Published in final edited form as:

Exp Neurol. 2008 May ; 211(1): 271–282. doi:10.1016/j.expneurol.2008.02.003.

Doublecortin-expressing cells are present in layer II across the adult guinea pig cerebral cortex: Partial colocalization with mature interneuron markers

Kun Xiong^{a,d}, Duan-Wu Luo^b, Peter R. Patrylo^c, Xue-Gang Luo^d, Robert G. Struble^e, Richard W. Clough^a, and Xiao-Xin Yan^a

^aDepartment of Anatomy, Southern Illinois University School of Medicine, Carbondale, IL 62901, USA

^bDepartment of Neurosurgery, Central South University Xiangya Hospital, Changsha, Hunan 410078, China

^cDepartment of Physiology, Southern Illinois University School of Medicine, Carbondale, IL 62901, USA

^dDepartment of Anatomy and Neurobiology, Central South University Xiangya Medical School, Changsha, Hunan 410013, China

^eDepartment of Neurology and Center for Alzheimer Disease, Southern Illinois University School of Medicine, Springfield, IL 62794, USA

Abstract

Doublecortin-immunoreactive (DCX+) cells were detected across the allo- and neo-cortical regions in the adult guinea pig cerebrum, localized to layer II specifically at its border with layer I. The density of labeled cells declined with age, whereas no apparent apoptotic activity was detectable over the cortex including layer II. DCX+ cells varied in somal size, labeling intensity, nuclear appearance, and complexity of processes. These cells were often arranged in clusters with cells of similar morphology sometimes packed tightly together. They exhibited complete colocalization with polysialylated neural cell adhesion molecule (PSA-NCAM) and neuron-specific type III β -tubulin (TuJ1). Medium to large-sized DCX+ cells had well-developed neuritic processes, and expressed neuron-specific nuclear protein (NeuN). Large mature-looking cells with weak DCX reactivity invariably displayed heavy NeuN reactivity, implicating a transitional stage of these labeled cells. These “transitional” cells also consistently exhibited weak reactivity for γ -aminobutyric acid (GABA), glutamate decarboxylase (GAD67), β -nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) and neuronal nitric oxide synthase (nNOS), suggestive of them being young GABAergic/nitrinergic interneurons. Our data indicate that DCX+ cells exist widely in the adult guinea pig cerebral cortex, with a predominant localization in upper layer II. The morphological variation and differential expression of neuronal markers in these cells implicate that they might be developing neurons, and that they are probably differentiating into GABAergic interneurons. This population of cells might be involved in interneuron plasticity in the adult mammalian cerebral cortex.

Keywords

Layer I; GABA; Nitric oxide synthase; Neuroplasticity; Neurogenesis; Neocortex

Introduction

In the developing brain, doublecortin (DCX) appears to play a crucial role in neuronal migration (Francis et al., 1999), including tangential migration of interneuron precursors into the cortex (Friocourt et al., 2007). A DCX gene mutation is related to X-linked subcortical laminar heterotopia (“double cortex” syndrome), a subclass of lissencephaly characterized by mental retardation and epilepsy ([des Portes et al., 1988] and [Gleeson et al., 1988]). In the adult brain, newly generated neurons in the hippocampal formation and the subventricular zone express DCX ([Magavi et al., 2000] and [Nacher et al., 2001]). Thus, DCX is transiently present in immature neurons that eventually develop into mature granule cells and integrate into functional hippocampal circuitry (Brown et al., 2003). Similarly, DCX immunoreactive (DCX+) neuroblasts in the subventricular zone migrate via the rostral migratory stream and differentiate into mature GABAergic interneurons in the olfactory bulb ([Magavi et al., 2000], [Nacher et al., 2001] and [Gritti et al., 2002]).

A novel population of DCX+ cells has been reported in the temporal and prefrontal cortices of mice, rats and primates. These cells reside primarily in layer II, and invariably express several other molecules enriched in immature neurons, including polysialylated neural cell adhesion molecule (PSA-NCAM) and neuron-specific tubulin-III (TuJ1) ([Seki and Arai, 1991], [Bonfanti et al., 1992], [O'Connell et al., 1997], [Fox et al., 2000], [Nacher et al., 2001] and [Varea et al., 2007a]). The true identity and origin of these cortical DCX+ cells remains elusive, and little is known regarding their existence in other mammalian species apart from those mentioned above (Bonfanti, 2006). Also, unlike adult-born subgranular and subventricular cells that express the very same set of immature neuronal markers, considerable controversy remains as to whether these DCX+ cortical cells are newly generated. For example, findings of 5-bromo-2'-deoxyuridine (BrdU) incorporation in these cells have been mixed ([O'Connell et al., 1997], [Fox et al., 2000], [Nacher et al., 2001], [Nacher et al., 2004], [Bernier et al., 2002], [Varea et al., 2005], [Bonfanti, 2006], [Pekcec et al., 2006], [Shapiro et al., 2007a] and [Shapiro et al., 2007b]).

In the present study, we have identified DCX+ cells in layer II throughout the allo- and neo-cortical areas in the adult guinea pig. Based on their morphology and differential expression of immature and mature neuronal markers, these DCX+ cells appear to be immature neurons undergoing differentiation. Moreover, some mature-looking DCX+ cells co-express γ -aminobutyric acid (GABA), glutamate decarboxylase (GAD67), β -nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) and neuronal nitric oxide synthase (nNOS). The existence of this population of neurons in the adult cerebral cortex might have important functional implications, potentially regarding interneuron plasticity under physiological conditions as well as interneuron alterations under certain pathophysiological circumstances.

Materials and methods

Animal and tissue preparation

Male Hartley guinea pigs weighing 560–1250 g were purchased from Charles River Laboratories (Wilmington, MA). Animals at 3, 6, 12, 24 and 36 months-old ($n = 4$ per age) were used to quantify the densities of DCX+ cells. Colocalization studies were carried out in brain sections from 6 and 12 months-old animals. Additional 3 ($n = 3$) and 12 ($n = 3$) months-old animals were used for western analyses and in situ detection of DNA cleavage,

together with frozen adult (2 months-old) rat brains ($n = 3$) and olfactory bulb sections ($n = 3$) available from a recent study serving as materials for positive controls (Yan et al., 2007).

For immunohistological studies, guinea pigs were perfused via the ascending aorta with 4% paraformaldehyde with (for GABA immunolabeling) or without 0.5% glutaraldehyde in 0.01 M phosphate-buffered saline (pH 7.4, PBS) under overdose anesthesia (sodium pentobarbital, 100 mg/kg, i.p.). Brains were removed, postfixed overnight, and soaked in 30% sucrose in PBS at 4 °C until they sunk. One cerebral hemisphere from each brain was sectioned coronally using a cryostat. Twenty consecutive sections at 30 μ m thickness in every 1 mm of brain were serially collected into culture plates. From the septal to mid-hippocampal levels, approximately 20 sets of 8 μ m sections were also collected, and thaw-mounted on positively charged microslides (VWR International, West Chester, PA). Tangential cortical sections were prepared from some brains using the other hemisphere. The cortex was separated from the striatum and hippocampal formation, and flat-embedded between Parafilm pieces lining inside two microslides. The flattened cortex was cut at 8 or 30 μ m from the pial surface down. All 30 μ m sections were used for immunolabeling using the peroxidase method, while double immunofluorescence was carried out on 8 μ m sections. One set of the 30 μ m sections was processed with Nissl stain for histological orientation. For western analyses and in situ detection of DNA cleavage, brains were rinsed briefly by perfusion with cold PBS. Semi-brains (forebrain hemispheres) from each animal were then snap-frozen for protein extraction and slide-mounted cryostat sections at 20 μ m (coronal), respectively.

Animal use was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All experimental procedures in the present study were approved by the Institutional Animal Care and Use Committee of Southern Illinois University Carbondale.

Immunohistochemistry

To visualize immunoreactivity with the peroxidase method, sections were treated initially with 1% H₂O₂ in PBS for 30 min, and then in 10% normal rabbit serum in PBS with 0.3% Triton X-100 for 1 h at room temperature. Subsequently, sections were incubated in PBS containing goat anti-DCX (1:2000) (see Table 1), 5% normal rabbit serum and 0.1% Triton X-100 overnight at 4 °C. Sections were further reacted with biotinylated rabbit anti-goat IgG at 1:400 for 2 h, and subsequently with ABC reagents (1:400) (Vector Laboratories, Burlingame, CA) for 1 h. Immunoreaction products were visualized using 0.003% hydrogen peroxide and 0.05% diaminobenzidine. Three 10-minute washes with PBS were used between incubations. Sections were mounted on slides, allowed to air-dry, and then were dehydrated, cleared and coverslipped.

For immunofluorescence, slide-mounted sections (8 μ m thick) were incubated in PBS containing 5% donkey serum, 0.3% Triton X-100 and a pair of primary antibodies raised in different species overnight at 4 °C (see Table 1). Sections were further incubated for 2 h at room temperature in PBS containing Alexa-Fluor® 488 and Alexa-Fluor® 568 conjugated donkey antibodies against mouse, rabbit or goat IgGs (1:200, Invitrogen, Carlsbad, CA). Sections were finally counter-stained with bisbenzimidazole (Hoechst 33342, 1:50,000), washed and coverslipped with anti-fading medium (Vector Laboratories).

The primary antibodies applied in this study have been used in multiple previous reports (See Results). In the present study, omission of the antibody in the assay buffer or substitution with a corresponding normal serum yielded no specific labeling.

NADPH-diaphorase histochemistry

β -Nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry was carried out as described previously (e.g., Yan et al., 1994). In brief, sections were incubated in a solution containing 0.3% Triton X-100, 1 mM β -NADPH-d (N7505, Sigma-Aldrich, St. Louis, MO), 0.8 mM nitroblue tetrazolium (N6639, Sigma-Aldrich) and 5% dimethyl sulfoxide in 0.05 M Tris-HCl buffered saline (pH 8.0, TBS) for 15 or 45 min at 37 °C. The reaction was stopped by rinsing sections in PBS. Some sections were further processed for DCX or NOS immunolabelings using the peroxidase method as described above.

In situ detection of DNA cleavage

A modified terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay was performed as described (Yan et al., 2001). Fresh-frozen guinea pig cortical and rat olfactory bulb sections (20 μ m) were fixed with 4% paraformaldehyde at room temperature in PBS for 20 min. The olfactory bulb sections were from rats surviving 1 month post unilateral naris-occlusion (Yan et al., 2007), serving as ideal positive control for TUNEL since apoptosis exists in normal rodent bulbs but increases significantly following functional deprivation (Najbauer and Leon, 1995). Slide-mounted sections were treated with 0.3% Triton X-100 in PBS for 30 min, rinsed in distilled water for several minutes, and incubated with 0.06 U/ml terminal deoxynucleotidyl transferase (TDT, Amersham Biosciences, Piscataway, NJ, E2230Z), and 4 mM biotin-16-dUTP (Roche Diagnostics, Indianapolis, IN, 11-093-070-910) in TDT buffer (pH 7.4, containing 140 mM sodium cacodylate, 1 mM cobalt chloride in PBS) at 37 °C for 3 h. The DNA terminal labeling was visualized with Alexa-Fluor 568-conjugated streptavidin (Invitrogen, 1:500), followed by bisbenzimidazole counterstain for laminar orientation.

Western blot

The piriform and entorhinal cortices of the guinea pigs and rats were dissected out and homogenized with a sonication device in T-PER buffer (10 $\times$ w/v) (Pierce, Rockford, IL) containing a cocktail of protease inhibitors (Roche). Tissue lysates were centrifuged at 8000 $\times$ g at 4 °C for 10 min. Supernatants were collected and protein concentrations determined by DC protein assay (Bio-Rad Laboratories, Hercules, CA). Equal quantities of protein (30 μ g) were run on 10% SDS-PAGE gels (Hoefer Scientific Instruments, San Francisco, CA). The polypeptides were electrotransferred to Trans-Blot® pure nitrocellulose membrane (Bio-Rad Laboratories). Non-specific binding was blocked with PBS containing 5% nonfat milk and 3% bovine serum albumin. Nitrocellulose membranes were blotted overnight with goat anti-DCX (1:2000) and rabbit anti-doublecortin-like kinase 1 (1:500) antibodies, respectively (see Table 1), followed by incubations in HRP-conjugated secondary antibodies (1:5000, Bio-Rad Laboratories) for 1 h. Protein bands were visualized with an ECL Plus™ Western Blotting Detection kit according to manufacturer's instruction (GE Healthcare Life Sci., Piscataway, NJ), and images captured immediately in a UVP Biodoc-it™ system (UVP, Inc, Upland, CA). Equal protein loading was evaluated by re-blotting β -tubulin-III signal (1:20,000) on the same membranes.

Imaging and densitometry

Sections were examined and imaged on an Olympus fluorescent BX60 microscope equipped with a digital imaging system (MicroFire® CCD Camera and software, Optronics, Goleta, CA). For cell counts, three coronal sections at comparable rostrocaudal levels were selected. Camera lucida drawings of labeled cells were made along the pial surface. Using the rhinal fissure (RS) and lateral sulcus (LS) as landmarks, the cortex was divided into parietal, temporal, entorhinal and piriform areas. Dots representing labeled cells were counted in each cortical segment, with the lengths of the pial surface measured for corresponding

cortical segments. Cell density was calculated and expressed as the number of cells per millimeter length of pial surface. Images of representative sections were captured using a 10× objective, and subsequently montaged with Photoshop 7.0.

Immunofluorescent labelings in coronal or tangential sections were examined with 20× and 40× objectives. Images of the same cellular profiles were taken using green (DCX labeling), red (other immunolabelings) and blue (bisbenzimidazole stain) fluorescent filters. Some sections were also examined with a confocal microscope (Olympus Fluoview, Japan), and overlapping of labeled cells was rarely encountered in the 8 μ m sections.

Statistical testing and figure preparation

Means of cell densities were calculated for individual and groups of animals. Statistical comparisons were conducted using one-way ANOVA followed by Bonferroni's pair-wise comparisons, yielding *p* values between individual groups (Prism GraphPad 4.1, San Diego, CA). The minimal significance level was set at *p* < 0.05. All illustrations were prepared with Corel Draw 10 (Corel Corp., Ontario, Canada). Whole panel images were converted into a TIFF format, with contrast/brightness adjusted as needed.

Results

Antibody characterization in guinea pig cerebral cortex

To confirm the specificity of the goat anti-DCX antibody used in the present study, western analyses were carried out using both guinea pig and rat cerebral extracts (piriform/entorhinal cortex). As with previous reports ([Brown et al., 2003] and [Capes-Davis et al., 2005]), this antibody recognized a single band migrated at ~ 40 kd in cortical lysates from both species (Fig. 1). In the same cortical samples, a putative doublecortin-like kinase-1 protein visualized with an antibody from Chemicon (Table 1) migrated at ~ 85 kd, consistent with the predicted molecular size of this latter protein (Capes-Davis et al., 2005). Thus, the expression of DCX in the brain appeared similar for rat and guinea pig, and the goat antibody appeared to be highly specific to DCX in these two species.

Overall distribution and age-related reduction of cortical DCX+ cells

DCX+ cells were found in multiple forebrain areas of all adult guinea pigs examined in the present study (Fig. 2). Consistent with previous findings in other species, DCX+ cells occurred in the rostral migratory stream (Fig. 2A, C), the subventricular zone as well as the nearby white matter (Fig. 2D, F, H), and the subgranular zone of the hippocampal dentate gyrus (Fig. 2H, J).

Distinct DCX+ cells were also seen in the cerebral cortex in the superficial region of the cortical mantle, forming a cellular band deep to layer I throughout the allo- and neo-cortical areas (Fig. 2A, D, H). Radially this band included a few cells in layer II, primarily at the border with layer I. Individual DCX+ cells were also noted in the upper part of layer III (especially in the piriform and entorhinal cortices) (Fig. 2B, E, I, K). For descriptive simplicity and consistency with literature, we refer to these cells collectively as layer II DCX+ cells in this current study. Tangentially this cellular band continued beneath the pial surface over the medial, dorsal and lateral cerebral aspects (Fig. 2 and Fig. 3). Within a given brain, the abundance of DCX+ cells in various cortical regions varied, with an approximate order of labeling being from high to low: piriform, entorhinal, temporal/frontal, and parietal/occipital areas (Fig. 2 and Fig. 3).

To determine whether there was an age-dependent change in DCX+ cell population, the density of immunoreactive cells was quantified in the parietal, temporal, entorhinal and

piriform areas using 3 sections (~ 2 mm apart) at the rostral to middle hippocampal levels from each animal (Fig. 3). To accomplish this, sections from individual animals were processed in parallel using multiple-chambered incubators. An overall decline of DCX+ cells was found with age in all neo-and allo-cortical areas analyzed ($F = 19.29$ for piriform, 32.80 for entorhinal, 51.37 for temporal and 39.32 for parietal cortices, respectively, with $p < 0.0001$ and $df = 4, 10$ in all cases, one-way ANOVA) (Fig. 3B). In all areas except the parietal cortex ($p < 0.01$), the mean densities of DCX+ cells in 3 months-old animals were not statistically different than that seen at 6 months, although differences were noted relative to older animals ($p < 0.001$ to $p < 0.0001$). Similarly, the mean densities at 6 months were not significantly different from that at 12 months, but again, were significantly different relative to those at 24 and 36 months. Further, the densities at 12 months did not reach statistical significance as compared to those of 24 ($p = 0.067$) and 36 months ($p = 0.052$).

Lack of overt apoptosis in cortical DCX+ cells

To explore whether the age-related decline in layer II DCX+ cells might involve obvious cell death or apoptosis, we carried out TUNEL assay in cortical sections from 3 (Fig. 4A, B) and 12 (not shown) months-old guinea pigs. At both ages, TUNEL profiles were occasionally encountered over the cortex and white matter, without noticeable laminar preference (Fig. 4A, B). In particular, there was no apparent indication of elevated TUNEL activity in layer II and its vicinity relative to the rest laminae of cortex. In contrast, in the rat olfactory bulb sections processed under identical conditions, TUNEL activity was evident across the sections especially the granule cell layer. Further, labeled profiles were apparently denser in the deprived side relative to the non-deprived side (Fig. 3C), as demonstrated previously (Najbauer and Leon, 1995). Because of the rare occurrence of TUNEL profiles across the normal guinea pig cortex (especially in layer II), a comprehensive search and analysis for double labeling of TUNEL and DCX was not performed in this study.

Morphology of cortical DCX+ cells

Overall, layer II DCX+ exhibited a heterogeneous morphology (Fig. 5). Cell bodies exhibited various shapes, including round, oval, bipolar, multipolar, pyramidal-like and irregular (Fig. 5A–E). The smallest cells were approximately 5 μm in somal diameter, weakly stained, and had no, one/two processes that extended from one or opposite poles of the somata. Other small cells were often bipolar with stronger labeling and longer processes (Fig. 5 and Fig. 6). Medium-sized (5–10 μm) cells displayed moderate to heavy DCX reactivity, with 2–4 processes radiating from the somata and extending for up to 100 μm into layer II and the lower part of layer I. Large-sized DCX+ cells ranged from approximately 10–20 μm in somal diameter and had branched dendrite-like processes that extended tangentially, obliquely or radially for several hundred micrometers (Fig. 5A–E). These branches could often be traced to the pial surface (Fig. 2 and Fig. 5). Thin, axon-like processes were identifiable on many medium and large-sized DCX+ cells. These axons extended into the deeper cortical layers frequently reaching as far as layer IV (Fig. 5B, C). Most of the large DCX+ cells showed moderate staining intensity, whereas a subpopulation of these cells displayed apparently reduced immunoreactivity in both the somata and processes (Fig. 5 and Fig. 6). Overall, small DCX+ cells appeared to outnumber medium and large-sized cells in any given region, which was most noticeable at the interface between layers I and II in tangential sections (Fig. 5D).

DCX+ cells appeared to occur in clusters, which was apparent in tangential sections. No obvious regularity or consistency was recognizable with regards to cell number, size, shape, or the border and distance between individual clusters (Fig. 2 and Fig. 5). However, within a given cluster cells of different sizes and shapes often co-existed (Fig. 5D, E). In many cases, a pair or several labeled somata were tightly packed together or appeared to be physically

attached. These closely apposed cells often displayed comparable morphological features (i.e., similar somal diameter, staining intensity, dendritic arbor size and nuclear appearance) (Fig. 2, Fig. 5 and Fig. 6).

In bisbenzimidazole stained sections, small DCX⁺ cells had small but brightly stained nuclei that were almost as big as their somata (Fig. 6A–D, H). Medium to large-sized DCX⁺ cells had larger but less intensely stained nuclei that were surrounded by a greater amount of cytoplasm (Fig. 6 and Fig. 7). The nuclei of large cells with reduced or faint DCX labeling had a similar appearance to neighboring mature neurons as assessed by other markers (see below) (Fig. 6 and Fig. 7).

Colocalization of immature and mature neural markers in DCX⁺ cells

Eight micrometer slide-mounted sections that exhibited minimal cellular overlapping were used to determine colocalizations of DCX and other molecules. In general, all layer II DCX⁺ cells were immunoreactive for PSA-NCAM ([Seki and Arai, 1991] and [Nacher et al., 2002]). PSA-NCAM immunoreactivity tended to outline the somata and dendrites of DCX⁺ cells. Thus, colocalization of these two markers was most obvious near the plasmalemma (Fig. 6A–D). Complete DCX colocalization with neuron-specific type III β -tubulin (TuJ1 or Tu-20) was also noted in this population of cells (Fig. 6E) (Yan et al., 2001).

To determine whether Cajal–Retzius cells or astrocytes could contribute to the layer II DCX⁺ cells, double labeling for DCX and reelin or GFAP was performed (Gong et al., 2007). As illustrated in Fig. 6F, reelin and DCX did not colocalize in layers I and II. Also, no colocalization of DCX with GFAP was noted in any cell (Fig. 6G).

DCX⁺ cells showed variable immunoreactivity for NeuN (Fig. 6H–K) (Yan et al., 2001). Specifically, small DCX⁺ cells, whether individually distributed or tightly packed, did not express NeuN (Fig. 6H–K). In contrast, larger DCX⁺ cells consistently expressed NeuN (Fig. 6I–K). Among the medium-sized DCX⁺ cells, weak NeuN labeling was seen in the nuclei of some cells but not others (Fig. 6I–K). Thus, it appears that elevated NeuN levels are concordant with a decrease in DCX staining in medium to large somal sized cells.

Colocalization of principal and local circuit neuron markers in DCX⁺ cells

Many DCX⁺ cells exhibited a mature-looking neuronal morphology and expressed high levels of NeuN. Thus, we examined whether specific markers of principal or local circuit neurons were present in these cells. Neurogranin, a neuron-specific protein that is heavily and exclusively expressed in cerebral principal neurons (Singec et al., 2004), was not colocalized with DCX⁺ cells, including those with weak or faint DCX reactivity (Fig. 7A–D).

A subpopulation of DCX⁺ cells however displayed weak to moderate GABA labeling (Yan et al., 1996b). In general, GABA immunoreactivity was not observed in small DCX⁺ cells, but became consistently detectable in most medium and large-sized cells (Fig. 7E–G). In fact, strong and distinct GABA reactivity occurred in virtually all large cells with weak DCX reactivity. Similarly, large-sized mature-looking cells with reduced DCX levels co-expressed GAD67 (Fig. 7H–N) or GAD65/67 (not shown but with similar patterns as GAD67) (Yan et al., 1998). Of note, GAD reactivity in DCX⁺ cells was weak relative to nearby, presumably mature, interneurons that displayed only GAD but no DCX labeling. Also, colocalization of DCX and GAD was better viewed in tangential sections (at the immediate interface of layer I and II) over areas with less crowded neurons and less dense GAD labeling in the neuropil (Fig. 7K–N). No clear colocalization of DCX and calcium-binding proteins (parvalbumin, calbindin and calretinin), markers of mature GABAergic subpopulations, was however noted (not shown).

Colocalization of NADPH-diaphorase and NOS in DCX+ cells

Nitric oxide synthase (NOS) is expressed in subpopulations of cortical interneurons, and can be detected by NADPH-d histochemistry or NOS immunolabeling (Dawson et al., 1991). Type I nitrinergic neurons have a large somal size and exhibit heavy NADPH-d staining (thus giving the cells a Golgi-like appearance). These cells are few in number and are localized overwhelmingly to the subcortical white matter. Type II neurons have small somal size, light to moderate NADPH-d reactivity, and are numerous in the supragranular cortical layers ([Yan et al., 1996b] and [Smiley et al., 2000]; review by Judas et al., 1999).

Some layer II DCX+ cells displayed weak yet consistent NADPH-d or neuronal NOS (nNOS) reactivity (Fig. 8). As with GAD67, NADPH-d or nNOS labeling was observed in large-sized mature-looking neurons that exhibited weak DCX reactivity (Fig. 8A–I). Double-labeled neurons were sometimes close to or apparently attached to each other, or to cells displaying strong and distinct reactivity for either NADPH-d/nNOS or DCX (Fig. 8C–F, I). Of note, all of these NADPH-d/nNOS reactive cells, whether or not expressing DCX, fell into the category of type II nitrinergic neurons. Colocalization of NADPH-d and nNOS in type I and type II nitrinergic neurons was verified in the present study using double labeling (Fig. 8J, K). Type I cells were visualized following a short (15 min) NADPH-d incubation (longer reaction results in heavy nitroblue tetrazolium deposits that mask immunoreaction products).

Most type II NADPH-d neurons also express calbindin ([Yan et al., 1996b], [Yan and Garey, 1997] and [Smiley et al., 2000]). To investigate why there can be a partial colocalization of DCX with NADPH-d but no colocalization with calbindin, the cellular pattern of calbindin/NADPH-d colocalization was examined. Calbindin was found to occasionally colocalize in weak NADPH-d reactive neurons cells, especially those in layers II and III (Fig. 8L). For quantitative analysis, cell counting was conducted in 3 coronal sections from the septal to mid-hippocampal levels in each brain over the parietotemporal areas by scanning along the border of layers I and II at 40× (covering ~ 200 µm cortical depth, roughly corresponding to layer II). These analyses yielded a small degree of calbindin colocalization in NADPH-d neurons (mostly weak reactive) in this lamina, about 9.3% (57/612) and 8.3% (43/516) at 6 ($n = 4$) and 12 ($n = 4$) months of age, respectively. In contrast, calbindin reactivity was present in virtually all NADPH-d (mostly moderate reactive) type II neurons in deeper cortical layers (Fig. 8 L, M), as characterized previously ([Yan and Garey, 1997] and [Smiley et al., 2000]).

Discussion

In the present study a population of DCX+ cells has been characterized in the cerebral cortex of adult guinea pigs. These DCX+ cells are predominately localized to upper layer II (occur from deep layer I to upper layer III). The number of these cells declines from juvenile to older adult stages. These cells co-express PSA-NCAM and TuJ1, two additional molecules that are typically associated with immature neurons. They also differentially express selective mature neuronal markers, but do not express glial marker proteins. These data support the general notion that these DCX+ cells have a neuronal identity ([Seki and Arai, 1991], [Bonfanti et al., 1992], [O'Connell et al., 1997], [Fox et al., 2000], [Nacher et al., 2001] and [Bonfanti, 2006]).

DCX+ cells show a trend of development and maturation

Layer II DCX+ cells exhibited remarkably heterogeneous yet apparently correlated morphological characteristics. From small to larger cells, somal shape ranges from round/oval to bipolar, multipolar or irregular; nuclei evolve from small to large and from bright to

pale in bisbenzimidazole stain; dendrite-like processes increase in number, length and branching complexity; and axon-like processes elongate and extend to deeper cortical layers. These morphological variables observed across DCX+ cells are largely comparable to those characterized in developing cortical neurons *in vivo* and *in vitro* ([del Rio et al., 1992], [de Lima and Voigt, 1997] and [Ang et al., 2003]). Thus, it is plausible that layer II DCX+ cells may be immature and developing.

Consistent with this hypothesis, the relative levels of DCX and NeuN in individual cells correlate with somal size and the complexity of neuritic processes. Thus, DCX expression appears to increase as the cells become larger and more mature-looking, until some peak point which is followed by downregulation. On the other hand, NeuN expression emerges in the cells approximately around the stage of peak DCX expression, and increases as DCX levels subsequently decline. DCX and NeuN expression patterns among the labeled cells are consistent with a transient expression of immature and increase of mature neuronal markers in developing neurons ([Brown et al., 2003] and [von Bohlen Und Halbach, 2007]). Taken together, these data suggest that layer II DCX+ cells are undergoing differentiation and maturation.

Also in supportive of a developmental nature of these cells is the lack of apparent indication of elevated apoptosis in layer II in TUNEL assay. Thus, it appears that layer II DCX+ cells might not be inevitably vulnerable to cell death under normal physiological conditions. As positive TUNEL activity is probably only detectable during a short period of time in the course of cell death, one cannot exclude the possibility that a few or some of layer II DCX+ cells may die. Regardless, apoptosis does not appear to be the major or immediate fate of these cortical DCX+ cells.

DCX+ cells may differentiate into GABAergic interneurons

Layer II DCX+ cells in rodent and primate cortex have been designated as neurogliaform, fusiform, semilunar, semilunar-pyramidal transitional and multipolar neurons, common morphological terms of major cortical interneuron subtypes ([Seki and Arai, 1991], [Nacher et al., 2001], [Bernier et al., 2002] and [Bonfanti, 2006]). We show here that a subpopulation of these DCX+ cells in the adult guinea pig cortex display neurochemical properties of mature cortical interneurons. Specifically, GABA, GAD67, NADPH-d and nNOS reactivities occur in large-sized and morphologically mature-looking cells that exhibited attenuated levels of DCX. In contrast, these interneuron markers are not detectable in small and morphologically primitive cells. In fact, when somal size and the complexity of neuritic branches are taken into account, the differential colocalization pattern of GABA and DCX is largely comparable with that of NeuN and DCX across individual cells. These data implicate that these DCX+ cells are undergoing differentiation into GABAergic/nitrinergic during morphogenesis. The intensity of GABA, GAD67, NADPH-d or nNOS labeling is apparently weaker in DCX double-labeled cells relative to neighboring mature interneurons lacking DCX. Also, calcium-binding proteins (parvalbumin, calbindin and calretinin) are not colocalized with DCX in layer II. Taken together, DCX+ cells co-expressing selective interneuron markers are probably at a transitional stage of development, evolving from immature neurons to young GABAergic interneurons.

In general, more than 90% of type II NADPH-d neurons in mammalian neocortex contain calbindin ([Yan et al., 1996b], [Yan and Garey, 1997] and [Smiley et al., 2000]). As calbindin is not detected in DCX+ cells, we speculate an infrequent colocalization of calbindin in NADPH-d reactive neurons in layer II relative to the overall population of these nitrinergic neurons in the cortex, which is confirmed by quantitative analysis in the present study. One may hypothesize that DCX+/NADPH-d+ cells may further develop into NADPH-d+/calbindin+ neurons in the guinea pig cortex.

Are layer II DCX+ cells recently generated?

BrdU incorporation in layer II DCX+ cells or alike (i.e., PSA-NCAM+ cells) has been described in rodent piriform cortex and monkey temporal lobe cortex ([Bernier et al., 2002], [Pekcec et al., 2006], [Shapiro et al., 2007a] and [Shapiro et al., 2007b]). However, others report little BrdU incorporation in this population of cells ([O'Connell et al., 1997], [Fox et al., 2000], [Nacher et al., 2001], [Nacher et al., 2002], [Nacher et al., 2004], [Varea et al., 2005] and [Bonfanti, 2006]). Of interest, significant changes in the number or density of this group of cells are reported in the piriform, temporal and prefrontal cortex under various experimental conditions, implicating a likelihood of alteration in cell population ([O'Connell et al., 1997], [Fox et al., 2000], [Tonchev et al., 2003], [Nacher et al., 2002], [Nacher et al., 2004], [Sairanen et al., 2007], [Shapiro et al., 2007a], [Varea et al., 2007b] and [van der Borgh and Brundin, 2007]). Future studies with improved birth-dating methodology might potentially clarify whether these DCX+ cells are newly-formed in the adult mammalian cortex. Alternatively, they may represent a reservoir of early-formed cells somehow maintaining at relatively immature status over time but capable for structural plasticity under various physiological and perhaps pathophysiological conditions ([Nacher et al., 2001] and [Bonfanti, 2006]).

Where do layer II DCX+ cells come from?

In the present study, DCX+ cells are found broadly across the guinea pig cerebral cortex including all neo-cortical areas. Morphologically primitive to fairly mature-looking DCX+ cells are essentially restricted to layer II, with virtually no similar cells detectable in deep cortical layers. Therefore, there exists little indication for a direct radial migration of these DCX+ cells from the subventricular zone before they reside in layer II.

During the past decade, it has become clear that cortical interneurons originate mostly from subcortical structures (medial, caudal and lateral ganglionic eminences, and olfactory primordium), with their precursors invading the cortex by tangential migration, including via layer I ([Lavdas et al., 1999], [Wichterle et al., 1999], [Anderson et al., 2001], [Zecevic and Rakic, 2001] and [Ang et al., 2003]). Several studies also show that some interneuron subgroups (including type II nitrinergic neurons) may populate over the cortical plate outside-in ([Soriano et al., 1992], [Yan et al., 1996a], [Wichterle et al., 1999], [Hevner et al., 2004] and [Rymar and Sadikot, 2007]). Importantly, the marginal zone/layer I may play a role in neurogenesis in the cortex during development, potentially contributing GABAergic interneurons to the cortical plate ([Zecevic and Rakic, 2001] and [Costa et al., 2007]). It is interesting to speculate that layer II DCX+ cells may have a local origin, being related to intrinsic layer I neuronal progenitors or interneuron precursors arrived by tangential migration via layer I. This hypothesis appears in harmony with their novel laminar location (at the border of layers I and II) and their potential to differentiate into type II NADPH-d neurons, possibly a phylogenetically and ontogenetically late-appearing GABAergic subpopulation ([Yan et al., 1996a], [Yan et al., 1996b] and [Yan et al., 1996b]).

In summary, this study describes an intriguing group of layer II cells over the adult guinea pig cerebral cortex that expresses DCX and other markers characteristic of immature neurons. There is a trend for morphological maturation of these DCX+ cells that is correlated with neurochemical differentiation into presumptive GABAergic interneurons. The precise origin and destiny of these cells, and their significance in cortical development, maintenance and function, remain a compelling question in neurobiology.

Acknowledgments

This study was supported by Southern Illinois University (X.X.Y.), Illinois Department of Public Health (X.X.Y., R.W.C., R.G.S.) and Epilepsy Foundation (P.R.P). We thank Dr. Charles E. Ribak at University of California Irvine for his constructive comments on the manuscript.

References

- Anderson SA, Marin O, Horn C, Jennings K, Rubenstein JL. Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development* 2001;128:353–363. [PubMed: 11152634]
- Ang ES, Haydar TF, Gluncic V, Rakic P. Four-dimensional migratory coordinates of GABAergic interneurons in the developing mouse cortex. *J. Neurosci* 2003;23:5805–5815. [PubMed: 12843285]
- Bernier PJ, Bedard A, Vinet J, Levesque M, Parent A. Newly generated neurons in the amygdala and adjoining cortex of adult primates. *Proc. Natl. Acad. Sci. U. S. A* 2002;99:11464–11469. [PubMed: 12177450]
- Bonfanti L. PSA-NCAM in mammalian structural plasticity and neurogenesis. *Prog. Neurobiol* 2006;80:129–164. [PubMed: 17029752]
- Bonfanti L, Olive S, Poulain DA, Theodosis DT. Mapping of the distribution of polysialylated neural cell adhesion molecule throughout the central nervous system of the adult rat: an immunohistochemical study. *Neuroscience* 1992;49:419–436. [PubMed: 1436474]
- Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG. Transient expression of doublecortin during adult neurogenesis. *J. Comp. Neurol* 2003;467:1–10. [PubMed: 14574675]
- Capes-Davis A, Tolhurst O, Dunn JM, Jeffrey PL. Expression of doublecortin (DCX) and doublecortin-like kinase (DCLK) within the developing chick brain. *Dev. Dyn* 2005;232:457–467. [PubMed: 15614772]
- Costa MR, Kessaris N, Richardson WD, Götz M, Hedin-Pereira C. The marginal zone/layer I as a novel niche for neurogenesis and gliogenesis in developing cerebral cortex. *J. Neurosci* 2007;27:11376–11388. [PubMed: 17942732]
- Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH. Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc. Natl. Acad. Sci. U. S. A* 1991;88:7797–7801. [PubMed: 1715581]
- de Lima AD, Voigt T. Identification of two distinct populations of gamma-aminobutyric acidergic neurons in cultures of the rat cerebral cortex. *J. Comp. Neurol* 1997;388:526–540. [PubMed: 9388013]
- del Rio JA, Soriano E, Ferrer I. Development of GABA-immunoreactivity in the neocortex of the mouse. *J. Comp. Neurol* 1992;326:501–526. [PubMed: 1484122]
- des Portes V, Pinard JM, Billuart P, Vinet MC, Koulakoff A, Carrie A, Gelot A, Dupuis E, Motte J, Berwald-Netter Y, Catala M, Kahn A, Beldjord C, Chelly J. A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. *Cell* 1988;92:51–61. [PubMed: 9489699]
- Fox GB, Fichera G, Barry T, O'Connell AW, Gallagher HC, Murphy KJ, Regan CM. Consolidation of passive avoidance learning is associated with transient increases of polysialylated neurons in layer II of the rat medial temporal cortex. *J. Neurobiol* 2000;45:135–141. [PubMed: 11074459]
- Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N, Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P, Chelly J. Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. *Neuron* 1999;23:247–256. [PubMed: 10399932]
- Friocourt G, Liu JS, Antypa M, Rakic S, Walsh CA, Parnavelas JG. Both doublecortin and doublecortin-like kinase play a role in cortical interneuron migration. *J. Neurosci* 2007;27:3875–3883. [PubMed: 17409252]
- Gleeson JG, Allen KM, Fox JW, Lamperti ED, Berkovic S, Scheffer I, Cooper EC, Dobyns WB, Minnerath SR, Ross ME, Walsh CA. Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. *Cell* 1988;92:63–72. [PubMed: 9489700]

- Gong C, Wang TW, Huang HS, Parent JM. Reelin regulates neuronal progenitor migration in intact and epileptic hippocampus. *J. Neurosci* 2007;27:1803–1811. [PubMed: 17314278]
- Gritti A, Bonfanti L, Doetsch F, Caille I, Alvarez-Buylla A, Lim DA, Galli R, Verdugo JM, Herrera DG, Vescovi AL. Multipotent neural stem cells reside into the rostral extension and olfactory bulb of adult rodents. *J. Neurosci* 2002;22:437–445. [PubMed: 11784788]
- Hevner RF, Daza RA, Englund C, Kohtz J, Fink A. Postnatal shifts of interneuron position in the neocortex of normal and reeler mice: evidence for inward radial migration. *Neuroscience* 2004;124:605–618. [PubMed: 14980731]
- Sestan N, Kostovic I. Nitrinergic neurons in the developing and adult human telencephalon: transient and permanent patterns of expression in comparison to other mammals. *Microsc. Res. Tech* 1999;45:401–419. [PubMed: 10402267]
- Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG. The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. *J. Neurosci* 1999;19:7881–7888. [PubMed: 10479690]
- Magavi SS, Leavitt BR, Macklis JD. Induction of neurogenesis in the neocortex of adult mice. *Nature* 2000;405:951–955. [PubMed: 10879536]
- Nacher J, Crespo C, McEwen BS. Doublecortin expression in the adult rat telencephalon. *Eur. J. Neurosci* 2001;14:629–644. [PubMed: 11556888]
- Nacher G, Alonso-Llosa G, Rosell D, McEwen BS. PSA-NCAM expression in the piriform cortex of the adult rat. Modulation by NMDA receptor antagonist administration. *Brain Res* 2002;927:111–121. [PubMed: 11821005]
- Nacher G, Pham K, Gil-Fernandez V, McEwen BS. Chronic restraint stress and chronic corticosterone treatment modulate differentially the expression of molecules related to structural plasticity in the adult rat piriform cortex. *Neuroscience* 2004;126:503–509. [PubMed: 15207367]
- Najbauer J, Leon M. Olfactory experience modulated apoptosis in the developing olfactory bulb. *Brain Res* 1995;674:245–251. [PubMed: 7796103]
- O'Connell AW, Fox GB, Barry T, Murphy KJ, Fichera G, Foley AG, Kelly J, Regan CM. Spatial learning activates neural cell adhesion molecule polysialylation in a corticohippocampal pathway within the medial temporal lobe. *J. Neurochem* 1997;68:2538–2546. [PubMed: 9166750]
- Pekcec A, Loscher W, Potschka H. Neurogenesis in the adult rat piriform cortex. *NeuroReport* 2006;17:571–574. [PubMed: 16603913]
- Rymar VV, Sadikot AF. Laminar fate of cortical GABAergic interneurons is dependent on both birthdate and phenotype. *J. Comp. Neurol* 2007;501:369–380. [PubMed: 17245711]
- Sairanen M, O'Leary OF, Knuutila JE, Castren E. Chronic antidepressant treatment selectively increases expression of plasticity-related proteins in the hippocampus and medial prefrontal cortex of the rat. *Neuroscience* 2007;144:368–374. [PubMed: 17049169]
- Seki T, Arai Y. Expression of highly polysialylated NCAM in the neocortex and piriform cortex of the developing and the adult rat. *Anat. Embryol. (Berl.)* 1991;184:395–401. [PubMed: 1952111]
- Shapiro LA, Ng KL, Zhou QY, Ribak CE. Olfactory enrichment enhances the survival of newly born cortical neurons in adult mice. *NeuroReport* 2007;18:981–985. [PubMed: 17558281]
- Shapiro LA, Ng KL, Kinyamu R, Whitaker-Azmitia P, Geisert EE, Blurton-Jones M, Zhou QY, Ribak CE. Origin, migration and fate of newly generated neurons in the adult rodent piriform cortex. *Brain Struct. Funct* 2007;212:133–148. [PubMed: 17764016]
- Singec I, Knoth R, Ditter M, Volk B, Frotscher M. Neurogranin is expressed by principal cells but not interneurons in the rodent and monkey neocortex and hippocampus. *J. Comp. Neurol* 2004;479:30–42. [PubMed: 15389613]
- Smiley JF, McGinnis JP, Javitt DC. Nitric oxide synthase interneurons in the monkey cerebral cortex are subsets of the somatostatin, neuropeptide Y, and calbindin cells. *Brain Res* 2000;863:205–212. [PubMed: 10773208]
- Soriano E, Del Rio JA, Ferrer I, Auladell C, De Lecea L, Alcantara S. Late appearance of parvalbumin-immunoreactive neurons in the rodent cerebral cortex does not follow an 'inside-out' sequence. *Neurosci. Lett* 1992;142:147–150. [PubMed: 1454208]

- Tonchev AB, Yamashima T, Zhao L, Okano HJ, Okano H. Proliferation of neural and neuronal progenitors after global brain ischemia in young adult macaque monkeys. *Mol. Cell. Neurosci* 2003;23:292–301. [PubMed: 12812760]
- van der Borgh K, Brundin P. Reduced expression of PSA-NCAM in the hippocampus and piriform cortex of the R6/1 and R6/2 mouse models of Huntington's disease. *Exp. Neurol* 2007;204:473–478. [PubMed: 17187781]
- Varea E, Nacher J, Blasco-Ibanez JM, Gomez-Climent MA, Castillo-Gomez E, Crespo C, Martinez-Guijarro FJ. PSA-NCAM expression in the rat medial prefrontal cortex. *Neurosci* 2005;136:435–443.
- Varea E, Castillo-Gomez E, Gomez-Climent MA, Blasco-Ibanez JM, Crespo C, Martinez-Guijarro FJ, Nacher J. PSA-NCAM expression in the human prefrontal cortex. *J. Chem. Neuroanat* 2007;33:202–209. [PubMed: 17467233]
- Varea E, Blasco-Ibanez JM, Gomez-Climent MA, Castillo-Gomez E, Crespo C, Martinez-Guijarro FJ, Nacher J. Chronic fluoxetine treatment increases the expression of PSA-NCAM in the medial prefrontal cortex. *Neuropsychopharmacology* 2007;32:803–812. [PubMed: 16900104]
- von Bohlen Und Halbach O. Immunohistological markers for staging neurogenesis in adult hippocampus. *Cell Tissue Res* 2007;329:409–420. [PubMed: 17541643]
- Wichterle H, Garcia-Verdugo JM, Herrera DG, Alvarez-Buylla A. Young neurons from medial ganglionic eminence disperse in adult and embryonic brain. *Nat. Neurosci* 1999;2:461–466. [PubMed: 10321251]
- Yan X, Garey LJ, Jen LS. Development of NADPH-diaphorase activity in the rat neocortex. *Dev. Brain Res* 1994;79:29–38. [PubMed: 8070062]
- Yan XX, Garey LJ, Jen LS. Prenatal development of NADPH-diaphorase-reactive neurons in human frontal cortex. *Cereb. Cortex* 1996;6:737–745. [PubMed: 8921208]
- Yan XX, Jen LS, Garey LJ. NADPH-diaphorase-positive neurons in primate cerebral cortex colocalize with GABA and calcium-binding proteins. *Cereb. Cortex* 1996;6:524–529. [PubMed: 8670678]
- Yan XX, Garey LJ. Morphological diversity of nitric oxide synthesising neurons in mammalian cerebral cortex. *J. Hirnforsch* 1997;38:165–172. [PubMed: 9176729]
- Yan XX, Toth Z, Schultz L, Ribak CE, Baram TZ. Corticotropin-releasing hormone (CRH)-containing neurons in the immature rat hippocampal formation: light and electron microscopic features and colocalization with glutamate decarboxylase and parvalbumin. *Hippocampus* 1998;8:231–243. [PubMed: 9662138]
- Yan XX, Najbauer J, Woo CC, Dashtipour K, Ribak CE, Leon M. Expression of active caspase-3 in mitotic and postmitotic cells of the rat forebrain. *J. Comp. Neurol* 2001;433:4–22. [PubMed: 11283945]
- Yan XX, Xiong K, Luo XG, Struble RG, Clough RW. β -Secretase expression in normal and functionally deprived rat olfactory bulbs: inverse correlation with oxidative metabolic activity. *J. Comp. Neurol* 2007;501:52–69. [PubMed: 17206602]
- Zecevic N, Rakic P. Development of layer I neurons in the primate cerebral cortex. *J. Neurosci* 2001;21:5607–5619. [PubMed: 11466432]

Fig. 1.

Representative western blots of adult guinea pig (G. pig) and rat cortical lysates (30 μ g protein/lane) with goat anti-doublecortin (DCX, Santa Cruz) and rabbit anti-doublecortin-like kinase-1 (DCLK, Chemicon). Western bands are comparable between the two species, migrating at $\sim$ 40 kD (DCX) and $\sim$ 85 kD (DCLK), respectively.

Fig. 2.

Doublecortin-immunoreactive (DCX+) cells in representative forebrain sections (30 μ m) from a 3 months-old guinea pig. Section levels are indicated in the low-right corner of (A), and framed areas in the low magnification images are enlarged as designated separate image panels. DCX+ cells are present in all typical adult neurogenic sites including the rostral migratory stream (RMS) (A, C); the subventricular zone over the striatum and deep to cortical white matter (WM) (D, F), and the subgranular zone deep to the granule cell layer (GCL) of the dentate gyrus (DG) (H, J). A cellular band of DCX+ cells, approximately corresponding to layer II, but mostly close to its upper border with layer I, extends along the entire cerebral surface except for the most ventral aspect (A, D, H, G, K). Cells include numerous lightly stained small ones and less abundant but more distinctly stained large ones. A plexus formed by labeled processes from these cells is present in layer I. Labeled cells are noticeably denser in the entorhinal (K), temporal (G, K) and prefrontal (B) areas, relative to the parietal area (D, E). Arabic numbers and short bars designate cortical layers. LV: lateral ventricle; LS: lateral sulcus; RF: rhinal fissure; CA1: hippocampal CA1 sector; ML: molecular layer; H: hilus; L and D with arrows designate lateral and dorsal directions relative to anatomic position. Scale bar = 1 mm in A; equal to 50 μ m for C, F; 200 μ m for B, E, I, J; 300 μ m for G, K; 400 μ m for D, H.

Fig. 3.

Quantitative analysis of DCX+ cell densities in representative neo- and allo-cortical areas (30 μm sections) in the adult guinea pigs at various ages. Panel A shows an example of camera-lucida drawing of labeled cells in layer II over the cortex of a 12 months-old animal. Image montage of the corresponding cortical hemisphere is placed side-by-side. Level of the section is indicated at the upper-left corner. The lateral sulcus (LS) and rhinal fissure (RF), borders dividing cortical areas, and scale bars for the drawing and image are indicated. Panel B plots mean cell densities, expressed as number of cells per 1 mm pial distance (or under $1000 \times 30 \mu\text{m}^2$ of pial surface), in different areas as a function of age. Note the parallel decreases of cell density over the piriform, entorhinal, temporal and parietal areas from 3 months to 3 years. Statistic analysis data are detailed in the text.

Fig. 4.

In situ detection of DNA cleavage in the cortex of a 3-months-old guinea pig (A, B) by fluorescent terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL). Olfactory bulb section (C) from a rat surviving 1 month after unilateral naris-occlusion (functional deprivation) serves as positive assay control. TUNEL profiles appear as small bright dots, which occur infrequently over the piriform (Pir.) and entorhinal (Ent.) (A) or temporal (Te.) and parietal (Pt.) (B) cortex (Ctx) and white matter (WM), with seemingly a random laminar distribution. Note the dense TUNEL profiles in the bulbs with an increase of labeling in the deprived side (C). The border between layers I and II is marked with a dash line (the upper one in B), same for the border between the cortex and white matter in (B) (lower line). RF: rhinal fissure; SEZ: subependymal zone; GCL: granule cell layer; EPL: external plexiform layer; GL: glomerular layer. D; dorsal; L: lateral. Scale bar = 500 μ m in A applying to B, and equivalent to 1 mm for C.

Fig. 5.

Closer views of DCX+ cells in representative coronal (A–C) and tangential (D, E) cortical sections (30 μm) from a 6 months-old guinea pig. Labeled cells display greatly diverse morphology, with cell size ranging from very small to considerably large, and cell body in round/oval, unipolar, fusiform/bipolar, triangular/multipolar and irregular shapes. Note that staining intensity, number and length of processes tend to increase with cell size, and that some large cells with well-formed processes have otherwise weak or faint DCX immunoreactivity (indicated by arrows). Labeled cells are mostly localized to layer II, especially at or near the junction of layers I and II (A–C, D). A few cells are found in upper layer III, especially in the entorhinal cortex (A). Small-sized cells generally outnumber larger cells in cross sections over a given region (A–C). In tangential sections, smaller cells are much more numerous than the larger ones at the immediate interface between layers I and II (D), but slightly deeper, larger cells appear to increase in proportion among existing cells (E). Labeled cells tend to arrange in clusters (cycles with broken lines in D, E), but some cells are tightly apposed (D, E). Note that closely apposed cells often show comparable morphological characteristics including similar somal shape and size, staining intensity and length/territory of dendrite-like processes (D and E, small circled areas and enlarged insets). Thin axon-like processes are detectable on many medium- to large-sized DCX+ cells, which run radially to deeper cortical layers (B, C, arrowheads). Cortical layers are marked with numbers and short bars, with 1/2 indicating the interface of layers I and II. EC: entorhinal cortex; FC: frontal cortex; TC: temporal cortex; PC: parietal cortex. Scale bar = 50 μm in A, applying for B and C; equal to 75 μm for D and E; and 25 μm for inserts at the right corners of D and E.

Fig. 6.

Colocalizations of DCX with several general immature and mature neural cell markers. Images are from coronal (A–C, E–I) and tangential (D, K) sections of parietal (somatosensory area) cortex from 6 months-old guinea pigs. Major image panels are merged views of 2 or 3 types of labeling. Inserts with thick borders are enlarged (1.5 to 2 times) or same-magnification (J) views of the boxed areas with thin borders in major panels, to show separate labelings. Arrows point to the same cells in different stainings. Labelings in different colors are indicated on the left side of individual image or image set. There exists a complete colocalization of DCX with polysialylated neural cell adhesion molecule (PSA-NCAM) (A–D) or the neuron-specific type III β -tubulin (TuJ1) (E), but no colocalization of DCX with reelin (F) or glial fibrillary acidic protein (GFAP) (G). Note reelin+ cells in layer I with their somata oriented parallel to the pial surface (F), and GFAP+ astrocytes near or at the cortical surface with processes extending inward (G). Neuron-specific nuclear protein (NeuN) is not expressed in small and lightly stained DCX+ cells, but does so in many medium and all large DCX+ cells (H–K). Note that relatively large labeled cells with weak or faint DCX reactivity show intense and distinct NeuN labeling (J, K, arrows). The small DCX+ cells have small nuclei with bright bisbenzimidazole (Bis) stain, whereas other DCX+ cells have larger but lightly stained nuclei (B, D, H). Two typical clusters, each consisting of 4 tightly apposed small cells, are illustrated in D and H. A pair of closely apposed large DCX+ cells is seen in D (center). Cortical layers are marked with numbers and short bars, with 1/2 indicating the interface of layers I and II. Scale bar = 50 μ m in A applying for major panels.

Fig. 7.

Colocalizations of principal and interneuron markers in DCX+ cells. Images are from coronal (A–C, E–J) or tangential (D, K–N) sections of the parietal cortex from 12 months-old guinea pigs. Panels A–D illustrate no colocalization of neurogranin in DCX+, including weakly stained (white arrows), cells. Panels E–G show γ -aminobutyric acid (GABA) immunoreactivity in medium to large (black and white arrows) but not in small DCX+ cells. GABA reactivity is heavier in large cells with weak DCX labeling (white arrows) relative to those with strong DCX labeling (black arrows). Single GABA labeling over the boxed areas with thin lines is shown in inserts with thick borders. Panels H–N depict absence of GAD67 reactivity in small DCX+ cells, but presence in large-sized cells with weak DCX labeling (white arrows). GAD67 labeling in medium to large-sized cells with strong DCX reactivity (black arrows) is either undetectable (K–N) or fairly weak (H–J). Two mature interneurons show strong GAD67 labeling in K–N (black arrowheads). Scale bar = 50 μ m in A, applying to other panels.

Fig. 8.

Colocalization of β -nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) or neuronal nitric oxide synthase (nNOS) with DCX and calbindin in coronal (A–F, L, M) and tangential sections (G–K) of the parietal cortex from 12 months-old animals. Panels A–F show weak NADPH-d reactivity in lightly stained DCX+ cells. Cells expressing either or both (white arrows) DCX and NADPH-d are tightly apposed (C–F). Panels G–I show partial colocalization of nNOS in DCX+ cells at upper layer II. nNOS immunoreactivity preferentially occurs in large-sized neurons with weak DCX labeling (white arrows). Panels J and K show colocalization of NADPH-d and nNOS in type I (J) and type II (K) nitrinergic neurons in the subcortical white matter and approximately layers I/II junction, respectively. Type I cells are indicated with fat black arrows (J, M), those in (J) are visualized following a short NADPH-d reaction. Panels L and M demonstrate partial colocalization of NADPH-d and calbindin in superficial cortical layers. Most weakly stained NADPH-d neurons in layer II are not calbindin-immunoreactive (black arrowheads) (M), whereas moderate reactive NADPH-d neurons commonly stain for calbindin (white arrows) (M, L). Cortical layers are marked with numbers and short bars. WM/6: border of the white mater and layer VI. Scale bar = 50 μ m in A, applying to other major panels, but equal to 25 μ m for the bottom inserts in A–F and 33 μ m for the upper-right inserts in H and I.

Table 1

Primary antibodies used in the present study

Antibody and product code	Source	Dilution
goat anti-doublecortin; sc-8066	Santa Cruz Biotech.	1:2000
rabbit anti-doublecortin-like 1, AB15584	Chemicon	1:500
mouse anti-PSA-NCAM ^a ; MAB5324	Chemicon	1:4000
mouse anti-neuron-specific tubulin-III; MAB1637	Chemicon	1:4000
mouse anti-neuron-specific nuclear protein; MAB377	Chemicon	1:4000
rabbit anti-glial fibrillary acidic protein; G9269	Sigma-Aldrich	1:4000
mouse anti-reelin; MAB5366	Chemicon	1:2000
mouse anti-gamma-aminobutyric acid; A0310	Sigma-Aldrich	1:10,000
mouse anti-GAD ^b 67; MAB5406	Chemicon	1:2000
rabbit anti-GAD 65/67; G5163	Chemicon	1:2000
mouse anti-calbindin; C9848	Sigma-Aldrich	1:4000
mouse anti-parvalbumin; P3088	Sigma-Aldrich	1:4000
rabbit anti-calretinin; C7479	Sigma-Aldrich	1:2000
rabbit anti-neurogranin; AB5620	Chemicon	1:2000
rabbit anti-nitric oxidase (neuronal isoform), AB5380	Chemicon	1:1000

^aPSA-NCAM: polysialic acid-neuronal cell adhesion molecule.

^bGAD: glutamic acid decarboxylase.