both cDNAs to be independently translated into two distinct proteins (Ghattas *et al*., 1991; Martinez-Salas, 1999). Reportedly, the mRNA upstream to the IRES is translated at roughly twice the level of the downstream mRNA. As a control to show that both gene products are efficiently translated in the adenoviral constructs that we used, we made a dicistronic expression construct with two distinct reporters, GFP and β-gal, by inserting cDNA encoding *GFP* in place of *Emx2*, upstream to the IRES, and leaving the downstream *LacZ* intact. In 293T cells transfected with the *CAG/GFP/IRES/LacZ* pA vector, the transfected cells express both GFP (Fig. 7*B*) and β-gal (Fig. 7*C*), indi-

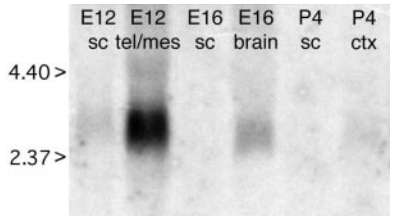

Figure 2. Northern blot analysis of the developmental expression of *Emx2*. Total-RNA samples were isolated from brain tissue derived from different embryonic (E) and postnatal (P) ages of rats. Agarose gel electrophoresis and Northern blotting were performed (see Methods). Northern blots were probed with ^{32}P labeled *Emx2* cDNA probes, revealing a single band of ~2.8 kb at E12 and E16. Controls were done to show that RNA loading was equivalent across lanes (not shown). Abbreviations: ctx, cortex; mes, mesencephalon; sc, spinal cord; tel, telencephalon.

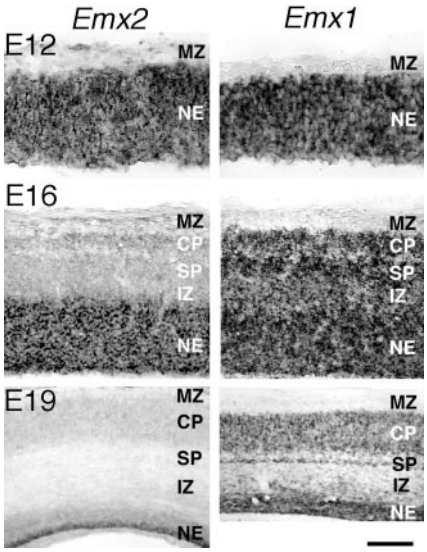

Figure 3. Laminar distribution of *Emx2* and *Emx1* in embryonic rat neocortex. *Emx2* (left panels) and *Emx1* (right panels) expression in E12, E16 and E19 neocortex was examined using digoxigenin-labeled cRNA probes. At E12, most, if not all, cells in the neuroepithelium (NE) were heavily labeled with both *Emx2* and *Emx1*. At E16, *Emx2* expression remained high in the neuroepithelium, but was not detected outside of it. *Emx1* was also found at high levels in the subplate (SP) and newly formed cortical plate (CP), and at moderate levels in the intermediate zone (IZ). At E19, *Emx2* expression was limited to the deepest aspect of the reduced neuroepithelium, adjacent to the ventricle, whereas a broader band of high *Emx1* expression was seen in the neuroepithelium, and moderate *Emx1* expression was evident in the cortical plate and subplate. Scale bar = 50μm at E12; 100 μm at E16; 200 μm at E19.

cating that the *GFP/IRES/LacZ* mRNA transcript is efficiently translated into two distinct proteins. Similarly, the *CAG/Emx2/IRES/LacZ* pA AdV gives strong expression of β-gal in both 293T cells (data not shown) and *in vivo* in neurons (Fig. 9). Although we do not directly localize EMX2 protein, because we have been unable to obtain a specific antibody, our control experiments, and those of others, show that the mRNA encoding the protein upstream to the IRES is translated more efficiently that the one downstream to the IRES (Ghattas *et al*., 1991), leaving no doubt that EMX2 protein is produced at least as abundantly, and likely at twice the levels, of β-gal in the infected cells.

Temporal–Spatial Parameters of Adenoviral Infection in Developing Cortex

We have previously shown that a replication defective recombinant AdV containing a *CAG/GFP* expression construct injected into postnatal rat cortex results in the infection and intense labeling of neural cells, including neurons and glia (Moriyoshi *et al*., 1996). However, in the present study, our aim was to overexpress *Emx2* in embryonic neocortex, preferably in progenitor cells within the neuroepithelium and their progeny.

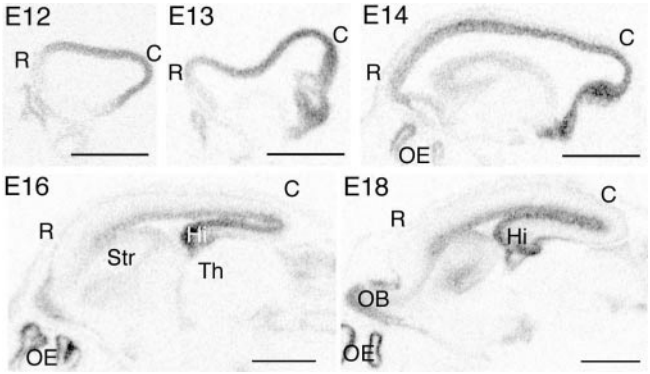

Figure 4. *Emx2* exhibits graded expression along the caudal–rostral axis of embryonic rat cortex. Autoradiograms were generated using an ^{35}S-labeled *Emx2* cRNA probe. These autoradiographic images demonstrate expression patterns of *Emx2* in sagittal sections of embryonic rat brain at the ages indicated. A high-caudal (C) to low-rostral (R) gradient in *Emx2* expression was apparent in the neuroepithelium of the neocortex, as well as the cerebral cortex as a whole, from E12 through E18. Smaller domains of *Emx2* expression are seen elsewhere, including the olfactory bulb (OB), the olfactory epithelium (OE) and the ganglionic eminence — the germinal zone of the striatum (Str). Hi, hippocampus. Scale bars = 200 μm.

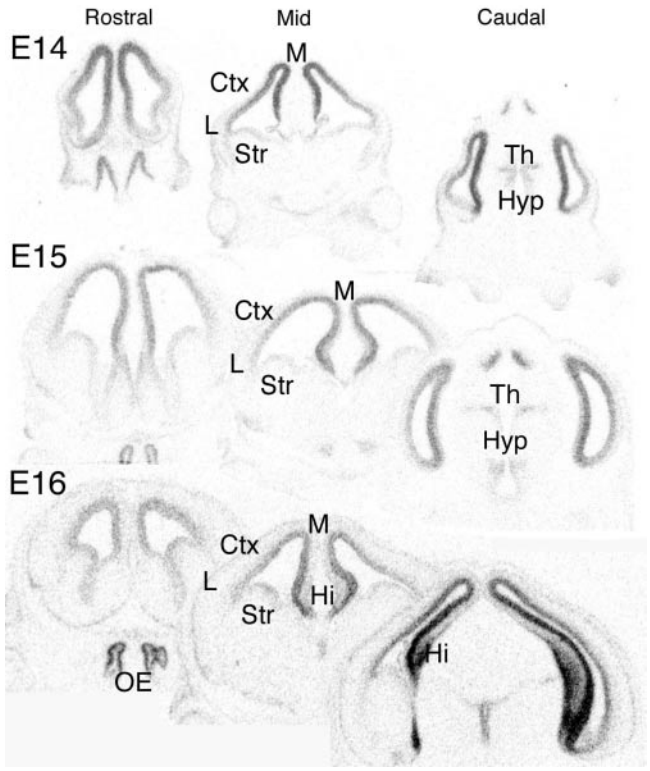

Figure 5. *Emx2* exhibits graded expression along the medial–lateral axis of embryonic rat cortex. Autoradiograms were generated using an ^{35}S-labeled *Emx2* cRNA probe. These autoradiographic images illustrate temporal and spatial patterns of *Emx2* expression in adjacent sections of rat brain at E14, E15 and E16 in coronal sections at three rostral to caudal levels. A high-medial (M) to low-lateral (L) graded expression of *Emx2* is apparent. Smaller domains of *Emx2* expression are seen elsewhere, including the olfactory epithelium (OE), the thalamus (Th), hypothalamus (Hyp), and the ganglionic eminence — the germinal zone of the striatum (Str). Hi, hippocampus. Scale bars = 200 μm.

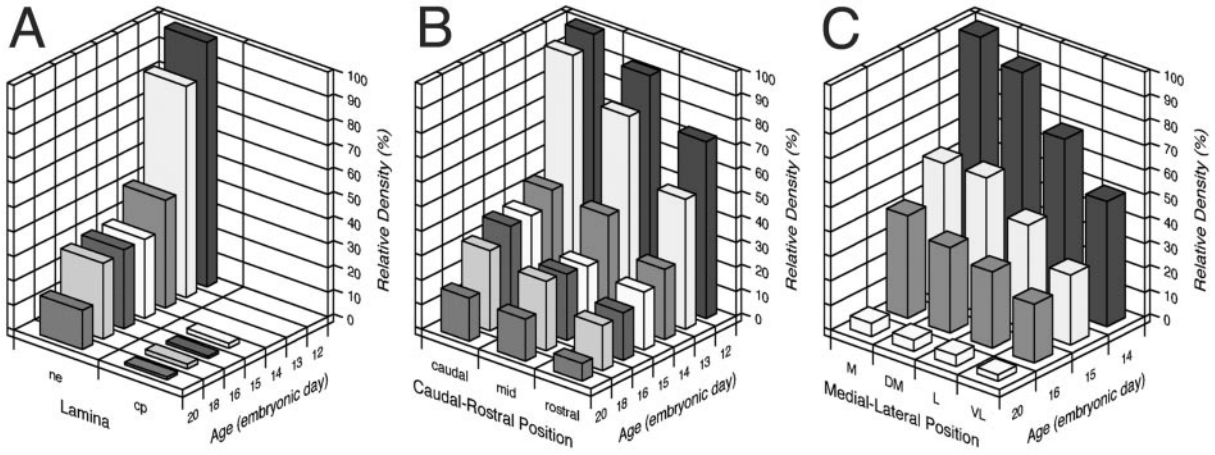

Figure 6. Densitometry analysis of *Emx2* expression in rat neocortex across embryonic development. To quantify the relative levels of *Emx2* expression across ages and axes of the neocortex, densitometry of the relative expression levels were done for sections processed at the same time in the same manner with the same probe, and apposed to the same film. (*A*) Expression levels of *Emx2* were highest in the ventricular neuroepithelium (ne) at E12, decreased precipitously at E14, and declined slowly thereafter. *Emx2* expression was mainly restricted to the neuroepithelium (ne) throughout development, with levels only slightly above background in the cortical plate (cp) and marginal zone. (*B*) Densitometric measurements in sagittal sections from equally spaced locations in the caudal, rostral and middle thirds of the developing neocortex from E12 to E20 embryos. *Emx2* is expressed in a high-caudal to low-rostral gradient between E12 and E18; by E20, when neurogenesis is concluding, graded expression is virtually absent. (*C*) Densitometry measurements from four equally spaced, positions along the medial–lateral axis of the neocortex (D, dorsal; DM, dorsal–medial; L, lateral; VL, ventral–lateral) indicate a M–L graded expression for *Emx2* at E14 to E16, but not at E20.

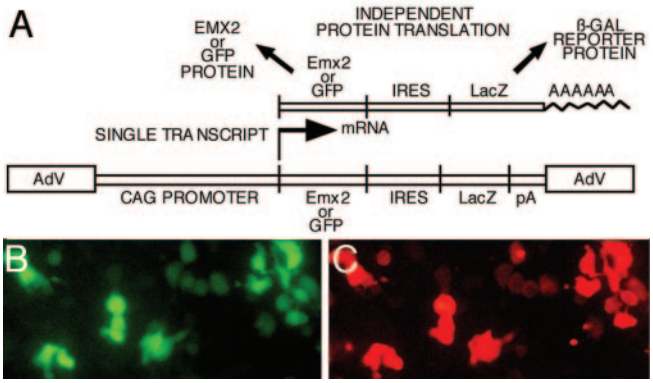

Figure 7. The dicistronic expression construct results in efficient translation of two distinct proteins. (*A*) Diagram of the dicistronic expression construct engineered into a replication defective recombinant AdV; expression was under the control of the strong and ubiquitous CAG promoter (Niwa *et al*., 1991). The use of the IRES to make a dicistronic expression construct results in a single transcript that is translated into two independent proteins in transfected or infected cells. Abbreviations: AdV, adenovirus DNA; IRES, internal ribosomal entry site; β-GAL, β-galactosidase; CAG, cytomegalovirus/chick β-actin hybrid promoter; GFP, green fluorescent protein. (*B*, *C*) The same field showing colocalization of GFP (*B*) and β-gal (*C*) proteins in 293T cells transfected with the dicistronic expression construct, *CAG/GFP/IRES/LacZ* pA. All cells that express GFP, also express β-gal (the protein product of the *LacZ* gene), and vice versa. Cells were transfected using liposomes (Lipofectamine) with 10 μg of the expression construct cDNA per 35 mm dish. After 3 days, cells were fixed, stained with a β-gal antibody and a rhodamine-conjugated secondary, and viewed with a fluorescence microscope using a fluorescein filter for GFP and a rhodamine filter for β-gal immunostaining.

As a prelude to our experimental AdV studies, we assessed the effectiveness and spread of cortical AdV infections. For these studies, we injected a replication defective recombinant AdV containing a *CAG/LacZ* pA expression construct unilaterally into the lateral ventricle of the cerebral hemispheres at sequential times over embryonic cortical neurogenesis. Injections were done at E13.5, E15.5, E16.5 and E17.5, and the brains were fixed at P4 or P12, and reacted for β-gal to localize AdV-infected cells. The distribution and density of labeled cells across the tangential axes of the cortex varied among cases, likely due to the effectiveness and placement of the AdV injection in the lateral ventricle. However, an injection at each of these ages often resulted in a high density of labeled cells broadly distributed tangentially across the cortex (Fig. 8). In contrast, the laminar distribution of infected cells varied in a reproducible manner that depended upon the age of AdV injection (Fig. 8).

An AdV injection at E13.5 resulted in a high density of infected cells in the subplate and the deeper layers of the cortical plate, and moderate to low density of infected cells in more superficial layers of the cortical plate (Fig. 8*A–D*). Interestingly, a low density of infected cells was seen in the marginal zone (Fig. 8*B*,*C*). These cells are likely to be Cajal–Retzius neurons, the only postmitotic cortical neurons reported to express *Emx2* (Mallamaci *et al*., 1998), which together with subplate neurons, are the earliest generated cortical neurons. These two populations of early-generated neurons initially form the preplate, which is later split by the cortical plate into the marginal zone and subplate. The pattern of infected cells in brains injected at E15.5 had similarities to the E13.5 cases, but the density of labeled cells in the subplate was lower, and few if any labeled cells were detected in the marginal zone. An AdV injection done at E16.5 (Fig. 8*H–K*) or E17.5 (Fig. 8*L–N*) resulted in labeling patterns very distinct from the earlier injections. In both, a high density of infected cells were localized to the superficial layers of the cortical plate, whereas the subplate, marginal zone and deep layers of the cortical plate were virtually devoid of infected cells. The high density band of infected cells in the superficial layers was somewhat broader with an E16.5 injection (Fig. 8*J*,*K*) than an E17.5 injection (Fig. 8*N*).

The laminar distribution of cells infected with AdV injected at successive ages reflects the inside-out generation of cortical neurons, and is reminiscent of the labeling patterns observed using birthdating markers injected at the same ages (Bayer and Altman, 1991). The early injections preferentially label the earliest generated cortical neurons, those that form the preplate (marginal zone and subplate neurons) and the deepest layers of the cortical plate, and later injections label the later

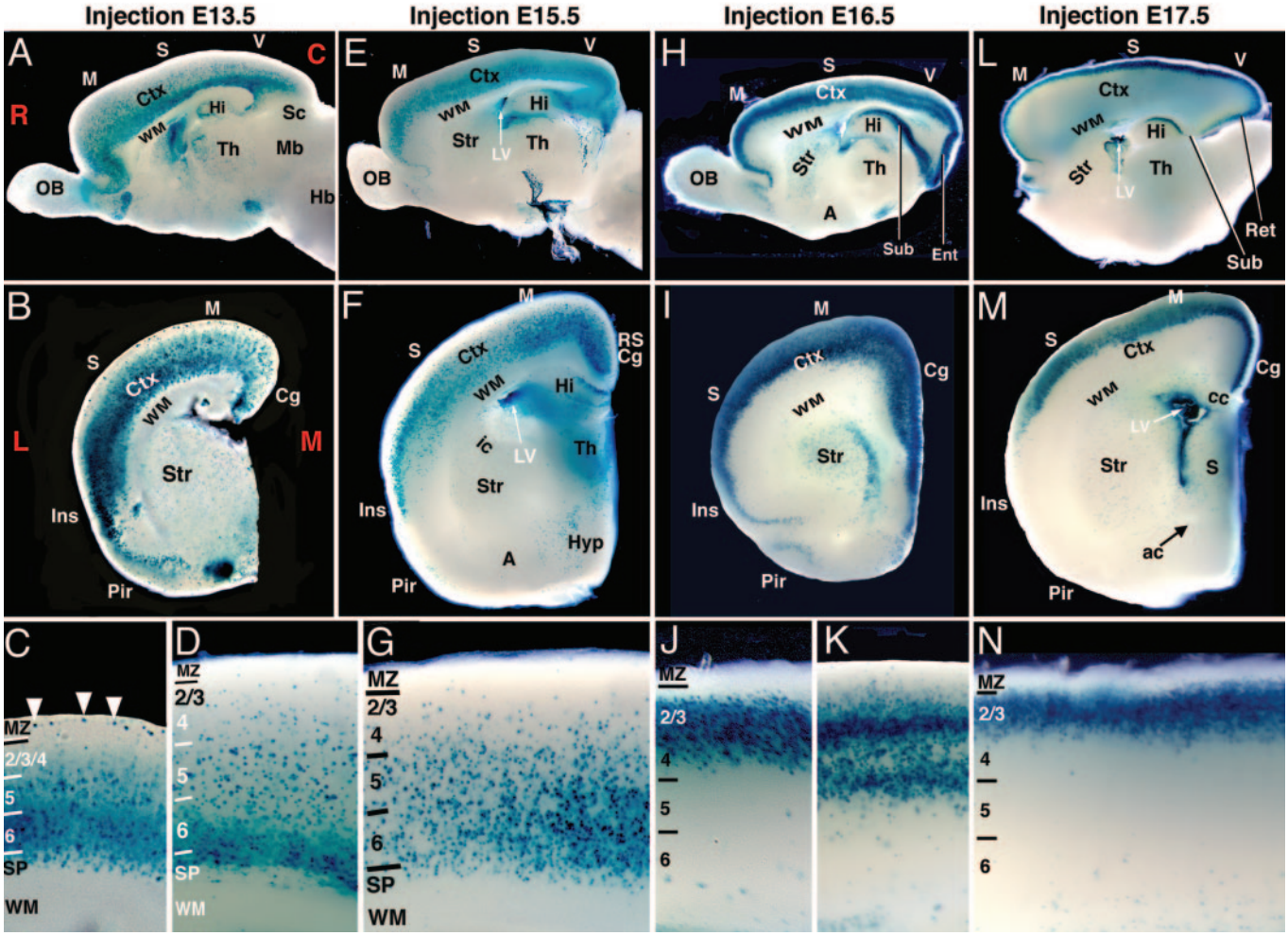

Figure 8. Transgene expressing cells have widespread tangential distribution but restricted laminar distribution dependent on age of AdV injection. A replication defective AdV containing a *CAG/LacZ* pA expression construct was injected unilaterally into the lateral ventricle of the cerebral hemispheres at E13.5 (*A–D*), E15.5 (*E–G*), E16.5 (*H–K*) or E17.5 (*L–N*). Brains were fixed and analyzed for LacZ expression at P12, with the exception of the case shown in panels *B–D*, which was fixed at P4. AdV-infected cells were identified by expression of the β-gal reporter. (*A*, *E*, *H*, *L*) Sagittal sections; (*B*, *F*, *I*, *M*) coronal sections. (*C*, *D*, *G*, *J*, *K*, *N*) High power views of sections through the neocortex. AdV injections performed at each age resulted in a high density of β-gal positive cells throughout the tangential extent of the cerebral cortex, from rostral (R) to caudal (C) (*A*, *E*, *H*, *L*) and from medial (M) to lateral (L) (*B*, *F*, *I*, *M*). The AdV transgene was usually predominantly expressed in cerebral cortex, including neocortex (Ctx) (e.g. S: somatosensory areas, M: motor areas; V: visual areas), cingulate cortex (Cg), entorhinal cortex (Ent), subiculum (Sub) and hippocampus (Hi), and was often only weakly expressed in most other brain regions. The choroid plexus was always heavily infected (see lateral ventricle; LV, in *E*, *F*, *L*, *M*). The radial expression pattern in neocortex varied in relation to the developmental stage of the AdV injection, roughly matching the temporal–spatial patterns of neurogenesis and laminar formation: injections at E13.5 (*A–D*) led to the most dense expression in subplate, layer 6 and to some extent layer 5, but only weak expression in the more superficial layers 2,3,4 (*C*, *D*). Cells in the marginal zone (MZ) cells (arrowheads in *C*), which are co-generated with subplate neurons, are also labeled. Injections at E15.5 (*E*, *F*, *G*) result in densest expression in layers 6 and 5, lower expression in superficial layers, no expression in the marginal zone, and weak to no expression in the subplate (*G*). In contrast, injections at E16.5 (*H*, *I*, *J*, *K*) led to a high density of expressing cells in superficial layers 2/3 and 4, but virtually no expression in deeper layers or the marginal zone (*J*, *K*). In caudal and medial cortex [e.g. visual cortex (V) and cingulate cortex (Cg)], we often saw two stripes of heavy expression, separated by a stripe of weak expression (*H*, *I*, *K*). Injections at E17.5 (*L*, *M*, *N*) led to an even more restricted laminar expression pattern with a high density of expressing cells in superficial layers 2/3 and to some extent in layer 4 (*N*). Abbreviations: A, amygdala; ac, anterior commissure; Hb, hindbrain; Ins, insular cortex; Mb, midbrain; Pir, piriform cortex; RS, retrosplenial cortex; OB, olfactory bulb; ic, internal capsule; Hyp, hypothalamus; cc, corpus callosum; LV, lateral ventricle; S, septum; Sc, superior colliculus; Str, striatum; Th, thalamus; wm, white matter.

generated superficial layer neurons but not the earlier generated cells. These findings indicate that AdV injected into the lateral ventricle has limited spread, if any, into the cortical wall. In addition, they suggest that such AdV injections preferentially infect progenitors in the ventricular zone, which leads to a preferential labeling of their immediate progeny, and possibly cells that have recently become postmitotic but have yet to move far from the ventricular surface.

Role for EMX2 in Regulating Areal Targeting of Thalamocortical Axons

To address the role of EMX2 in regulating the area-specific targeting of TCAs, we used a replication defective, recombinant AdV containing the *CAG/Emx2/IRES/LacZ* pA dicistronic expression construct (*Emx2*–AdV), and as a control, recombinant AdVs containing either a *CAG/GFP/IRES/LacZ* pA dicistronic expression construct or a *CAG/LacZ* pA expression construct (Fig. 7*A*). We hypothesized that ectopic domains of *Emx2*–AdV expression would perturb the areal targeting of TCAs in a manner that would reflect that high levels of EMX2 'caudalize' rostral areas of neocortex. We focused on the TCA projection of the dorsal lateral geniculate nucleus (dLG), which normally projects specifically to the primary visual area (V1), a caudal-medially located area.

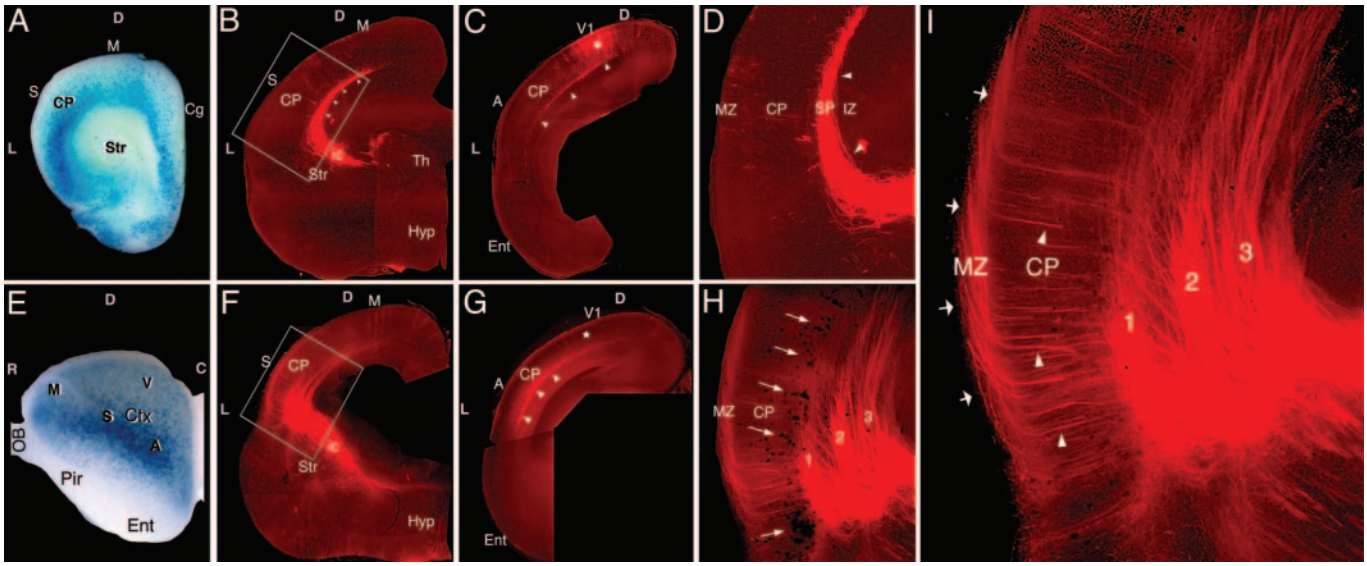

Figure 9. Aberrant area-specific thalamocortical projections correlate with domains of ectopic *Emx2*–AdV infection. Recombinant AdV, containing a *CAG/LacZ* pA expression construct as a control (*A–D*) or a *CAG/Emx2/IRES/LacZ* pA dicistronic expression construct (*E–I*), was injected unilaterally into the lateral ventricle of the cortical hemisphere of E13.5 rat embryos. Pups were delivered normally, and later their brains were fixed and processed for X-gal histochemistry (blue) to β-gal protein product of *LacZ*. In cases infected with the *CAG/Emx2/IRES/LacZ* pA AdV, β-gal positive cells express both β-gal and EMX2 proteins. DiI was injected into the dorsal lateral geniculate nucleus (dLG) to anterogradely label its TCA projection that normally targets the primary visual area (V1). Later, the brains were coronally sectioned and photographed. β-gal labeled cells were not detected in X-gal processed, uninfected control brains (data not shown). (*A–D*) A control virus injected case fixed at P2 exhibits normal TCA pathfinding and targeting as typically observed in uninfected control brains. (*A*) After fixation, the part of the brain rostral to the internal capsule was cut off from the rest of the brain and processed for X-gal histochemistry. The rest of the brain was postfixed and used for DiI tracing. Strong blue staining demonstrates heavy infection with control AdV (S: somatosensory, M: motor, Cg: cingulate cortex). A high density of infected cells are seen in the subplate and deep layers of the cortical plate (CP). This high expression near the level of the internal capsule suggests heavy labeling throughout most of the tangential extent of the neocortex, as shown in control AdV infection studies (Fig. 8*A*,*B*). (*B*) A coronal section at a mid rostral–caudal level demonstrates DiI labeled dLG axons extending through the internal capsule (ic) and entering the cortex. TCAs from the dLG turn dorsally, accumulate in the subplate, and grow tangentially dorsally and caudally toward V1. (*C*) A more caudal coronal section shows labeled dLG axons growing along their tangential path (arrowheads) towards their final target, V1. The axons heavily invade the cortical plate of the primary visual area (V1) (asterisk). (*D*) A higher power view of the boxed area in *B*. The labeled dLG TCAs accumulate as a dense bundle centered on the subplate (SP; arrowheads); no labeled dLG axons invade the cortical plate (CP) at this lateral part of the neocortex, the location of the primary somatosensory area (S1). (*E–I*) An example of aberrant dLG TCA pathfinding and areal targeting in an *Emx2/IRES/LacZ* AdV injected case perfused shortly after time of birth (P0) and processed similar to the control case. (*E*) The entire brain was processed for X-gal histochemistry and photographed as a whole mount prior to sectioning to analyze DiI labeling. A high density of β-gal positive cells is found in lateral neocortex along its entire rostral–caudal extent; medially the density of β-gal positive cells is more moderate. (*F*) Labeled TCAs from the dLG extend normally through the internal capsule (ic), but upon entering the cortex, they exhibit highly aberrant pathfinding. (*G*) A more caudal section at a level similar to panel *C*. Relatively few TCAs reach their target area, V1 (asterisk). (*H*) Higher power view of the boxed area in *E*. This single exposure photo combines darkfield illumination with rhodamine fluorescence. Some of the more heavily labeled *Emx2/IRES/LacZ* AdV-infected cells can be seen in the deep cortical layers (clusters of black dots marked by arrows). The DiI labeled dLG TCAs fail to accumulate as a single dense bundle on the subplate, as in controls (see *B*, *D*), but instead form multiple bundles (marked as 1, 2 and 3) that extend tangentially within what appears to be (1) the deep layers of the cortical plate, (2) the subplate and (3) the intermediate zone. This aberrancy, and others, can be better seen in panel *I*. (*I*). A higher magnification view of the same region in panel *H* but photographed with only with rhodamine fluorescence. Many labeled TCAs extend from the aberrant bundles (1, 2, 3) radially across the cortical plate (CP, arrowheads), enter the marginal zone (MZ), turn dorsally, and extend tangentially within it (arrows). Abbreviations: A, auditory areas; C, caudal; CP,cortical plate; Ctx, neocortex; D, dorsal; Ent, entorhinal cortex; Hyp, hypothalamus; IZ, intermediate zone; L, lateral; M, motor areas; MZ, marginal zone; Pir, piriform cortex; OB, olfactory bulb; ic, internal capsule; R, rostral; S, somatosensory areas; SP,subplate; Str, striatum; Th, thalamus; V, visual areas; V1, primary visual area.

Recombinant AdV injections were targeted *in utero* to the lateral ventricle of E13.5 rats (developmentally equivalent to E11.5–E12 mice), during the generation of subplate and deep layer cortical neurons (Bayer and Altman, 1991; Koester and O'Leary, 1993). The infected animals were fixed between hours before birth (E22/P0) to P2, and processed for X-gal histochemistry to visualize the β-gal reporter that marks the AdV-infected cells. Cortical domains of AdV infection were correlated with the intracortical pathfinding and targeting of TCAs revealed by anterograde labeling using crystals of DiI placed into the dLG, or into the ventroposterior (VP) thalamic nucleus that projects to the primary somatosensory area (S1). In some AdV-infected cases, the cortex was reduced in radial thickness and the lateral ventricles were expanded. This effect was independent of which of the three recombinant AdVs was injected, and likely non-specific, since it was not evident in most of the brains heavily infected with any of the three recombinant AdVs used. These brains were not included in this analysis.

Infection with the control recombinant AdVs had no effect on the pathfinding and area-specific targeting of TCAs. We analyzed 11 control infected cases with good DiI labeling of TCA axons; six of these had high levels of AdV infection in the cortex and five had moderate levels of cortical AdV infection. Laminar patterning and differentiation of the cortex appeared unaffected in these brains (data not shown). An example of a control case with high levels of AdV infection in the cortex and good DiI labeling of the TCA projection from the dLG is illustrated in Figure 9*A–D*. The labeled TCAs passed through the striatum within the internal capsule, entered the cortex as a relatively tight bundle, turned dorsally, accumulated in the subplate and extended tangentially within it caudally to the occipital cortex (Fig. 9*B*). At the rostral–lateral location where they first entered the cortex, the labeled dLG axons did not invade the cortical plate of presumptive S1 (Fig. 9*B*,*D*). However, within the occipital cortex, the labeled TCAs heavily invaded the cortical plate of presumptive V1 in an area-specific manner indistinguishable from normal (Fig. 9*C*).

In contrast, infection with the *Emx2*–AdV had a substantial

effect on the pathfinding and area-specific targeting of TCAs. We analyzed 15 cases with high levels of *Emx2*–AdV infection in the cortex; four of these cases had good DiI labeling and 11 had sparse DiI labeling of the TCA projection. All four cases with good DiI labeling exhibited aberrant TCA projections within the cortex; two were strongly aberrant and two had similar defects but less pronounced. Nine of the 11 sparsely labeled cases had similarly aberrant TCA projections, but again less pronounced than the strongly aberrant cases; we suspect that at least in some, if not all, of these instances the milder appearance of the aberrancy may be due to fewer TCAs labeled. As in the control AdV-infected cases, no obvious defects were apparent in the laminar patterning and differentiation of the cortex in the *Emx2*–AdV infected brains (data not shown).

An example of a case with high levels of *Emx2*–AdV infection in the cortex and good DiI labeling of the TCA projection from the dLG is illustrated in Figure 9*E–I*. In this case, the *Emx2*–AdV infection was particularly high in lateral cortex (Fig. 9*E*) at the position that TCAs pass from the striatum into the cortex (Fig. 9*F*,*H*). The appearance of the labeled TCAs as they extend through the striatum within the internal capsule appeared normal (Fig. 9*F*). However, upon entering the cortex, their pathfinding became highly aberrant in several respects (Fig. 9*F–I*). First, rather than turning dorsally and accumulating on the subplate as a tight bundle, the TCAs turned but splayed out into several bundles of fascicles that extended tangentially with an abnormally broad distribution that included not only their appropriate pathway centered on the subplate, but also layer 6 and deep into the intermediate zone (Fig. 9*F*,*H*,*I*). In addition, many of the labeled TCAs left these aberrant bundles, turned into the cortical plate, extended radially through it without branching, and accumulated in the marginal zone. Within the marginal zone, the labeled TCAs turned dorsally, and extended tangentially within it (Fig. 9*H*,*I*). These aberrancies were coincident with dense domains of deep layer cells infected with the *Emx2*–AdV (Fig. 9*H*). Only a proportion of the labeled TCAs continued caudally toward their target area, V1, and only a small proportion appeared to reach it (Fig. 9*G*). These findings indicate that TCAs labeled from the dLG aberrantly invade an inappropriate cortical area coincident with high levels of deep layer *Emx2*–AdV infection. This targeting inaccuracy is not seen during normal development, and none of these intracortical pathfinding defects in the *Emx2*–AdV infected brains are seen at any stage of development for any population of TCAs in normal or control AdV-infected brains.

In six additional rats injected with the *CAG/Emx2/IRES/LacZ* pA AdV, good DiI labeling of TCAs was obtained and *Emx2*–AdV infection was detected in the cortex, but the density of β-gal positive, *Emx2*–AdV infected cells was low relative to the moderate to high *Emx2*–AdV infected cases described above with aberrant TCA targeting. The pathfinding and areal targeting of the labeled TCAs in these cases were indistinguishable from control infected and normal rats (data not shown). In conclusion, our findings show that dLG TCAs aberrantly invade the cortical plate of rostral–lateral areas (S1) coincident with domains of moderate to high levels of ectopic *Emx2*–AdV expression that they pass beneath en route to V1; they do not invade domains of low levels of ectopic *Emx2*–AdV expression or domains of high levels of control AdV infection. These results suggest that higher levels of EMX2 in the ectopic domains in rostro-lateral cortical areas can specify them to have characteristics, such as thalamic innervation from the dLG, that are normally associated with caudo-medial cortical areas, such as V1.

Discussion

A major goal of our study was to use a gain-of-function test of the role of EMX2 in regulating the area-specific development of TCA projections. For this, we used a replication defective recombinant AdV with a dicistronic expression construct under the control of the strong and ubiquitous CAG promoter (*CAG/Emx2/IRES/LacZ* pA AdV), to ectopically express *Emx2*. Similar viral constructs lacking cDNA for *Emx2* were used as controls. Partial cDNA sequence for *Emx2*, and the related gene *Emx1*, the vertebrate homologues of the *Drosophila empty spiracles* gene, were originally reported by Simeone *et al.* (Simeone *et al.*, 1992a,b). Because at the time we initiated this study, cDNA for the full-length coding region of mammalian *Emx2* was not available, we cloned and sequenced rat *Emx2* cDNA. The clone was ~1500 kb and contained an open reading frame of 780 bp, as well as 5′ and 3′ untranslated regions. Northern analysis of total RNA from E16 rat cortex using radiolabeled probes of the full-length coding region for *Emx2* reveals a transcript size of ~2.8 kb, as previously reported for mouse (Simeone *et al.*, 1992b). Based upon the rat sequence, we defined a predicted sequence for the coding region of mouse *Emx2* (Fig. 1*A*) through analysis of a mouse genomic DNA database. Sequence has been recently published for the full-length coding region of *Emx2* for human (Noonan *et al.*, 2001), zebrafish (Morita *et al.*, 1995), chicken (Bell *et al.*, 2001) and dogfish (Derobert *et al.*, 2002). Within the homeodomain of these six species, the predicted amino acid sequence is 100% identical; they are 80% identical to the homeodomain of *Drosophila empty spiracles*. For the full-length coding region, the predicted amino acid sequence of rat and mouse EMX2 is 100% identical; each is 98.8% identical to human, 96.8% to chick, 93.1% to zebrafish and 86.6% to dogfish.

Our *in situ* hybridization analyses in embryonic rat confirm previous reports in mouse that *Emx2* is predominantly expressed in the forebrain (Simeone *et al.*, 1992a,b), and that within the cortex, it exhibits a graded expression pattern (Gulisano *et al.*, 1996; Mallamaci *et al.*, 1998). Throughout cortical neurogenesis in rat and mouse, *Emx2* is expressed in a gradient with highest levels caudally and medially, and lowest levels rostrally and laterally. As cortical neurogenesis ends, *Emx2* expression ceases. In addition, the laminar expression patterns in rat neocortex of both *Emx2* and *Emx1* are similar to reports in mice [for a review, see Cecchi (Cecchi, 2002)]. *Emx2* is restricted to progenitors in the neocortical neuroepithelium (except for Cajal–Retzius neurons), and *Emx1* is expressed in progenitors as well as in the postmitotic neurons that they generate (present study) (Gulisano *et al.*, 1996; Mallamaci *et al.*, 1998). Thus, *Emx2* is expressed highest in progenitors in the ventricular zone that will generate caudal and medial areas of neocortex, such as visual areas, and lowest in rostral and lateral areas, such as sensorimotor areas. Interestingly, the strongest graded expression of *Emx2* appears to be at earlier stages of cortical neurogenesis, during the generation of subplate, marginal zone, and the deep layers of the cortical plate. This finding suggests that EMX2 regulated positional information may be more prominent in the neurons of these layers. Intuitively, these layers would seem to most require such information because the subplate is likely involved in establishing area-specific TCA projections, and layers 6 and 5 are the sources of area-specific cortical efferent projections to subcortical targets.

In the adult rodent, TCAs from the principal dorsal thalamic sensory nuclei project to specific primary sensory neocortical areas (Hohl-Abrahao and Creutzfeldt, 1991). The development of area-specific TCA projections occurs in two phases: (i) the targeting phase: after entering the cortex, TCAs grow tan-

gentially along an intracortical pathway centered on the subplate layer to reach their appropriate cortical area (Ghosh and Shatz, 1993; Miller *et al*., 1993; Bicknese *et al*., 1994); and (ii) the invasion phase: after reaching their appropriate target area, TCAs extend collaterals superficially into the overlying cortical plate (Catalano *et al*., 1991; Ghosh and Shatz, 1993). TCAs from the principal sensory thalamic nuclei target and invade their appropriate cortical areas in a precise area-specific manner; TCAs rarely overshoot their appropriate cortical areas or make gross directional errors, and only invade the cortical plate of the appropriate cortical area (Crandall and Caviness, 1984; Miller *et al*., 1993). In addition, TCAs grow past, rather than invade, the cortical plate overlying regions of the subplate pharmacologically depleted of neurons (Ghosh *et al*., 1990; Ghosh and Shatz, 1993). These findings suggest that the axon guidance molecules that control the area-specific targeting of TCAs are associated with the subplate.

In addition, the 'handshake hypothesis' postulates a subplate axon-mediated mechanism for TCA guidance, whereby orderly arrays of subplate axons and TCAs meet in the internal capsule, interact with their corresponding area-specific partners, and serve as topographic scaffolds for one another (Molnar *et al*., 1998). Recent evidence supporting this possibility comes from the analysis of *Tbr1* and *Gbx2* mutants (Hevner *et al*., 2001, 2002), as well as *Emx1/Emx2* double mutants (Bishop *et al*., 2003). *Gbx2* expression in the dorsal thalamus is required for the production of a normal TCA projection (Miyashita-Lin *et al*., 1999). Despite the lack of *Gbx2* expression in the cortex, corticothalamic axons fail to reach the dorsal thalamus in *Gbx2* mutants (Hevner *et al*., 2002). *Emx1* and *Emx2*, as well as *Tbr1*, are expressed in the embryonic cortex and not in the dorsal thalamus. In Emx double mutants (Shinozaki *et al*., 2002; Bishop *et al*., 2003) and *Tbr1* mutants (Hevner *et al*., 2001) cortical axons do not reach the dorsal thalamus and TCAs fail to reach the cortex. Regardless of whether the guidance information for TCAs is present on subplate axons, within the subplate layer itself, or both, the precise mapping of TCA connections is likely to involve guidance molecules expressed in graded or areal patterns. Regulatory proteins such as EMX2 are candidates to control the differential expression of these guidance molecules.

The modified AdV5 used in our studies infects cells through a receptor-mediated mechanism that involves the Coxsackie Adenovirus Receptor (CAR) and the αvβ3 or αvβ5 integrin receptors (Nemerow, 2000). CAR is highly expressed in progenitors in the cortical ventricular zone, and is moderately expressed by postmitotic cortical neurons (Tamamaki *et al*., 2001). Interestingly, it has been reported that only one daughter cell of an AdV-infected progenitor in the cortical ventricular zone will inherit the AdV vector (Tamamaki *et al*., 2001), which does not replicate during cell division due to the episomal localization of the AdV vector (Verma and Somia, 1997). This is consistent with our finding that the laminar distribution of cells infected with the replication defective recombinant AdV injected at successive embryonic ages reflects the temporal sequence of generation of cortical neurons (Bayer and Altman, 1991). The early AdV injections done at E13.5, the age that we injected the *Emx2*–AdV to study the area-specific targeting of TCAs, preferentially labeled the earliest generated cortical neurons, including subplate neurons, neurons in the marginal zone, and the deepest layers of the cortical plate. These findings indicate that AdV injected into the lateral ventricle preferentially infects cells that have recently become postmitotic and have yet to move far from the ventricular surface, and/or progenitors in the ventricular zone, which leads to a preferential labeling of their immediate progeny. AdV vectors can infect cells *in vivo*, causing them to express very high levels of the transgene within hours after infection (Moriyoshi *et al*., 1996; Verma and Somia, 1997). In somatic tissue, this expression usually lasts for 5–10 days postinfection due in part to a virally mediated immune response. Thus, the time course of high expression of the *Emx2* transgene would be appropriate to alter area-specific infor- mation that EMX2 may regulate in subplate and cortical plate cells.

We hypothesized that the establishment of area-specific TCA projections is controlled by the graded position-dependent expression of regulatory genes, including *Emx2*. Higher levels of EMX2 expression impart more caudal cortical positional values, such as those normally associated with visual areas. Our findings show that TCAs labeled from the dLG, which would normally target the primary visual area (V1), aberrantly invade an inappropriate, rostral–laterally located cortical area (S1) coincident with high levels of deep layer *Emx2*–AdV infection. These results suggest that higher levels of EMX2 in the ectopic domains of *Emx2*–AdV expression in rostral–lateral cortical areas specify them to have properties normally associated with caudal–medial cortical areas, such as V1. This finding is consistent with loss-of-function analyses of the role of EMX2 in arealization in mutant mice deficient for *Emx2* (Bishop *et al*., 2000, 2002; Mallamaci *et al*., 2000). Changes in the patterns of gene expression suggest that rostral–lateral areas are expanded, whereas caudal–medial areas are reduced in the mutant. Alterations in the organization of area-specific TCA projections are also consistent with this interpretation. Retrograde labeling from the neocortex of *Emx2* mutants indicates an orderly expansion and caudal shift of the topographic TCA projection of VP, indicative of an expansion of its target area, S1, a caudal shift of the S1 border, and a contraction of V1 (Bishop *et al*., 2000; Mallamaci *et al*., 2000). Unfortunately, *Emx2* mutant mice die soon after birth; thus, the adult anatomical and functional organization of the mutant cortex cannot be studied in these mice.

These findings suggest that during normal development, the graded expression of *Emx2* controls through a regulatory cascade the differential expression across the neocortex of genes encoding cell surface or ECM molecules that target TCAs to their appropriate areas. Our findings suggest that the ectopic expression of *Emx2* alters this regulation of guidance molecules for TCA targeting. In the *Emx2*–AdV infected brains, not only do dLG TCAs aberrantly invade rostral–lateral areas that ectopically express *Emx2*, but they exhibit other aberrancies as well. For example, within the ectopic domain of *Emx2* expression, TCAs extend radially directly through the cortical plate and turn and extend dorsally within the marginal zone. This behavior is never seen during normal development. It may be due to persistent *Emx2* expression in postmitotic deep layer neurons, such as layer 5 and 6 neurons, all of which at the ages analyzed extend their apical dendrites radially through the cortical plate and into the marginal zone (Koester and O'Leary, 1992). Thus, the ectopic expression of *Emx2* within these neurons may lead to the overexpression of cell surface proteins that promote the growth of TCAs and provide a substrate for their extension through the cortical plate, which at early ages normally has inhibitory effects on the growth of TCAs (Tuttle *et al*., 1995).

EMX2 regulates arealization presumably by conferring positional identities to cortical cells and regulating their expression of axon guidance molecules that control the area-specific targeting of TCAs. A potentially analogous scenario is the graded expression in the developing chick optic tectum and rodent superior colliculus of the vertebrate engrailed genes, *En-1* and

En-2 (Gardner *et al*., 1988; Martinez and Avarado-Mallart, 1990; Martinez *et al*., 1991), which, like EMX2, are homeodomain transcription factors. Ectopic expression of *En-1* and *En-2* using recombinant retrovirus in embryonic chick tectum shows that these genes regulate the topographic targeting of retinal axons along the rostral–caudal axis of the tectum (Friedman and O'Leary, 1996; Itasaki and Nakamura, 1996), in part through their regulation of two membrane associated proteins, ephrin-A2 and ephrin-A5 that have a graded expression that parallels that of the engrailed genes (Logan *et al*., 1996; Shigetani *et al*., 1997). Ephrin-A2 and ephrin-A5 act as repellents for retinal axons and branches (Nakamoto *et al*., 1996; Monschau *et al*., 1997; Yates *et al*., 2001) and are required for the proper topographic mapping of retinal projections (Frisén *et al*., 1998; Feldheim *et al*., 1998, 2000). Although considerable progress has been made in recent years in defining the genetic and molecular control of TCA pathfinding from dorsal thalamus to the neocortex (Kawano *et al*., 1999; Miyashita-Lin *et al*., 1999; Tuttle *et al*., 1999; Zhou *et al*., 1999; Braisted *et al*., 2000; Bishop *et al*., 2003; Hevner *et al*., 2002; Lopez-Bendito *et al*., 2002), characterization of the area-specific targeting of TCAs within the neocortex has lagged. As in the visual system (O'Leary *et al*., 1999), area-specific TCA targeting is likely primarily controlled by graded guidance molecules, and may also be influenced by neural activity, since blockade of neural activity in cats results in aberrant areal targeting of TCAs (Catalano and Shatz, 1998).

Members of the cadherin family of cell adhesion molecules have been suggested to influence the development of area-specific TCA projections because the principal sensory thalamic nuclei and their target primary sensory areas show matching expression of *cadherin-6*, *-8* and *-11* (Korematsu and Redies, 1997; Suzuki *et al*., 1997; Inoue *et al*., 1998). Other candidates to control TCA targeting are the ephrins and their Eph receptors, which as described above, act as axon guidance molecules in many systems, in ways that resemble the mapping of area-specific TCA projections (O'Leary and Wilkinson, 1999). In the rhesus monkey, primary (V1) and secondary (V2) visual areas form reciprocal connections with adjacent dorsal thalamic nuclei, the dLG and pulvinar, respectively. In embryonic monkeys before TCA projections are established, *EphA3*, *EphA6* and *EphA7* and *ephrin-A5* are expressed at higher levels in V1 than V2; these three EphA receptors are also highly expressed in overlapping graded patterns in pulvinar and to a lesser extent in dLG, whereas *ephrin-A5* is highly expressed in the ventrolateral complex that projects to S1 rather than visual areas (Sestan *et al*., 2001). Similarly, in mice, *ephrin-A5* is expressed in a medial to lateral gradient across S1, and *EphA4* is expressed in a matching gradient across VP, which provides TCA input to S1 (Mackarehtschian *et al*., 1999; Vanderhaeghen *et al*., 2000). *In vitro*, ephrin-A5 repels VP axons. The *ephrin-A5* expression in S1 has also been suggested to repel axons from medial dorsal thalamic nuclei, which express *EphA5* and pass under S1 *en route* to their target areas in limbic cortex, which do not express *ephrin-A5* (Gao *et al*., 1998). Another candidate TCA targeting molecule is the neurotrophin receptor (NTR), *p75*, which is expressed by subplate and layer 6 neurons throughout the period of TCA targeting in a graded pattern that resembles that of *Emx2* (Mackarehtschian *et al*., 1999).

Interestingly, the restricted or graded patterns of expression of *p75*, *EphA7*, *ephrin-A5*, and *cadherin-6* and *cadherin-8*, are all shifted or altered in *Emx2* mutant mice in a manner consistent with a role for them in controlling the area-specific targeting of TCAs (Bishop *et al*., 2000, 2002; Mallamaci *et al*., 2000). The appropriate analyses have not been done to confirm the roles of some of these candidate TCA targeting molecules. Surprisingly, though, in *ephrin-A5* knockout mice, VP axons properly target S1 and form an orderly map of the body, albeit one that exhibits a graded, topographic distortion with medial representations contracted and lateral representations expanded (Vanderhaeghen *et al*., 2000). In contrast, mice lacking *p75NTR* have diminished or absent innervation of V1 by TCAs from the dLG, a defect that is coincident with an altered morphology of subplate growth cones, and the mistargeting of some subplate axons (McQuillen *et al*., 2002). TCA projections to auditory and somatosensory areas of the cortex are normal in the *p75NTR* mutants, consistent with their lower levels of graded *p75NTR* expression.

Further studies will be required to confirm the roles of EMX2 in controlling not only the area-specific targeting of TCAs, but also the process of arealization of the neocortex. An important aspect of these future studies will be to identify the downstream targets of EMX2, and in particular cell surface ligands and receptors that control the pathfinding and area-specific targeting of TCAs.

Notes

This work was supported by NIH grant NS31558. A.L. was supported by a DFG fellowship, C.A. by the Sankyo Foundation of Life Science, and L.J.R. was a Lucille P. Markey Postdoctoral Fellow. We thank J.J.A. Contos and J. Chun for comments, advice, and *Emx1* cDNA. We thank Inder Verma and members of his laboratory for advice on adenoviral vectors.

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