Expression of Cux-1 and Cux-2 in the Subventricular Zone and Upper Layers II–IV of the Cerebral Cortex

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ABSTRACT
Little is known about how neurons in the different layers of the mammalian cerebral cortex are specified at the molecular level. Expression of two homologues of the *Drosophila* homeobox *Cut* gene, *Cux-1* and *Cux-2*, is strikingly specific to the pyramidal neurons of the upper layers (II–IV) of the murine cortex, suggesting that they may define the molecular identity of these neurons. An antibody against *Cux-1* labels the nucleus of most of the postmitotic upper layer neurons but does not label parvoalbumin-positive cortical interneurons that derive from the medial ganglionic eminence. *Cux-1* and *Cux-2* represent early markers of neuronal differentiation; both genes are expressed in postmitotic cortical neurons from embryonic stages to adulthood and in the proliferative regions of the developing cortex. In precursors cells, Cux-1 immunoreactivity is weak and diffuse in the cytoplasm and nucleus of ventricular zone (VZ) cells, whereas it is nuclear in the majority of bromodeoxyuridine (BrdU)-positive subventricular zone (SVZ) dividing cells, suggesting that Cux-1 function is first activated in SVZ cells. *Cux-2* mRNA expression is also found in the embryonic SVZ, overlapping with BrdU-positive dividing precursors, but it is not expressed in the VZ. A null mutation in *Pax-6* disrupts *Cux-2* expression in the SVZ and *Cux-1* and *Cux-2* expression in the postmigratory cortical neurons. Thus, these data support the existence of an intermediate neuronal precursor in the SVZ dedicated to the generation of upper layer neurons, marked specifically by *Cux-2*. The patterns of expression of *Cux* genes suggest potential roles as determinants of the neuronal fate of the upper cortical layer neurons. J. Comp. Neurol. 479:168–180, 2004. © 2004 Wiley-Liss, Inc.

Indexing terms: Cux; CDP; cortex; transcription factors; homeodomain

The mammalian cortex is organized into six layers, with each layer containing neurons that share common morphologies, patterns of connectivity, and development. The cortex develops from an early neuroepithelial precursor population that lines the ventricle of the two telencephalic vesicles. During cortical developmental, these precursors divide to either expand the precursors population or give rise to the postmitotic neurons that will form the cortical plate (Fishell and Kriegstein, 2003). Laminar cortical organization arises as a consequence of the sequential birth and orderly migration of these neurons during development. Neurons of the deeper cortical layers are born first, followed by cells of more superficial layers, which then migrate through

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the existing layers of neurons (Angevine and Sidman, 1961). Hence, late-born neurons populate the superficial layers of the cortex and are also referred to as upper layer neurons, whereas early-born neurons are found in deeper layers.

It is still unknown how cortical precursors chose between an upper and a deeper layer fate. Two precursor populations can be distinguished in the germinative cortical neuroepithelium: the ventricular zone (VZ) and the subventricular zone (SVZ). Cortical precursor cells tightly attached to each other at the ventricular surface form the VZ. During midneurogenesis, VZ cells give rise to the secondary progenitors of the SVZ, which do not contact the ventricle (Boulder Committee, 1970). Traditionally, it has been thought that all neurons are born in the VZ, with the SVZ being a source of glial cells (Bayer and Altman, 1991; Noctor et al., 2001; Malatesta et al., 2003). However, this view has been challenged by studies proposing that upper layer neurons originate in the SVZ based on the expression of the noncoding gene *Svet* (Tarabynkin, 2001) and, more recently, by time-lapse imaging studies showing that neurons arise from symmetrically dividing cells located in the SVZ (Noctor et al., 2004; Haubensak et al., 2004).

Transcription factors have been shown to regulate different aspects of cortical fates, including laminar identity (review in Rubenstein and Beachy, 1998; Shuurmans and Guillemot, 2002). Transcription factors expressed in postmitotic neurons regulate late aspects of specific laminar neuronal differentiation, and genes expressed in cortical precursors regulate early differentiation programs. *Otx-1* and *Tbr-1*, for example, are expressed in neurons of the cortex, regulating their correct molecular specification and the development of axonal projections (Weimann et al., 1999; Hevner et al., 2001). Even within the same layer, the expression of different transcription factors, such as *Otx-1* and *Er81* in layer V, correlates with projection to specific subcortical targets (Hevner et al., 2003). With respect to early laminar determinants, initial transplantation experiments showed that laminar fate is already determined in precursor cells at the time of the last mitotic division and predicted that genes regulating early programs of laminar specification should already be found in precursor cells, where they would be sequentially activated as cells of each lamina are generated (for review see McConnell, 1995). In accordance with this model, the expression of Foxg1 in cortical precursors suppresses the generation of the earliest-born neurons, the Cajal-Retzius cells (Hanashima et al., 2004). However, to this day, very few other such genes have been found (review in Shuurmans and Guillemot, 2002). No transcription factor specifically expressed in the SVZ that could account for the intrinsic changes in cell fate that may occur in these precursors has been reported.

Homeobox-containing genes encode transcription factors involved in embryonic patterning and cell type specification and are candidates as genes that specify cell fates in the cortex. CDP/Cux/Cut transcription factors are an evolutionarily conserved family of proteins containing several DNA binding domains: one homeodomain and one, two, or three Cut repeats (CR1, CR2, CR3). In *Drosophila*, Cut functions as a determinant of cell type specification in several tissues (for review see Nepveu, 2001). In the peripheral nervous system, *Cut* determines the neuronal fate of external sensory organ precursor cells (Bodmer et al., 1987), and its expression is maintained in postmitotic neurons, where it regulates their dendrite morphology (Grueber et al., 2003). Murine Cux and human CDP proteins complement certain *Drosophila Cut* mutants, suggesting some evolutionarily conserved function of Cut proteins (for review see Nepveu, 2001).

Two mammalian homologs of *Drosophila* Cut, *Cux-1*/CDP and *Cux-2*, have been identified in several vertebrate species, including human and mouse (Neufeld et al., 1992; Quaggin et al., 1996; Tavares et al., 2000). *Cux-1* is widely expressed in several tissues in the embryonic and adult mouse. In contrast, *Cux-2* is mostly restricted to the nervous system (Quaggin et al., 1996), although it is also expressed in the developing limb buds and the urogenital system (Iulianella et al., 2003). Although the functions of *Cux-1* have been quite extensively studied in other organs, such as kidney (for review see Nepveu, 2001), less is known about the specific functions of *Cux-2*, and, so far, very little is known about the roles of either gene in the nervous system. In this regard, it is known that *Cux-1* has a wide range of transcription activities, it functions both as a transcriptional repressor and as an activator, and its transcriptional activity is regulated by posttranslational mechanisms involving phosphorylation, acetylation, and proteolysis (for review see Nepveu, 2001). Notably, several reports involve *Cux-1* in the regulation of cell cycle, and expression of both *Cux-1* and *Cux-2* largely overlaps with regions undergoing proliferation (Coquerot et al., 1998; Nepveu et al., 2001; Ledford et al., 2002; Iulianella et al., 2003). Here we describe the specific and dynamic expression patterns of *Cux-1* and *Cux-2*in the cerebral cortex, which suggest potential roles for these genes in neuronal development. Expression of *Cux-1* and *Cux-2* in the adult cortex is confined almost exclusively to layers II/III and IV, making them ideal candidates as regulators of their neuronal identity. Embryonically, *Cux* genes are found in the proliferative regions of the developing cerebral cortex, as well as in the emerging population of postmitotic upper layer neurons in the cortical mantle. Most remarkably, we found that *Cux-2* mRNA specifically labels SVZ cells. *Pax-6* mutant mice show a loss of *Cux-2*-positive cells in the SVZ as well as a loss of *Cux-1*- and *Cux-2*-positive upper layer neurons. These data support the SVZ origin of the pyramidal neurons on layer II–IV of the neocortex and provide excellent markers that define the different molecular identities of these neuronal populations and their intermediate SVZ neuronal precursors.

MATERIALS AND METHODS

Animals and tissue

Swiss Webster timed-pregnant animals were obtained from Taconic Farms (Germantown, NY). The day of the vaginal plug was considered embryonic day (E) 1. Reeler ($Reln^{rl}$) mice were obtained from Jackson laboratory (Bar Harbor, ME). The *Small-eye* (*Sey)* allele was maintained in C57BL/6J × DBA/2J mice. All animal use procedures were reviewed and approved by the Harvard Medical School Standing Committee on Animals and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All mice were overdosed with sodium pentobarbital. Embryos were fixed at 4°C in 4% paraformadehyde overnight. Adult animals were perfused first with phosphate-buffered saline (PBS; pH 7.5), then with 10% formalin in PBS, and postfixed for

overnight in 10% formalin. Fixed tissue was equilibrated in 30% sucrose in PBS and embedded in OCT (Tissue-Tek; Miles, Elkhart, IN). Sections were cut at 10–30 μm thickness on a cryostat. For digoxigenin (DIG) in situ hybridization (ISH), tissue was snap frozen in liquid nitrogen, mounted, and cut at 10 μm in the cryostat.

RNA ISH

Cux-1 probe corresponds to nt 3,496–4,575 from the cDNA (GenBank accession No. X75013). *Cux-2* probe (nt 3,026–3,832; GenBank accession No. U45665) was a gift from Dr. Peter Igarashi. Nonradioactive DIG-labeled cRNA in situ hybridization was performed as described elsewhere (Berger and Hediger, 2001). ^{35}S radioactive RNA ISH was performed as described previously (Marcus et al., 2001). No specific labeling was observed with sense probes.

Generation and purification of anti-Cux-1 rabbit polyclonal antibody

Anti-Cux-1 rabbit polyclonal antibody was generated using a 332-amino-acid (aa) synthetic polypeptided (SP8). DNA sequence encoding for aa 611–942 of Cux-1 was amplified by PCR with the primers TA CGC CTC GAG AAA CAG TTC CTC TCC GAT GAG (nt 1,874–1,894), and CG GAA TTC TCA CTC CTG GTA CAT GTA GAC CTC (nt 2,872–2,849). PCR products were digested with XhoI and EcoRI, purified from agarose gel, and ligated into XhoI-EcoRI site of pRSETA (Invitrogen, La Jolla, CA). This resulted in the expression construct of SP8 fused with 6xhis, pRSETA/SP8. By using Ni-NTA superflow (Qiagen, Chatsworth, CA), the SP8 polypeptide was purified from *Escherichia coli* according to the manufacturer's protocol. The purified SP8 was sent to Babco for production of a rabbit antiserum.

Affinity purification of anti-Cux-1 antibody

The purified SP8 polypeptide described above was coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) and used to affinity purify anti-Cux-1 polyclonal antibodies from the immunized rabbit serum. Western blotting of cerebral cortical lysates showed a major band of the expected molecular weight (200 kDa) and three minor bands of 130, 110, and 90 kD that may correspond to other observed isoforms of Cux-1 (for review see Nepveu, 2001). No bands were observed on tissue homogeneizates from the hippocampus, where Cux-1 protein is not expressed (not shown).

This purified rabbit polyclonal antibody did not give any specific staining on brain sections of Cux-1 mutant mice (not shown), ensuring specificity of the staining observed in the wild-type animals (see Results). This also suggests that the antibody does not cross-react with Cux-2, whose RNA expression is not affected in this mutant mouse (see Results). This antibody did not stain tissue sections of the embryonic spinal cord, which express *Cux-2* mRNA but not *Cux-1* mRNA (not shown).

Immunofluorescence

Ten- to twenty-micrometer cryosections were blocked with 5% goat serum in TBST (Tris 0.1 M, NaCl 150 mM, pH 7.5, Triton 0.1%; blocking solution), then incubated for 1 hour at room temperature (r.t.) with primary antibodies diluted in blocking solution. Secondary antibodies diluted in TBS 5% goat serum were applied for 1 hour at r.t., and sections were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR), and mounted with Aqua Polymount mounting media (Poly-Labo).

Polyclonal antibody anti-Cux-1 was used at a final concentration of IgG 3 mg/ml. Antiparvalbumin mouse monoclonal (IgG1) clone PARV-19 (1:1,000; Sigma, St. Louis, MO), mouse antineuronal nuclei (NeuN) monoclonal antibody (IgG1 1 mg/ml; Chemicon, Temecula, CA), Cy3 conjugated anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), and highly cross-adsorbed Alexa 488 anti-goat (Molecular Probes) secondary antibodies were applied at 1:500.

Confocal microscopy

Confocal microscopy was performed with a Nikon E800 microscope coupled to a Bio-Rad Radiance confocal system and with a Zeiss LSM 510 laser scanning microscope and 3D analysis software for multiplanar reconstruction (Zeiss, Thornwood, NY). Briefly, serial sections were acquired each 0.3 μm to generate multiplanar Z stacks of E16 cerebral cortical sections. Then, orthogonal views of individual cells depicting xyz axes were generated and analyzed for colocalization and intensity of nuclear expression of Cux-1, BrdU, and NeuN. More than 50 VZ and 50 SVZ cells were individually analyzed. Multiple images and profile of the covariance of intensity of expression were obtained and plotted to demonstrate the levels of expression of Cux-1 in any given dividing (BrdU$^+$) progenitor. All photomicrographs were manipulated with Adobe Photoshop software and stored as TIFF files.

RESULTS

Expression of *Cux-1* and *Cux-2* in the mouse adult cortex

We found that expression of *Cux-1* and *Cux-2*, two mammalian homologues of Cut (for review see Nepveu, 2001), is restricted to layers II/III and IV of the adult mouse cortex (Fig. 1A,B). As shown in Figure 1, expression of *Cux-1* and *Cux-2* mRNA is found predominantly in cortical structures of the adult telencephalon. Telencephalic mRNA expression patterns of *Cux-1* and *Cux-2* are very similar and are found throughout the upper layers of the neocortex, the ventrolateral cortex, and the piriform cortex (Fig. 1A,B). Levels of expression of *Cux-1* mRNA, but not of *Cux-2* mRNA, appear reduced in the cingulate region compared with other areas of the cortex (Fig. 1A,B). *Cux-1* and *Cux-2* are also expressed in neurons that form the claustrum and endopiriform nucleus, which are also born over the time period overlapping neurogenesis of layers II–IV (Bayer and Altman, 1991; Fig. 1A,B). Only a few scattered neurons in the deep layers of the neocortex express *Cux-1* and *Cux-2* mRNA (Fig. 1A,B).

The expression of Cux-1 protein, characterized with an antiserum that specifically recognizes Cux-1, overlaps expression of *Cux-1* mRNA in the adult telencephalon (Fig. 1C,D) and also demonstrates the nuclear localization of the Cux-1 protein (Fig. 1C, inset). Cux-1 protein is found in the majority of cells of layers II–IV but only in a few cells of the deep layers (Fig. 1C). All cells of the marginal zone (MZ) are negative for Cux-1 staining (Figs. 1C, 2A–C). Double-labeling experiments with the pan-neuronal marker NeuN (Mullen et al., 1992) show that Cux-1 immunoreactivity localizes to the nucleus of most neurons of layers II–IV. In the majority of Cux-1-positive neurons,

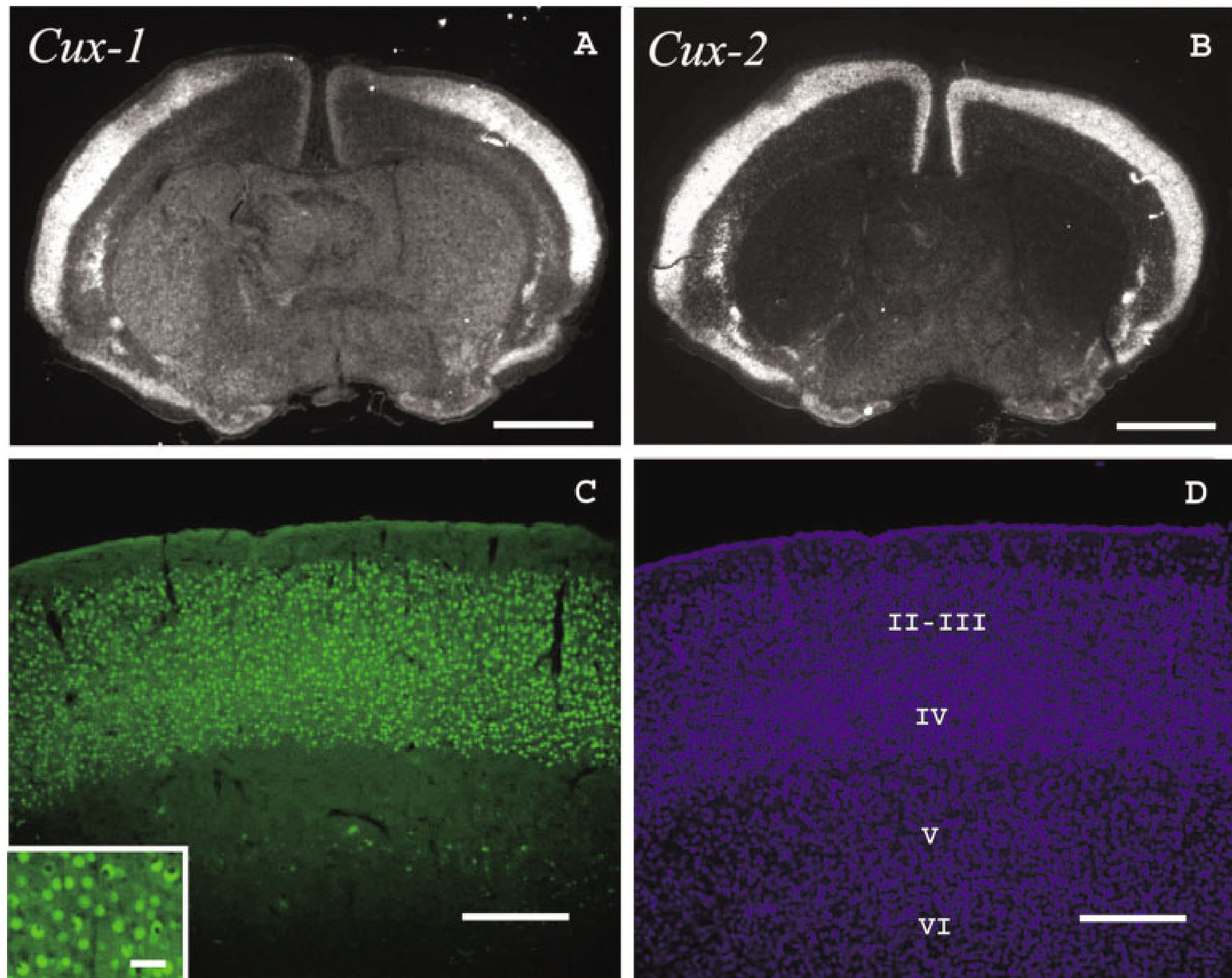

Fig. 1. Expression of Cux genes in adult telencephalon is restricted to upper layers. *In situ* hybridization with *Cux-1* and *Cux-2* mRNA probes (**A,B**) and staining with an antibody against Cux-1 (**C**) in adult telencephalon. Inset details the nuclear localization of Cux-1 protein. *D* shows layers revealed by nuclear staining with Hoetsch. Scale bars = 2.5 mm in A,B; 100 μm in C,D; 10 μm in inset.

the staining with NeuN reveals a pyramidally shaped cell body and suggests that Cux-1 expression is found in pyramidal neurons (Fig. 2A–D). However, some subpopulations of upper cortical neurons do not express detectable levels of Cux-1 (Fig. 2D, arrowhead). These experiments also show that most, if not all, Cux-1-positive cells in the cortical plate correspond to neurons, and not glial cells, and that Cux-1 immunoreactivity is found in some, but not all, neurons in layers II–IV.

Most cortical neurons are generated from precursors of the dorsal neuroepithelium immediately adjacent to the cortical plate. On the other hand, a subpopulation of neurons that express the neurotransmitter γ-aminobutyric acid (GABA) are born from precursors located in the ventral subcortical telencephalon and then migrate tangentially to reach the cortical plate (Anderson et al., 1997a; Gorski et al., 2002; Marin and Rubenstein, 2003). To investigate whether the Cux-1-negative upper cortical neurons revealed by NeuN staining could correspond to GABAergic interneurons, we stained cortical sections with parvalbumin, which marks a subpopulation of GABAergic neurons (Hendry et al., 1989), and Cux-1. In these experiments, Cux-1 staining in parvalbumin-positive cells was analyzed by confocal microscopy in detail in individual cells. These experiments revealed that none of the ventrally born GABAergic neurons marked by parvalbumin immunoreactivity expressed Cux-1 (Fig. 2E–H). Thus, Cux-1 is expressed in the majority of NeuN-positive cells in layers II–IV, but not in parvoalbumin-positive cells. Although parvalbumin marks only a subpopulation of interneurons and we cannot rule out that other inhibitory neurons might express Cux-1, our data suggest that Cux-1 is restricted to pyramidal neurons that derive from the proliferative regions of the dorsal telencephalon.

Cux-1 as a layer-specific marker in the *reeler* mouse

We next examined the expression of Cux-1 protein in the cortex of *reeler* mutants, which show disrupted and partially inverted cortical layering (Caviness and Sidman, 1973). *Reeler* mice show very poor lamination and defects in neuronal migration, with a tendency for early-born

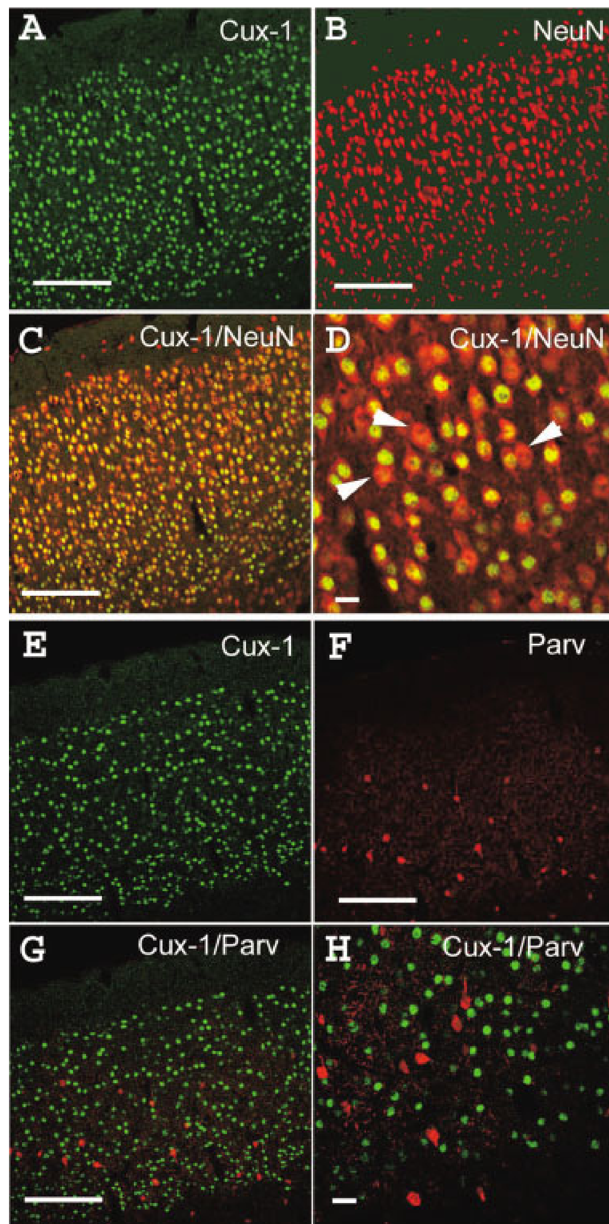

Fig. 2. Cux-1 labels a subpopulation of neurons of layers II–IV. **A–D** show confocal analysis of cerebral cortical sections stained for Cux-1 (green) and NeuN (red). D shows high magnification of cells shown in C. Arrowheads indicate NeuN$^+$ neurons that are negative for Cux-1. **F–H** show confocal studies of sections stained for parvalbumin (red), which labels a subpopulation of GABAergic neurons. H shows high magnification of cells shown in G. Parvalbumin-positive neurons do not express Cux-1. Scale bars = 100 μm in A–C,E–G; 10 μm in D,H.

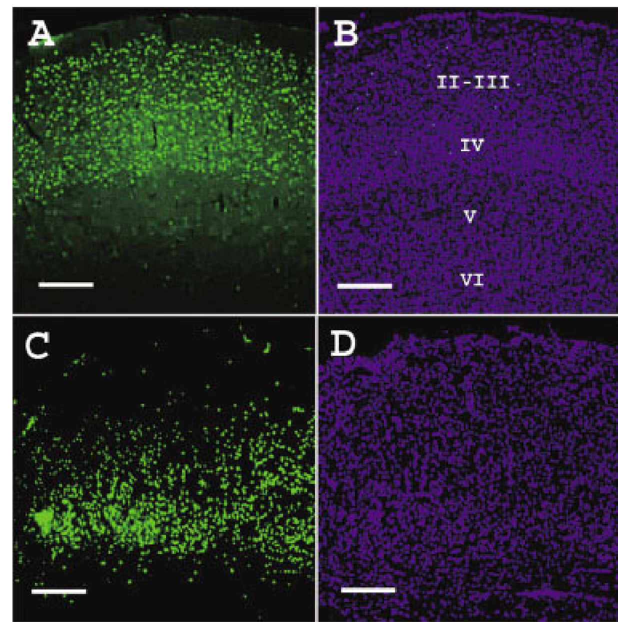

Fig. 3. Expression of Cux-1 in *reeler* cortex. Expression of Cux-1 protein in postnatal brain at 21 days old (P21) in wild-type (**A,B**) and *reeler* (**C,D**) cortex. In *reeler* cortex, Cux-1 immunoreactivity is found in the late-born neurons located in the deeper cortex (C), whereas it is found in the upper layers in the wild-type brain (B), suggesting that Cux-1 expression correlates with cell identity specification rather than migration or laminar localization. Scale bars = 100 μm.

neurons to localize to the upper cortical plate, whereas late-born neurons tend to be in the deep regions of the cortex. We found that, in the cortex of adult *reeler* mice, most Cux-1-positive neurons are found in the deeper cortex and do not form the strict laminar boundary that characterizes normal lamination. Some scattered, single Cux-1-positive neurons are also found throughout the superficial cortex (Fig. 3). Thus, Cux-1 staining in the *reeler* mutants is consistent with the abnormal pattern of migration observed in these mutants, which creates a rough inversion of cortical layering, as previously observed (Caviness and Sidman, 1973). This suggests that *Cux-1* marks the identity of upper layer neurons, rather than merely reflecting their migration or laminar position.

Expression of *Cux-1* during cortical development

Some aspects of cortical neuronal identity are determined already in the precursor cells in the proliferating zone before they give rise to the postmitotic neuron (McConnell and Kaznowski, 1991). Early determinants of laminar identity are thus expected to be expressed in some precursors, whereas expression of late fate markers may be activated in differentiating neurons after cortical migration is complete. We therefore asked whether *Cux* genes are expressed in precursors at the time when upper layer neurons are generated (E13–E17) or, on the contrary, whether expression of these genes is activated only once cells reach the cortical plate and differentiate (Takahashi et al., 1999). From E13 to E17, the proliferative neuroepithelium of the cerebral cortex is composed of two progenitor populations, the VZ and SVZ. We found *Cux-1* mRNA signal, but dim Cux-1 immunoreactivity, in the VZ and SVZ of the dorsal developing telencephalon (E14 and E16; Fig. 4A–E). In the cortical plate, the appearance of Cux-1 immunoreactivity correlates with birth of upper layer neurons: At E14, the majority of cells in the cortical plate represent cells that are destined to form the deep cortical layers, and these cells do not show positive Cux-1 immunoreactivity (Fig. 4A, arrow) or mRNA signal (Fig.

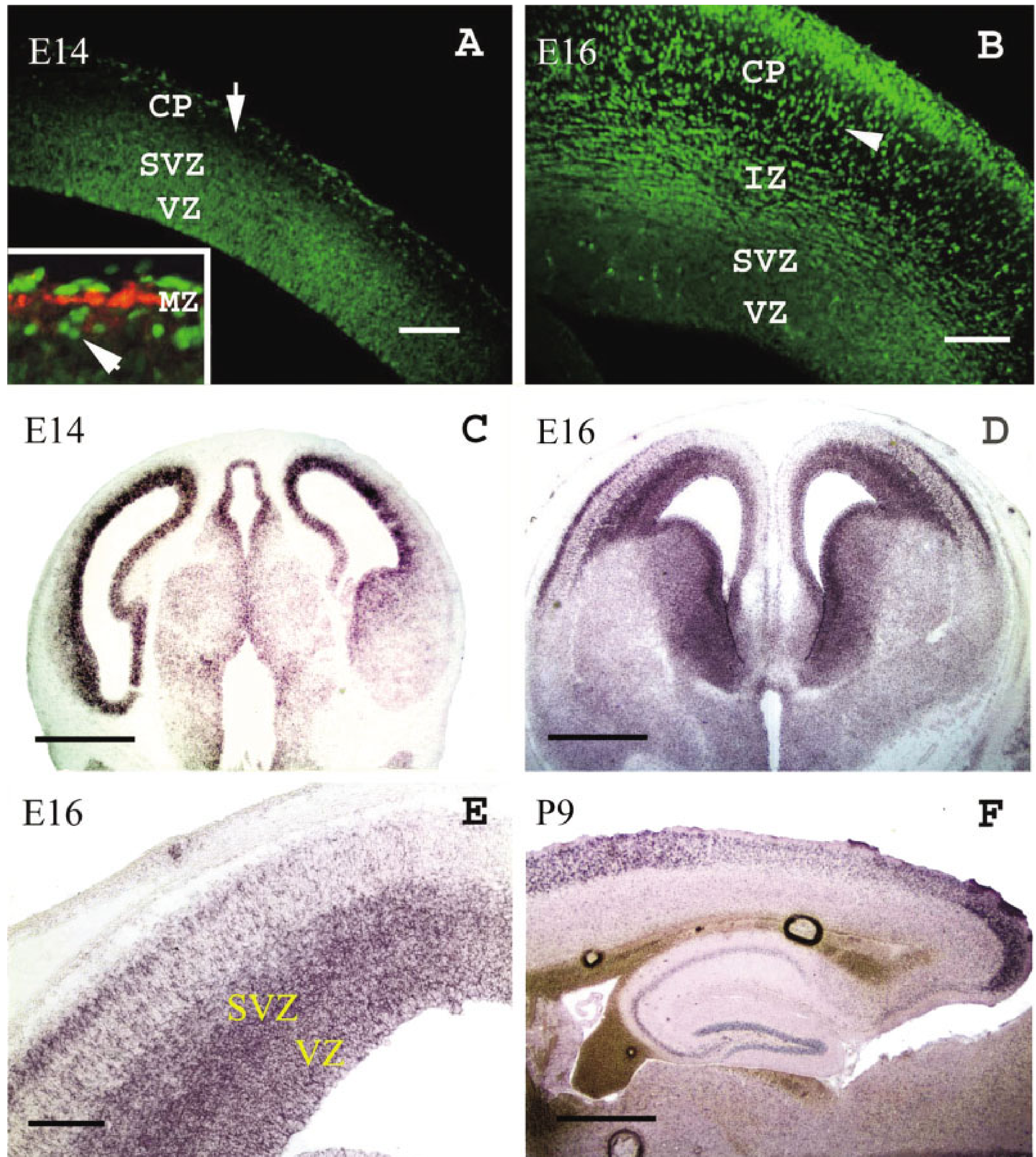

Fig. 4. Expression of *Cux-1* during cerebral cortical development. An antibody against Cux-1 reveals expression in the VZ and SVZ but not in the cortical plate at E14 (arrow, **A**). Inset shows double staining of Cux-1 (green) and Reelin (red) in E14 cortex. Arrowhead labels Cux-1 neuron below the marginal zone (MZ). At E16, Cux-1 protein is detected in the VZ-SVZ (**B**). Bright Cux-1-positive staining is found in presumptive neurons migrating toward the cortical plate and on top of it (E16; arrowhead in B). **C–E** show *Cux-1* patterns of expression at E14 and E16. E shows high magnification of the E16 cortex shown in D. **F** shows Cux-1 mRNA expression in a P9 brain, showing uniform expression along the anterior-posterior axis. Scale bars = 100 μm in A,B,E; 1,000 μm in C,D,F.

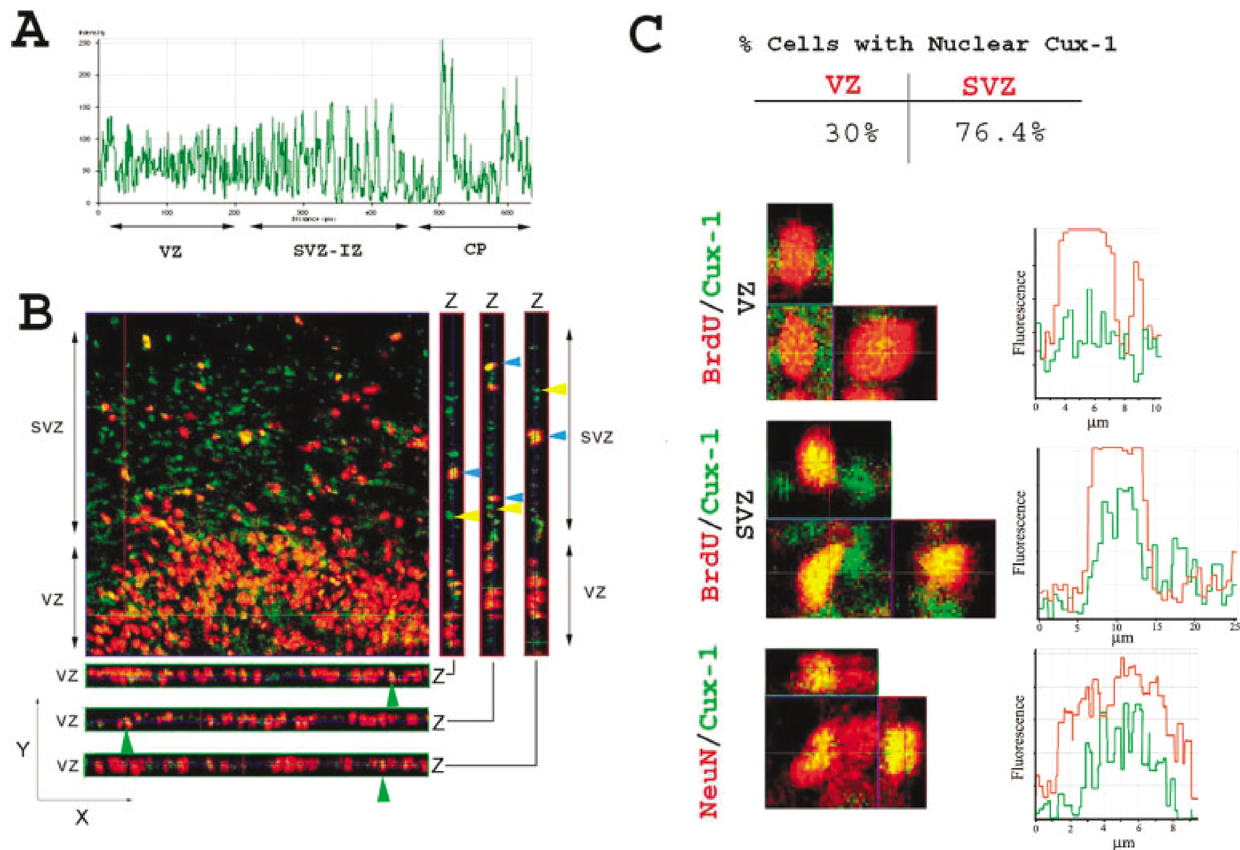

Fig. 5. Nuclear localization of Cux-1 protein in SVZ precursors and postmitotic neurons. **A** shows plot of the intensity of Cux-1 staining along the E16 dorsal telencephalon, measured from the VZ to the cortical plate, analyzed by confocal microscopy. Roughly, each peak corresponds to one cell. Cux-1 staining is more intense in the cortical plate and SVZ. **B** shows three-dimensional confocal analysis of E16 cortex doubly stained for Cux-1 and BrdU after a short pulse of the nucleotide. Cux-1 staining shown in green, Brdu in red. z Cut reconstruction of signal along the x and y axis is shown in the bottom and side, respectively. These z cuts show that Cux-1 staining is diffuse in VZ cell precursors and does not colocalize with BrdU signal in the nucleus (bottom z cuts). Green arrowheads highlight cells with some nuclear Cux-1 staining. High Cux-1 nuclear staining is observed in cells of the SVZ (blue arrowheads, three z cuts on right side). Yellow arrowheads point to BrdU-negative cells that express high levels of Cux-1. **C** shows three-dimensional reconstruction of individual cells along the xyz axis. Fluorescence intensity profiles are shown at right. In VZ precursor cells, Cux-1 signal is only slightly higher in the nucleus (red BrdU signal) than outside the nucleus. In SVZ precursor cells, a sharp, intense peak of Cux-1 fluorescence intensity is observed in the nucleus.

4C), only a few cells at the top of the cortical plate being Cux-1 positive. Double staining for Cux-1 protein and Reelin, which labels cells of the MZ (Schiffmann et al., 1997), shows that Reelin-positive neurons are negative for Cux-1 and identifies Cux-1-positive cells as cells of the pia, outside of the MZ, with just a few Cux-1-positive neurons below the MZ in the uppermost part of the developing cortical plate (Fig. 4A, inset, arrowhead). We infer that these Cux-1-positive neurons correspond to the first upper layer neurons arriving at the top of the cortex. At E16, when a great proportion of newly born upper layer neurons is migrating away from the proliferative areas, many Cux-1-positive cells can be observed migrating through the intermediate zone (IZ) and the cortical plate (Fig. 4B,D,E). Remarkably, Cux-1-positive cells in the cortical plate show much more intense immunoreactivity than cells of the VZ and SVZ (Fig. 4E). Thus, we conclude that *Cux-1* marks an early step in the acquisition of the neuronal identity of upper layer neurons, in that it is expressed both in the precursor population and in immature migrating upper layer neurons.

Expression of nuclear Cux-1 protein in dividing SVZ cells but not in VZ precursors

Because the expression of Cux-1 is apparently higher and clearly more strikingly nuclear in cortical plate cells than in progenitor cells of the VZ and SVZ, we wanted to analyze further the levels of nuclear Cux-1 expressed in precursor and neuronal populations. Immunofluorescence and confocal analysis of cortical E16 sections revealed a gradient of intensity of Cux-1 staining in the cortical mantle: low Cux-1 in the VZ and highest in the cortical plate (Fig. 5A). Although weaker than in neurons of the cortical plate, staining of Cux-1 in precursor cells appears to be specific, compared with the very low nonspecific binding of the antibody to cells of the deep layers (Fig. 5A,B). Furthermore, localization of Cux-1 protein in precursors cells in the VZ was not as obviously nuclear, and the staining not as bright, as it was in postmitotic neurons found in the IZ and the cortical plate. Many cells in the SVZ also show bright and well-defined nuclear Cux-1 staining (Fig. 4B, arrowhead).

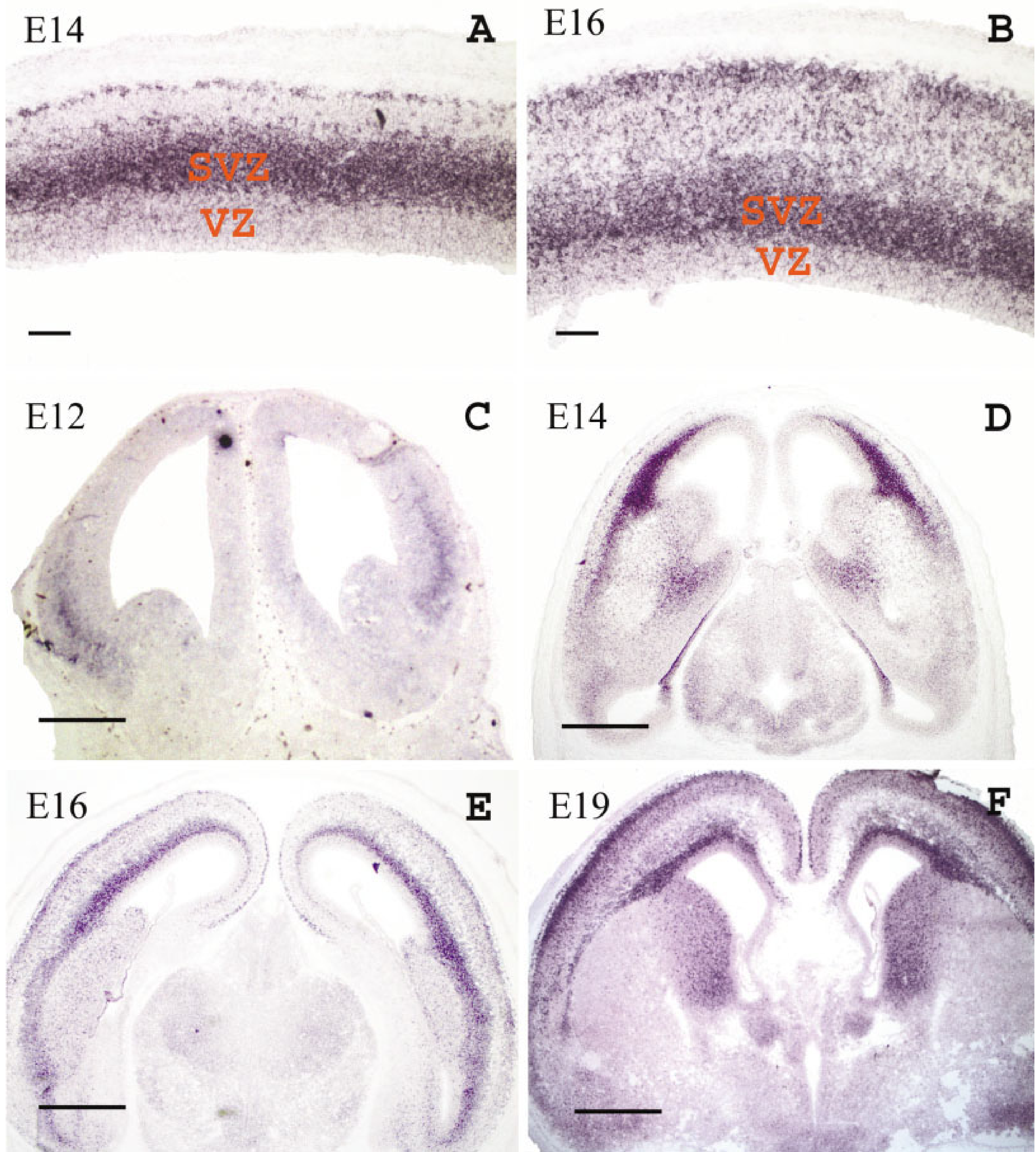

Fig. 6. Expression of *Cux-2* during cortical development. **A–F** show *Cux-2* expression in the telencephalon during development (E12–E19). A and B show high magnification of cortical plate at E14 (A) and E16 (B). The earliest expression of *Cux-2* is detected at E12 in the lateral cortex (C). D–F: From E14 to E19 *Cux-2* is restricted to the SVZ and to the emerging population of upper layer neurons migrating in and on top of the cortical plate. Scale bars = 50 μm in A,B; 2,000 μm in C; 1,000 μm in D–F.

Next, because the SVZ contains a mixed population of migrating neurons and precursors, we analyzed the subcellular localization of Cux-1 protein in dividing cells of an E16 brain labeled after a short pulse of BrdU (Fig. 5B,C). In these studies, Cux-1/BrdU double-positive cells of the VZ and SVZ were analyzed by three-dimensional recon-

struction of serial confocal sections to ensure nuclear localization. This analysis revealed that most (70%) BrdU-positive cells of the VZ show very low or undetectable levels of Cux-1 protein in the nucleus (Fig. 5C). Staining of Cux-1 in the cytoplasm of VZ cells was also weak and not evenly distributed (Fig. 5C). In contrast, the majority of precursor cells in the SVZ showed intense Cux-1 nuclear signal (76% high nuclear Cux-1), as did postmitotic cortical upper layer neurons (Fig. 5B,C). Thus, Cux-1 immunoreactivity becomes localized to the nucleus in dividing cells of the SVZ compared with the VZ, and this nuclear localization appears to be mantained in postmitotic migratory and differentiated upper layer neurons. This changing subcellular distribution of Cux-1 immunoreactivity suggests that the protein undergoes translocation to the nucleus and/or that its expression is up-regulated between VZ and SVZ cells. These experiments also highlight an important population of dividing cells in the SVZ that expresses high levels of nuclear Cux-1.

Expression of *Cux-2* in SVZ precursors and upper cortical layers during cortical development

We found that the expression of *Cux-2* mRNA is restricted to the specialized SVZ precursors and to the upper layer neurons during cortical development. We found that *Cux-2* mRNA is absent in the VZ of the developing dorsal telencephalon, although it highlights a population of cells in the SVZ that appears just before the time of birth of upper layer neurons. Expression of *Cux-2* in the cortical plate parallels the appearance of upper layer neurons in the cortical plate (Fig. 6) and coincides with the expression of Cux-1 (Fig. 4). We analyzed the expression of *Cux-2* mRNA at representative points of cortical development: before, during, and after the generation and migration of upper cortical neurons (E12–E19; Takahashi et al., 1999). As shown in Figure 6, *Cux-2* expression is first observed at E12 in the SVZ of the most lateral cortex, but not in cells of the cortical plate (Fig. 6C). At E12, deep layer neurons are being produced, and few or no upper layer neurons are born (Takahashi et al., 1999). At E14, the expression of *Cux-2* in the SVZ is expanded to the medial part of the telencephalon, reflecting the developmental neurogenic gradient that progresses from lateral to dorsomedial, and *Cux-2* is also found in some cells at the top of the cortical plate (Fig. 6A,D), in a location very similar to that of the earliest Cux-1-positive neurons of the cortical plate (Fig. 4A, inset). Insofar as the cortical plate is negative for *Cux-2* expression at E12 (Fig. 6C), the *Cux-2*-positive cells seen at E14 appear to correspond to the first cohort of upper layer neurons to arrive in the cortex (Fig. 6A,D). As neurogenesis advances and upper layer neurons are formed, increasing numbers of *Cux-2*-positive cells appear in the cortical plate, and *Cux-2* signal in the SVZ gradually disappears (E16–E19; Fig. 6E,F). At E19, when the vast majority of upper layer neurons are already born, *Cux-2* signal in the SVZ is greatly reduced (Fig. 6F), and it completely disappears at early postnatal stages (data not shown). Thus, the expression of *Cux-2* in the SVZ precedes and correlates in time with the birth and migration of postmitotic upper layer neurons and suggests that at least some *Cux-2* cells in the SVZ may be neuronal precursors that, after dividing one or more times in the SVZ, will give rise to the postmitotic upper layer neurons.

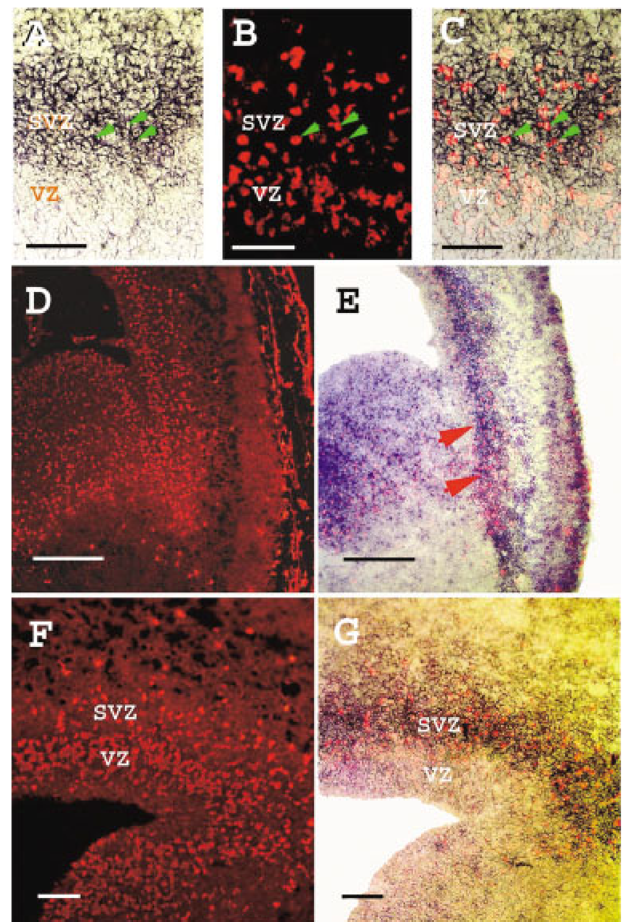

Fig. 7. *Cux-2*-positive SVZ cells incorporate BrdU. Double staining of *Cux-2* mRNA and BrdU after a short pulse of the nucleotide (45 minutes) in E16 cortex (**A–G**). A–C show a detail of the border of VZ and SVZ of staining shown in D,E. Arrowheads point to examples of cells doubly positive for *Cux-2* RNA and BrdU. F and G show *Cux-2* expression and BrdU staining after a 45-minute pulse of the nucleotide, demonstrating that the expression of *Cux-2* in the SVZ coincides with cells undergoing proliferation, including the SVZ cells of lateral cortex at the corticostriatal junction (arrows, G). Scale bars = 100 μm in A–C,F,G; 500 μm in D,E.

The idea that some of the *Cux-2*-positive cells in the SVZ may be precursor cells rather than *Cux-2* postmitotic upper layer neurons is supported by double-labeling experiments with in situ hybridization and BrdU. A short pulse of BrdU at E16 labels many dividing precursors in the SVZ, and these SVZ precursors extensively overlap the region of *Cux-2* expression (Fig. 7A–G). Although these experiments do not allow a precise double-label analysis of individual cells, these results are consistent with the suggestion that *Cux-2* is expressed in some progenitors of the SVZ. Thus, the pattern and timing of expression of *Cux-2* and the BrdU experiments strongly suggest that some *Cux-2*-positive cells are specific precursors for the upper layer neurons dividing in the SVZ.

Abnormal development of *Cux*-positive neurons and SVZ precursors in *Pax-6* mutants

We next turned to the *Pax-6 small-eye* (*Sey*) mutant mice, which show defects in cortical development, to

search for further links between the expression of Cux genes in the upper layer neurons and the SVZ precursors. In *Pax-6* mutants, neurons in the deep cortical layers are correctly specified, whereas late-born neurons do not migrate properly, accumulate in the SVZ, and do not express the upper-layer-specific marker *Svet* (Schmahl et al., 1993; Tarabykin et al., 2001; Heins et al., 2002). We found that *Pax-6* mutant mice show reduced numbers of *Cux-1*- and *Cux-2*-positive neurons in the cortical plate at E16, and more dramatically at E19, compared with wild-type littermates (Fig. 8A–D). The reduction in the number of *Cux* positive neurons correlates with a striking and progressive reduction of the *Cux-2*-positive SVZ cells at E14 and E16 compared with wild-type embryos (Fig. 8E–H). At E14, *Cux-2* SVZ cells are greatly reduced but still detectable (Fig. 8E,F), and, at E16, they have virtually disappeared, except for the most medial cortex (Fig. 8G,H). These data suggest that the generation of upper layer neurons is prematurely extinguished in the *Pax-6* mutant cortex and that *Cux-2*-positive SVZ cells depend on *Pax-6* activity for their generation and/or proliferation. These results also show that, in *Pax-6* mutants, during neurogenesis, some SVZ cells are not correctly specified, in that they fail to express *Cux-2*.

DISCUSSION

We found that expression of two homeodomain transcription factors homologous to *Drosophila Cut* is confined to the pyramidal neurons of layers II–IV of the murine cortex. Cux-1 inmunoreactivity is found in the nucleus of upper cortical postmitotic neurons of the adult and developing cortex. Among the precursor populations of the developing cortex, nuclear Cux-1 protein is found in most of the dividing cells of the SVZ, but not in VZ cells. Concurrently, *Cux-2* mRNA labels a population of cells in the SVZ and its expression is absent in the VZ. The timing of expression of *Cux-1* and *Cux-2* genes in neurons of the cortical plate and of *Cux-2* and nuclear Cux-1 protein in precursor cells of the SVZ and the correlated depletion of both populations in the *Pax-6* mutant cortex strongly suggest that a subpopulation of cells expresses *Cux-2* mRNA and nuclear Cux-1 protein and that these Cux-positive SVZ cells are precursors of upper layer cortical neuron.

Cux genes as markers of the upper layer fate

We describe two new molecular markers for cortical upper layer neurons and their precursors. Very few other transcription factors have been reported to be so dramatically restricted to specific cortical layers, and none of them gives these expression patterns identifying layers II/III and IV (for review see Schuurmans and Guillemot, 2002; Hevner et al., 2003). The expression pattern of *Cux* genes in the cerebral cortex, the deep localization of Cux-1-positive neurons in the roughly inverted cortex of *reeler* mouse (Caviness and Sidman, 1973), and particularly the role of Cut as a cell fate determinant in the *Drosophila* nervous system (Bodmer et al., 1987; Grueber et al., 2003) argue in favor of a potential role of *Cux* genes as regulators of the neuronal fate and cell identity of the upper layer of the cortex. The pattern of expression of murine Cux genes seems to parallel that in the fly. The *Drosophila* Cut is necessary for the early specification of neuronal precursor cells but also controls the phenotype of postmitotic neurons, where it regulates the dendritic morphology. In mouse, the expression of *Cux* genes in cortical precursors suggests that these genes may participate in the regulation of the early specification of upper layer neurons, whereas the maintenance of expression throughout adulthood would agree with potential roles in dendritic regulation.

In any case, the potential essential roles of *Cux* genes in neuronal precursors and postmitotic upper layer neurons remain to be investigated. The normal development of upper layer cortical neurons in *Cux-1* mutant mice (Luong et al., 2002; data not shown) indicates that *Cux-1* alone is not essential for cortical development. Moreover, cortical expression of *Cux-2* mRNA is maintained at normal levels in *Cux-1* mutant mice (data not shown), indicating that it could compensate for the loss of *Cux-1*. This, together with the overlapping expression patterns of *Cux-1* and *Cux-2* in the developing and adult cortex, suggests that *Cux-1* and *Cux-2* might have some redundant functions, but the essential roles of *Cux* genes can be investigated only by analyzing the results of mutations in *Cux-2* as well as *Cux-1*.

Expression of *Cux* genes in cortical precursors

Expression of Cux genes appears to define cortical SVZ precursor cells and suggests specific roles in these cells. We found that the transition from VZ precursor cells to SVZ cells correlates with the expression of high levels of nuclear Cux-1 protein and the onset of expression of *Cux-2 mRNA*. We do not know what mechanisms account for the appearance of Cux-1 protein in the nucleus of SVZ cells, but they could involve translocation to the nucleus from the cytoplasm, and perhaps up-regulation of protein expression, insofar as we show a gradient of expression in the cortical mantle. Nevertheless, regardless of the levels of expression of the Cux-1 protein, mRNA expression is down-regulated in early-born neurons derived from the VZ of the dorsal telencephalon and in cortical interneurons derived from the VZ of the medial ganglionic emineces.

Whereas *Drosophila* Cut has been shown to regulate aspects of neuronal cell fate, data on mammalian *Cux* genes extensively implicates them in the regulation of cell proliferation (for review see Nepveu et al., 2001; Ledford et al., 2002; Iulianella et al., 2003). For example, *Cux-1* negatively modulates the cell cycle inhibitors p27 and p21 (Ledford et al., 2002), which are expressed in a temporally dynamic fashion in cerebral cortical progenitors (Delalle et al., 1999). Nevertheless, several other transcriptional activities have been described (for review see Nepveu et al., 2001).

Thus, the known functions of *Cux* genes in regulating cell cycle, and their specific patterns of expression during cortical development, allow the speculation that *Cux* genes may be involved in the regulation of cortical cell precursor proliferation, specifically of SVZ cells, whereas the functions of *Drosophila* Cut suggest a role in cell fate specification. In the cerebral cortex, the acquisition of specific laminar fates correlates with the number of cell cycles completed by cortical precursors (Takahashi et al., 1999), and VZ and SVZ progenitors differ in their proliferative properties (Takahashi et al., 1995). Moreover, modifying the levels of p27 expression changes the proliferative rates and cell cycle length of precursors, altering

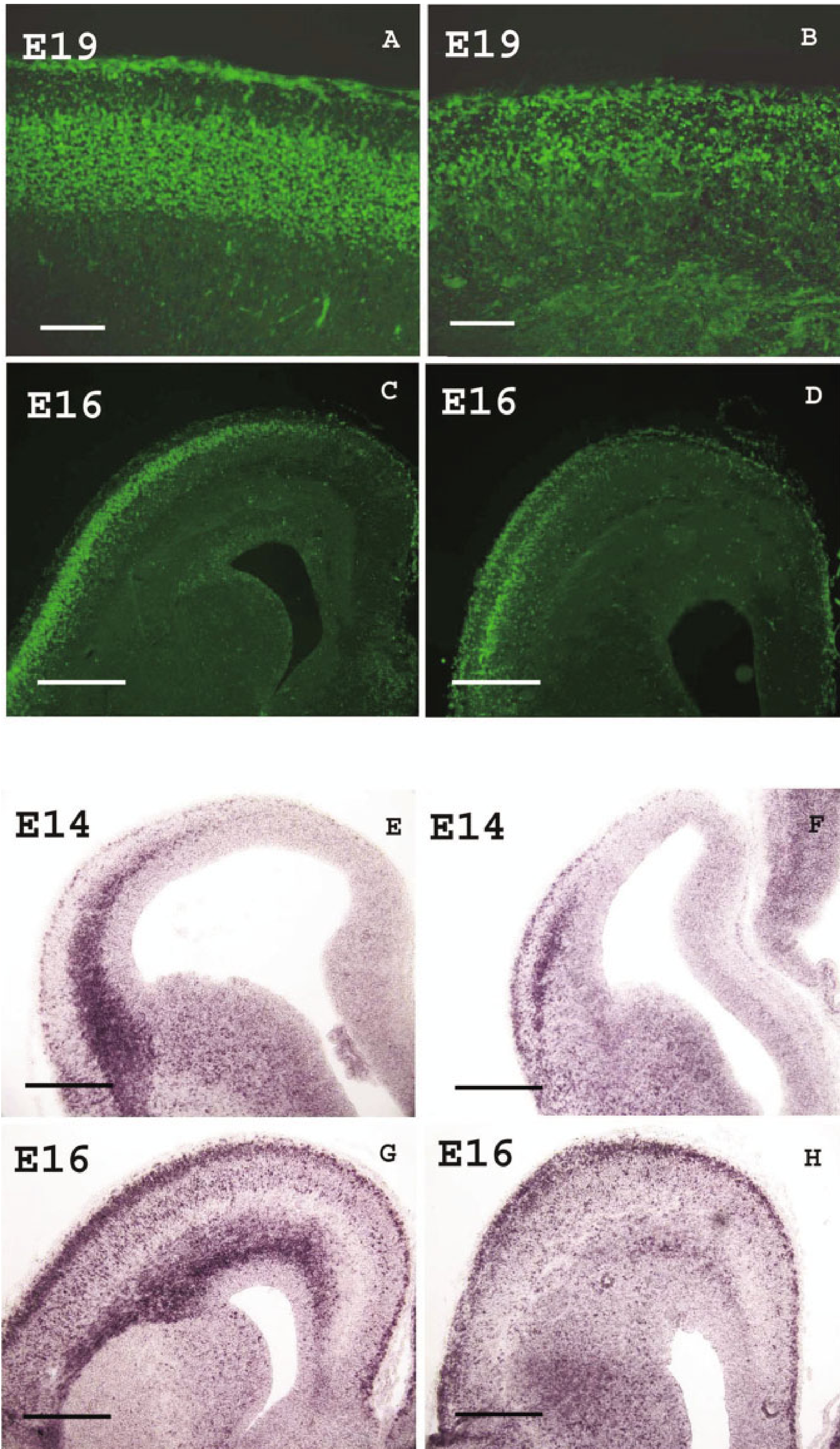

Figure 8

the laminar fate of the progeny (Caviness et al., 2003), which suggests a potential manner in which Cux expression might regulate cell cycle and cell fate through the modulation of p27. Thus, it would be interesting to investigate whether *Cux* genes can couple cell cycle and specification of upper laminar fates in the cortex and specifically regulate the proliferation of SVZ precursors. In this regard, it is worth mentioning that a role for *Cux* genes in cortical proliferation would not preclude a possible function of these genes in differentiated postmitotic neurons, in that, in transgenic mice, *Cux-1* overexpression promotes cell proliferation and results in organ hyperplasia but does not block differentiation; these organs are relatively normal and have the correct differentiated cell types (Ledford et al., 2002).

Expression of Cux genes in the SVZ suggests a distinct mode of specification of the upper layer neurons

Although it is increasingly accepted that radial glial cells in the VZ are neuronal precursors and that they ultimately give rise to most, or all, cortical neurons (Noctor et al., 2001; Malatesta et al., 2003), accumulating evidence has suggested that late-born neurons originate from an intermediate precursor in the SVZ (Smart and McCherry, 1982; Tarabykin et al., 2001). Recently, more definitive evidence has been provided from in vivo imaging studies showing neurons arising from intermediate neuronal precursors of the SVZ (Noctor et al., 2004; Haubensak et al., 2004).

We found that *Cux-2* mRNA expression, as well as nuclear Cux-1 protein, identifies a population of cells located in the SVZ that we propose serve as precursors of the upper layer neurons. Three main lines of evidence support this conclusion. First, BrdU labeling experiments demonstrate that dividing SVZ precursors express high levels of nuclear Cux-1 protein, as found in the postmitotic upper layer neurons; in contrast, VZ precursors express very low levels of Cux-1. BrdU and in situ double labeling also suggest that some *Cux-2*-expressing cells in the SVZ may be dividing. Second, the chronological appearance of *Cux-2* expression in the SVZ during development precedes the birth of upper layer neurons. The first upper layer neurons are born in the most rostrolateral cortex. Upper layer neurons appear subsequently in more dorsomedial and caudal regions, as neurogenesis advances from lateral to dorsal and from rostral to caudal (Takahashi et al., 1999). *Cux-2* in the SVZ is first detected at E12 in the lateral cortex (Fig. 3), when only deep layer neurons are born. Because deep layer neurons never express *Cux-2*, the *Cux-2* SVZ expression may correspond to precursors of the upper layer neurons. This is also clear at E14; the high number of cells expressing *Cux-2* in the lateral areas precludes the notion that they would all be postmitotic upper layer neurons (Fig. 3I). Finally, we observe that reduction of the number of *Cux-2*-positive cells in the SVZ in the *Pax-6* mutant mice correlates with defects in *Cux*-positive postmitotic cortical neurons in time and number. This links both *Cux-2*-positive populations in the SVZ and cortical plate and strongly suggests that *Cux-2* SVZ cells are neuronal precursors for the upper layer neurons.

Hence, *Cux-2* appears as an excellent marker for SVZ neuronal precursors specific for the upper layer neurons. Intermediate neuronal precursors have been identified in the cortex *in vivo* (Price et al., 1991; Noctor et al., 2004), but their molecular identity has remained elusive. Strong evidence for the existence of an SVZ precursor specific for upper layer neurons was recently provided by cloning of the noncoding RNA *Svet*, which is expressed both in the SVZ and in upper layer neurons (Tarabykin et al., 2001). It is interest to note that the hypothetical existence of a cortical SVZ precursor parallels the developmental processes that occur in the subcortical telencephalon, where late-born neurons of the striatum are born from SVZ neuronal precursors (Anderson et al., 1997b). In this case, two homeobox transcription factors, Dlx-1 and Dlx-2, have been shown to regulate specifically the differentiation of the striatal SVZ and the late-born neurons (Anderson et al., 1997b; Yun et al., 2002). Thus, it is feasible a that similar type of late precursor might exist in the dorsal telencephalon. However, to this day, no other molecular markers different from Svet have been reported to identify these populations and support this hypothesis, nor has the expression of any transcription factors reflected the changes in cell fate specification that must occur at the switch of VZ to SVZ cells. Remarkably, *Cux-2* and *Svet* show very similar expression patterns in the telencephalon, suggesting that they mark overlapping cellular populations. Accordingly, expression of both *Svet* and *Cux* genes is disrupted in *Pax-6* mutants. However, the expression of *Svet* is virtually lost in *Pax-6* mutants, whereas *Cux-2* is reduced but still detectable, indicating that *Cux-2* and *Svet* might be differentially regulated and perhaps that *Svet* could mark a more differentiated neuronal state than *Cux-2*.

In conclusion, *Cux-1* and *Cux-2* genes represent novel markers for the II/III and IV cortical layers, whereas the expression of *Cux-2* mRNA provides for an excellent additional marker for the switch between the VZ and SVZ precursors. Furthermore, the involvement of the *Drosophila Cut* in neuronal cell fate determination points to a possible functional role for *Cux* genes as part of the molecular mechanisms directing the specific differentiation of upper layer neurons, although this hypothesis can be definitively tested only by genetic analysis of *Cux-1* and *Cux-2* function.

Fig. 8. Production of Cux-positive neurons is prematurely extinguished in *Pax-6 Sey*/*Sey* mutants. Immunostaining with anti-Cux-1 antibody of E19 and E16 WT and *Sey*/*Sey* mutant brains (**A–D**). *In situ* hybridization with a *Cux-2* probe in E14 and E16 WT and *Sey*/*Sey* (E–H). In *Sey*/*Sey*, defects in *Cux*-positive neurons in the cortical plate correlate with a progressive reduction of *Cux-2* expression in the SVZ. Scale bars = 100 μm in A,B; 500 μm in C–H.

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