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DCX and PSA-NCAM Expression Identifies a Population of Neurons Preferentially Distributed in Associative Areas of Different Pallial Derivatives and Vertebrate Species

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In adult rodents, doublecortin (DCX) and polysialylated neural cell adhesion molecule (PSA-NCAM) expression is mostly restricted to newly generated neurons. These molecules have also been described in prenatally generated cells of the piriform cortex and, to a lesser extent, neocortex (NC) of the rat. In addition, PSA-NCAM1 cells have been identified in several telencephalic regions of the lizard. Here, through immunohistochemistry and 3-dimensional reconstruction, we have investigated distribution, morphology, and phenotype of DCX/PSA-NCAM--expressing cells in the pallium of different mammals and in lizard. In all species, a population of nonnewly-generated pallial DCX1/PSA-NCAM1 cells shows common morphological and phenotypic characteristics, including expression of Tbr-1, a transcription factor expressed in pallial projection neurons, and preferential distribution in associative areas. In the guinea pig and rabbit, DCX1/PSA-NCAM1 elements are also abundant in the NC, particularly in areas implicated in nonspatial learning and memory networks. In reptiles, DCX1/PSA-NCAM1 cells are located in the lateral and medial cortex and dorsal ventricular ridge but not in the dorsal cortex. These data support the fact that coexpression of DCX1/PSA-NCAM1/Tbr-11 in the adult brain identifies evolutionary conserved cell populations shared by different pallial derivatives including the mammalian NC.

Keywords: evolution, extraverted neurons, lizard, neocortex, neurogenesis, piriform cortex, plasticity

Introduction

Despite its remarkable plasticity at the behavioral level, the adult brain is usually considered a complex, hardwired organ with only limited capacity of structural remodeling, at least in mammals (Frotscher 1992). Accordingly, it is generally thought that neuronal cell morphology becomes fixed after specific time points or critical periods (Hensch 2004). Direct in vivo observations of neuronal processes in the adult primary visual and somatosensory cortex support this view. Although minor remodeling of some secondary dendritic branches and axons has been observed, the general picture shows relatively static tissue (De Paola et al. 2006; Lee et al. 2006).

In contrast with this view, adult neurogenic regions such as the olfactory bulb and hippocampal formation have recently drawn attention as the most evident exception to this rule (Alvarez-Buylla and Garcia-Verdugo 2002; Kempermann et al. 2004). Despite reducing their activity with age, neurogenic areas

continuously produce new neurons that undergo strong structural modifications until attaining a fully mature phenotype and integration into preexisting circuits. Accordingly, newly generated neuronal precursors and neurons retain the expression of molecules involved in structural remodeling during development (Bonfanti et al. 1992; Gleeson et al. 1999; Nacher et al. 2001; reviewed in Bonfanti 2006). The polysialylated form of neural cell adhesion molecule (PSA-NCAM) and the cytoskeleton-associated protein doublecortin (DCX) are both involved in different aspects of structural plasticity (Francis et al. 1999; Gleeson et al. 1999; Friocourt et al. 2003; Bonfanti 2006). They are widely expressed during central nervous system (CNS) development and become progressively restricted during adulthood, with high expression within regions of persistent neurogenesis (Francis et al. 1999; Gleeson et al. 1999; Nacher et al. 2001; Bonfanti 2006). In newly generated neurons, DCX and PSA-NCAM act in different cellular compartments, the microtubule cytoskeleton and the plasma membrane, respectively, to promote/allow migration and differentiation of immature elements (Gleeson et al. 1998; Bonfanti 2006; Koizumi, Higginbotham, et al. 2006; Koizumi, Tanaka, Gleeson 2006; Friocourt et al. 2007). To date, only very few examples of adult resident, nonnewly-generated neurons strongly coexpressing DCX and PSA-NCAM have been described. In mouse and rat brains, most of these cells are confined to layers II/III of the piriform cortex (PC) (Seki and Arai 1991; Nacher et al. 2001; Gomez-Climent et al. 2008), an allocortical domain which is highly conserved between vertebrates (Haberly 1990). In addition, very few DCX and/or PSA-NCAM--expressing cells have been identified in the mouse and rat neocortex (NC) and are mostly restricted to layers II/III of the perirhinal, agranular insular, and entorhinal cortices (Gomez-Climent et al. 2008). Recently, numerous DCX+ cells have been described throughout the allo- and neocortical regions in the adult guinea pig (Xiong et al. 2008). The concurrent expression of the 2 markers in these cortical populations suggests the occurrence of cells capable of global morphological remodeling. As recently hypothesized (Gomez-Climent et al. 2008), PSA-NCAM expression might also play an insulatory role. Nevertheless, the exact role of these forms of structural plasticity in the cortex are still unclear.

Comparative studies of molecular embryology have identified 4 pallial domains common to all tetrapods: the medial, dorsal, lateral, and ventral pallium (VP) (Puelles et al. 2000; Puelles 2001; Brox et al. 2004; Jarvis et al. 2005). Each domain contributes to specific pallial derivatives whose homology in different vertebrates has only been partially clarified. Similar to mammals, in the mature brain of the lizard, PSA-NCAM+ cells have been described in restricted regions, including the olfactory bulb, the medial cortex (medial pallium derivative), dorsal ventricular ridge (DVR; lateral/VP derivative), and the lateral cortex (lateral pallium derivative; Ramirez-Castillejo et al. 2002). The latter region is considered the reptilian homologue to the mammalian PC (Haberly 1990; Puelles 2001).

These data indicate that the expression of specific markers of structural plasticity are conserved between vertebrates, supporting the existence of common regulatory mechanisms in specific regions of the adult vertebrate brain. Furthermore, they suggest that expression of certain molecules might involve similar cell populations in homologue brain regions of different vertebrates.

Data regarding the occurrence, distribution, neurochemical, and

morphological profiles of DCX+ and PSA-NCAM+ cells in different species have been partially investigated (Seki and Arai 1991; Nacher et al. 2001; Gomez-Climent et al. 2008; Xiong et al. 2008). In order to address some of these issues, we performed a detailed analysis of DCX and PSA-NCAM expression in the telencephalon of 4 different mammalian species and in the lizard. In mammals, our results indicate that besides adult germinative regions, a population of resident, nonnewly-generated DCX and PSA-NCAM coexpressing cells occurs in several pallial derivatives, including the NC. In all species and pallial derivatives, these cells show common morphological and neurochemical profiles and are particularly abundant in higher order associative areas. These data support the fact that DCX, PSA-NCAM, and Tbr-1 coexpression identify phylogenetically conserved neuronal populations in mammals and reptiles. In addition, comparative analysis of the distribution patterns of DCX/PSA-NCAM-expressing elements in the different pallial derivatives of both mammals and the lizard leads to some speculation regarding the still debated origin of the NC.

Material and Methods

Animals, 5-Bromo-2'-Deoxyuridine Injections, and Tissue Preparation

Experiments were conducted in accordance with current European Union and Italian law under authorization of the Italian Ministry of Health number 66/99-A. All experiments were designed to minimize the number of animals used and their discomfort. Twenty-four postpubertal female (6--10 month old; Charles River, Milan, Italy) New Zealand White HY/CR rabbits (*Oryctolagus cuniculus*), 12 adult (2-5 month old; Morini, S. Polo d'Enza, Italy) guinea pigs (*Cavia porcellus*), 12 adult (3--4 month old, Charles River) CD-1 mice (*Mus musculus*), 12 adult (3--4 month old, Charles River) albino Wistar rats (*Rattus norvegicus*), and 3 adult (9--10 g of body weight) lizards (*Podarcis muralis*) were used in the present study. Seven rabbits and 3 guinea pigs were killed 2 h after a single intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU; Sigma, Steinheim, Germany) (40 mg/kg body weight in 0.1 M Tris). Seventeen rabbits, 9 guinea pigs, 9 mice, and 9 rats received one daily injection of BrdU for 5 consecutive days and were then killed 2 h (rabbit *n* = 5; guinea pig, mice, and rats *n* = 3), 10 days (rabbit, guinea pig, mice, and rats *n* = 3), 30 days (rabbit *n* = 5; guinea pig, mice, and rats *n* = 3), and 60 days (rabbit *n* = 4) after the last injection. Animals were deeply anesthetized with a ketamine-xylazine solution (100 and 33 mg/kg body weight, respectively) and transcardially perfused with ice-cold saline solution (0.9% NaCl), followed by a freshly prepared solution of 4% paraformaldehyde plus 2% picric acid in 0.1 M sodium phosphate buffer, pH 7.4. Brains were then postfixed overnight, cryoprotected, frozen at -80 °C, and cryostat (25 and 40 μ m) sectioned in series. The reptilian brains were taken from wild animals captured in the surrounding area of the department in Spring. They were fixed, cryoprotected, and sectioned as previously described.

Immunohistochemistry

Immunohistochemical reactions were performed either by using the biotin-avidin system or double-immunofluorescence methods on sections incubated for 24--48 h at 4 °C with the following antibodies: anti-BrdU, 1:2000 (mouse monoclonal; Harlan CPB, Zeist, The Netherlands); anti-Ki67, 1:300 (MIB1, mouse monoclonal; ScyTek Laboratories, Logan, UT); anti-Ki67 (rabbit

polyclonal; Novocastra, Benton Lane, UK); anti-class III β -tubulin, 1:600 (TU-J1, mouse monoclonal and polyclonal; Babco, Richmond, CA); anti-neuronal-specific nuclear protein (NeuN), 1:1000 (mouse monoclonal; Chemicon, Temecula, CA); anti-double-cortin (DCX), 1:500 (goat polyclonal sc-8066; Santa Cruz Biotechnology, Santa Cruz, CA); anti-PSA-NCAM, 1:4000 (monoclonal IgM; G. Rougon, Marseille, France); anti-parvalbumin, 1:2000 (rabbit polyclonal; Swant, Bellinzona, Switzerland); and anti-somatostatin, 1:2000 (α -SRIF, rabbit polyclonal; Dr R. Benoit, Montreal, Quebec, Canada); anti-Clabindin-D28K 1:2000 (mouse monoclonal; Swant); anti-GABA 1:1000 (rabbit polyclonal, Sigma, St Louis, MO); anti-GAD67 1:5000 (mouse monoclonal; Chemicon); anti-pan DLX (rabbit polyclonal; Panganiban et al. 1995); anti-Lhx6, 1:1000 (rabbit polyclonal; Grigoriou et al. 1998); and anti-Tbr1 (rabbit polyclonal; Chemicon). All the antibodies were diluted in a solution of 0.01 M PBS, pH 7.4, containing 0.5–1% Triton X-100. For BrdU staining, DNA was denatured in 2N HCl for 30 min at 37 °C. Sections were then rinsed in 0.1 M borate buffer, pH 8.5. When anti-BrdU was used in combination with other primary antibodies, sections were processed first for partner antibodies and only afterward for the BrdU reaction. For the biotin-avidin system, sections were rinsed in PBS, incubated with the appropriate biotinylated secondary antibodies for 1 h (1:250; Vector Laboratories, Burlingame, CA), rinsed, and then incubated in avidin-biotin complex (1:400; Vector Laboratories). The reaction product was visualized with 0.15 mg/ml 3,3'-diaminobenzidine in PBS with 0.01% H₂O₂. Sections were then serially mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), air dried, dehydrated in graded alcohols, cleared in xylenes, and coverslipped using DPX mounting medium (Aldrich, Milwaukee, WI). For dual immunofluorescence, following primary antisera incubation, sections were washed and incubated with appropriate solutions of secondary cyanine 3 (Cy3)-, Cy2-, and Cy5-conjugated antibodies (all diluted 1:800; Jackson ImmunoResearch, West Grove, PA). Sections were then coverslipped with antifade mounting medium Dabco (Sigma) and analyzed with a laser scanning Olympus Optical (Milan, Italy) Fluoview confocal system (Olympus Optical). All the antibodies used gave reliable immunoreactions on the analyzed species. Images were processed using NIH image J (<http://rsb.info.nih.gov/ij/>) and Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) and assembled into montages using CorelDraw 11 (Corel, Ottawa, Ontario, Canada). Only general adjustments to color, contrast, and brightness were made. The images were not otherwise manipulated.

Three-Dimensional and 2-Dimensional Models of DCX Staining

For 3-dimensional (3D) and 2-dimensional (2D) reconstruction (Fig. 2), equally spaced coronal sections (40 μ m thick) spanning the entire NC and PC of a rat (14 sections), a rabbit (18 sections), and a guinea pig (21 sections) were immunolabeled for anti-DCX and digitally captured. Using NIH image J, the pial surface of PC and NC of each section was divided into segments of the same length (~1.5 mm for the rabbit and ~1 mm for the guinea pig and the rat). Under each segment, the DCX staining in layer I and II was thresholded and the percentage of DCX-stained area was translated into a scale of red (0–5% is indicated in white to red, >5% yellow). The pial surfaces of each section were then traced, reciprocally aligned, and spaced using Reconstruct software version 1.1 (<http://synapses.bu.edu/tools>; Copyright 2004, John C. Fiala, Boston University, Boston, MA) and then imported in a 3D modeling software

(Rhino 3D version 3.0 software; Robert McNeel & Associates, Seattle, WA). In Rhino 3D, each pial segment was extended caudally as far as the level of the adjacent section and stained in accordance with the threshold analysis. For this reason, the section thickness which is visible in the 3D and 2D models (Fig. 2) equals the section spacing. To flatten the 3D model into a 2D model, each section was linearized and aligned on a 2D plane. The projections of the relative shifts in the dorsoventral plane of the PC--NC border (blue line Fig. 2) between subsequent 3D sections was used to align the linearized sections in the 2D plane. Subsequent sections were shifted medially or laterally from the PC/NC border following the dorsalward or ventralward orientations, respectively, of the 3D shifts. The 3D reconstruction of the lizard brain was entirely obtained in reconstruct 1.1 from a representative series of double-labeled sections for DCX and Tbr-1. The size of the dots representing the DCX/Tbr-1 double-labeled cells was increased in rendered image using Photoshop 7.0 (Fig. 5D).

Results

PSA-NCAM and DCX Expression in the Telencephalon of the Rabbit, Guinea Pig, Mouse, and Rat

DCX+ and PSA-NCAM+ cells distribution was studied in the forebrain of young--adult animals. In all species, the time course analysis of BrdU-treated animals at different survival times (5, 15, 35, and 65days) confirmed that in restricted regions of the adult CNS, such as the subventricular zone (SVB)-olfactory bulb (OB) system, dentate gyrus (DG) of the hippocampus, and striatum (Luzzati et al. 2003, 2006; Dayer et al. 2005), these molecules are mainly associated with the migratory and differentiating processes of newly generated neurons (data not shown). In these cells, there is a strong temporal coexpression of both molecules. Nonetheless, we also identified some populations of DCX+/PSA-NCAM+ cells that had never been associated with BrdU at any of the survival times considered, thus confirming that DCX and/or PSA-NCAM can also be retained in resident, nonadult-generated neurons (Gomez-Climent et al. 2008). In addition, as previously described (Luzzati et al. 2003), we found numerous DCX +/PSA-NCAM+ cells in the lateral nucleus of the amygdala of the rabbit but only few of them had newly generated. In general, the pattern of PSA-NCAM expression appears more widespread compared with DCX. Indeed, a low level of PSA-NCAM immunoreactivity was visible in scattered cells distributed over the entire telencephalon of all species considered. In contrast to PSA-NCAM, DCX expression in non-newly generated cells is much more restricted to neurons of layers II and, to a lesser extent, layer III of the PC and NC (Fig. 1). Virtually, all the cortical DCX+ elements are also positive for PSA-NCAM (Fig. 4E,E').

Comparative analysis showed many DCX+/PSA-NCAM+ cells in the PC of all animals (Fig. 1A,D). In the mouse and rat, only very few scattered DCX+/PSA-NCAM+ cells are visible outside the PC, mostly restricted to layers II/III of the perirhinal cortex (data not shown), as previously described in the rat (Gomez-Climent et al. 2008). On the other hand, in the rabbit and guinea pig, numerous DCX+/PSA-NCAM+ elements are present in layers II/III of 2 pallial domains neighboring the PC: the NC (laterally) and the amigdalopyriform transition area (APA, medially, Fig. 1). In these regions, the DCX/PSA-NCAM staining is often continuous with that of the PC, such that in coronal sections, the immunoreactive cells appear distributed along a line that encompasses

several pallial domains, from the NC to the PC and then to the APA (part of the pallial amygdala; Moreno and Gonzalez 2007), where the staining deepens in the temporal lobe toward the basal nucleus of the amygdala (Fig. 1A). Notably, in the PC and NC, most of the DCX+/PSA-NCAM+ cells are concentrated on the external border of the cellular layers (layer II), whereas in the APA, they are mostly found in the deeper layer (layer III). Within each pallial domain, the DCX+/PSA-NCAM+ cells are not homogeneously distributed along the anteroposterior and dorsoventral axes. In the APA, the positive cells are present only in its rostral part, whereas in the PC, they are concentrated in its

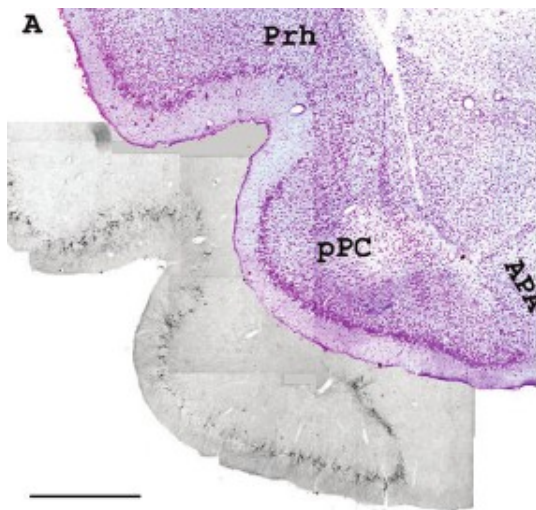
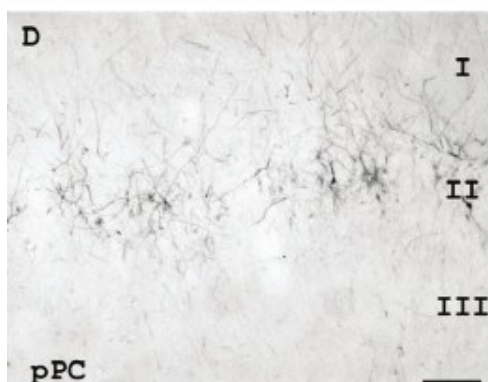
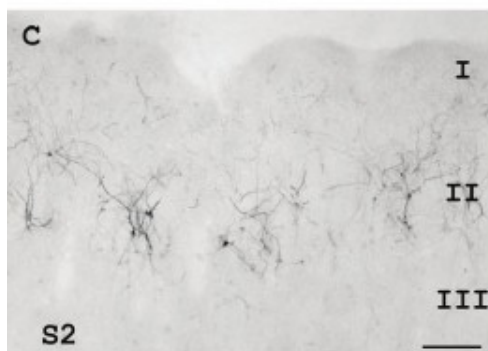
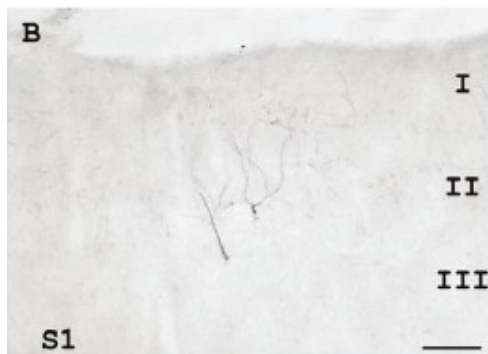


Figure 1. Distribution of DCX+ neurons in coronal sections of different pallial derivatives of the rabbit (B--D) and guinea pig (A). (A) Two consecutive sections of guinea pig brain taken at the level of posterior piriform cortex (pPC) and stained with cresyl violet and anti-DCX immunoreaction. DCX+ cells are distributed along an uninterrupted line through the NC, PC, and APA, where the staining deepens in the temporal lobe (A). (B--D) Higher magnification of DCX-stained sections in different cortical areas of the rabbit brain. DCX+ neurons are located in layers II and III of both NC (A, B) and PC (D). Prh, perirhinal cortex. Reference bars 1 mm in (A); 100 μ m in (B--D).



posterior part (Fig. 1D). In the NC, the pattern of DCX/PSA-NCAM labeling follows the thickness variations of layer II among the cortical areas. In conclusion, DCX and PSA-NCAM coexpression in the telencephalon of analyzed adult mammals involves 2 main cell populations 1) newly generated cells in adult neurogenic regions and 2) resident cells in layers II/III of different pallial domains.

Pattern of DCX Staining in the PC and NC

To investigate in detail the distribution of the cortical DCX+/PSA-NCAM+ cells in the rabbit, guinea pig, and rat, we produced 3D and 2D representations of DCX immunostaining in layers I/II by analysis of series of coronal sections representing the entire PC and NC (Fig. 2). The pial surface of the PC and NC of every section was divided into segments of the same length, and the DCX staining in the underlying layers I/II was thresholded. These models gave a comprehensive view of the relative distribution of the DCX+ cells among the cortical areas of the species considered.

DCX Staining in the PC

The PC is a 3-layered cortical domain located laterally to the rhinal sulcus (blue line in the 2D models, Fig. 2). As evident in both the 3D and 2D models, all the species show DCX labeling in the PC, although the staining level is lower in the rat. In all the animals, DCX staining shows a strong antero-caudal gradient being mostly absent in the anterior PC and reaching the highest level in the posterior PC (Fig. 2). Thus, in the PC of all the analyzed species, DCX staining mostly involves the posterior PC, which is generally considered an

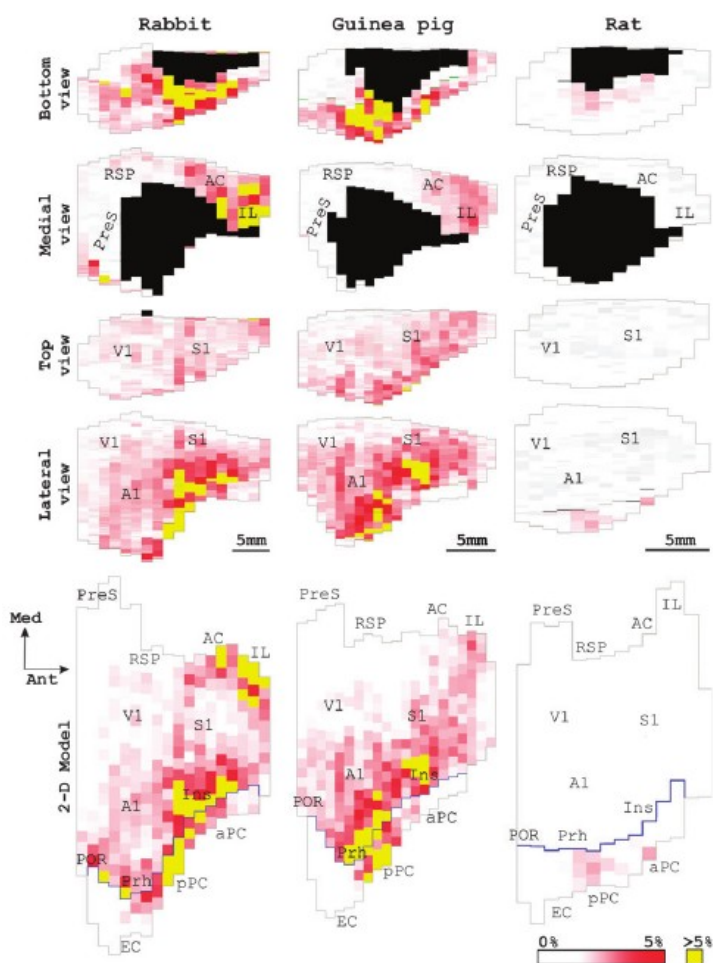


Figure 2. The 3D and 2D representations of DCX immunoreactivity in layers II and III of the rabbit, guinea pig, and rat PC and NC. The percentage of DCX-stained area is reported as a color scale (0--5% white to red; [5% yellow). In the PC of all species, DCX immunostaining is higher in its posterior part. In the NC of both the guinea pig and rabbit, the staining is distributed along a cortical stripe which extends from the anteromedial to the posterolateral aspects of the hemisphere. In the rat NC, the density of DCX+ cells is too low to be detected. In all cortical domains and species, DCX immunostaining mostly involves associative areas rather than primary and secondary sensory areas. The rhinal sulcus is indicated as a blue line in the 2D models. A1, primary auditory cortex; AC, anterior cingulate cortex; IL, infralimbic cortex; Ins, insular cortex; pPC posterior piriform cortex; aPC; anterior piriform cortex; PreS, presubiculum; POR, postrhinal cortex; Prh, perirhinal cortex; RSP, retrosplenial cortex.

association cortex (Haberly 2001).

DCX Staining in the NC

As for the PC, the DCX staining is not homogeneously distributed among the different areas of the 6-layered NC (Figs 1B,C and 2). In the rat, very few DCX+ elements were found in the NC (data not shown). These cells are restricted to the insular and perirhinal cortex. Both their number and level of DCX expression were too low to be detected by the threshold method. Therefore, the NC of the rat shows complete absence of staining in the 2D and 3D models (Fig. 2, rat column). By contrast, in the guinea pig and rabbit NC, a diffuse DCX staining was identified. In these species, a similar pattern of distribution involving DCX-positive and -negative regions is evident. The immunonegative regions are mostly located in the caudomedial aspect of the hemisphere and include the retrosplenial and presubicular and parasubicular cortices (Fig. 2, medial view and 2D model, Fig. 3). In addition, the entorhinal cortex (EC), which extends from the presubiculum and parasubiculum caudomedially to the postrhinal and perirhinal cortices and posterior PC anterolaterally, is mostly DCX negative apart from its border with the PC (Fig. 2, bottom view and 2D model, Fig. 3C,D).

The DCX-enriched regions form an oblique strip of cortical tissue extending from the anteromedial to the posterolateral aspect of the hemisphere. Within this pattern of distribution, an increase in DCX expression is evident at the most medial and lateral borders. Notably, the strong staining of the posterior PC appears as a lateral extension of the NC strip (see Fig. 2, lateral view and 2D model). Analyses of the 3D and 2D models indicate that the staining level in both species increases when moving out of primary sensory areas which lie in the center of the NC. These areas show an intermediate to low (particularly in the rabbit) staining level. Among the primary sensory areas, DCX expression is strongly reduced caudally in the primary visual cortex (V1). Medially to the primary sensory areas, DCX staining increases in the anterior part of the hemisphere (particularly in rabbit) in the infralimbic, prelimbic, and anterior cingulate area (Figs 2 and 3B). In the caudal part of the hemisphere, a small increase is visible only at the medial border of the rabbit V1 (Fig. 2, medial view and 2D model, Fig. 3B).

Laterally to the primary sensory areas, a progressive increase in DCX staining involves the entire rostrocaudal axis of the brain. In the rabbit, the highest DCX expression is found laterally to primary somatosensory cortex (S1). At this level, the intensity of DCX staining rises, passing from S1 to secondary somatosensory cortex (S2), and then increases further, close to the rhinal sulcus where the granular, dysgranular, and agranular insula are located (Fig. 2, rabbit column). In the guinea pig, the highest value of DCX expression is found laterally to primary auditory cortex in the posterior insula and anterior perirhinal cortex (Figs 1A and 2, guinea pig column). In both species, the level of DCX expression is reduced caudally although the posterior perirhinal and postrhinal cortex show the highest values of DCX expression at their relative anteroposterior levels (Figs 2 and 3D).

In conclusion, DCX expression in the NC, as in the PC, is enriched in specific associative regions.

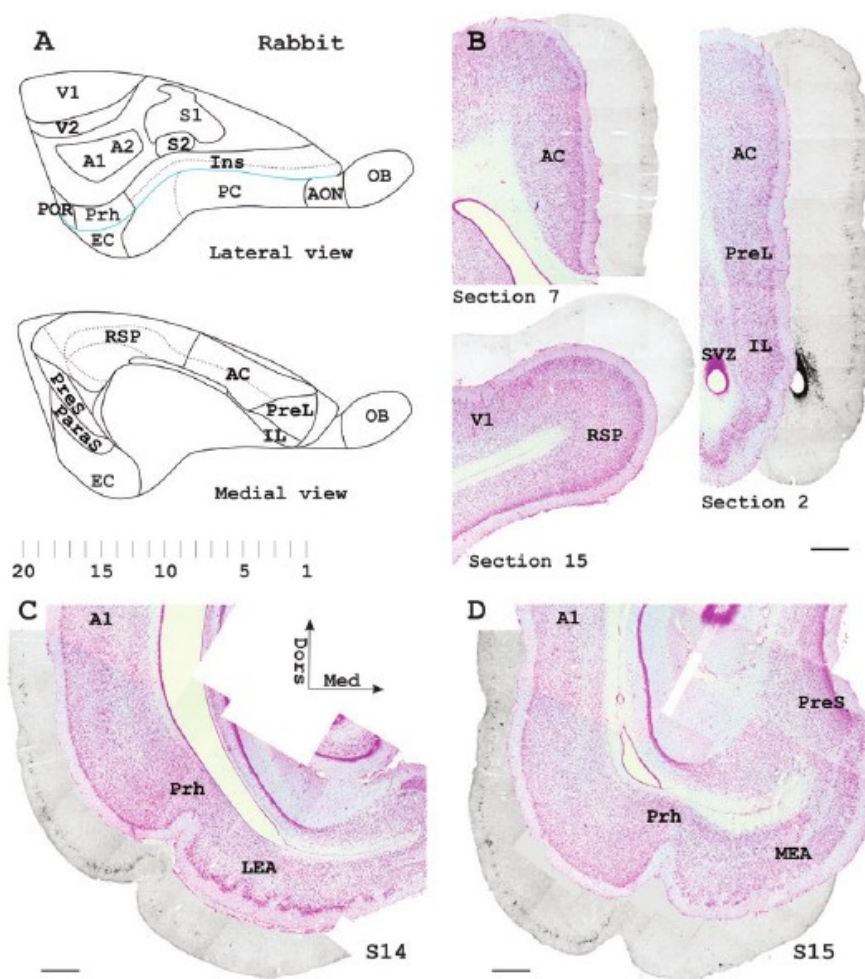


Figure 3. Distribution of DCX+ neurons in coronal sections of PC and NC of the rabbit. (A) Representation of some cortical areas of the telencephalon of the rabbit in lateral and medial views. (B--D) Couples of consecutive sections stained with cresyl violet and anti-DCX immunoreaction at different anteroposterior levels (see number of section and reference in A). In the medial aspect of the hemisphere (B), most of the DCX+ cells are located anteriorly (Section 2) in the IL, prelimbic cortex (PreL), and anterior anterior cingulate cortex (AC). In the lateral aspect of the hemisphere (C, D), the staining increases from A1 to Prh. A few DCX+ cells are visible in the LEA (C) but not in the MEA (D). A1, primary auditory

cortex; IL, infralimbic cortex; Ins, insular cortex; pPC posterior piriform cortex; aPC; anterior piriform cortex; PreS, presubiculum; POR, postrhinal cortex; Prh, perirhinal cortex; RSP, retrosplenial cortex. Reference bars: 1 mm.

Characterization of DCX+ Cells in the PC and NC

The occurrence of similarities in the distribution of cortical DCX cell populations in different pallial domains suggest these cells might represent a common neuronal type in the PC and NC. To unravel similarities and/or differences among DCX+ cells in these regions, we performed neurochemical and morphological analyses.

Neurochemical Analysis

Two main cell types are found in both the NC and PC: glutamatergic projection neurons and GABAergic interneurons (Peters and Saint Marie 1984; DeFelipe and Farinas 1992). To establish the neurochemical profile of the DCX+ cells, we first investigated the coexpression of typical markers of cortical projection neurons and/or interneurons. In both regions, the DCX+ cells were negative for major markers of cortical interneurons, such as parvalbumin, calbindin, and somatostatin (data not shown). We could very rarely observe, by anti-GABA staining, few elements faintly colabeled for DCX (Supplementary Fig. 1A). These data strongly suggest that the large majority of DCX+ cells were not interneurons. To confirm this hypothesis, we analyzed the DCX+ cells for the expression of transcription factors discriminating the embryonic origin of cortical cells. It is generally thought that most, if not all,

cortical interneurons originate in the subpallium, whereas projection neurons are born in pallial germinative layers (Parnavelas 2000; Kriegstein and Noctor 2004). Some of the embryonic molecular determinants maintain their expression through differentiation, being detectable in mature neurons (Stuhmer et al. 2002; Molnár and Cheung 2006). In accordance with previous studies performed on the mouse, many cortical neurons in all species considered here were immunolabeled by a pan distalless (DLL) antibody and anti-Lhx6, 2 markers of subpallium-derived interneurons (Anderson et al. 1997; Stuhmer et al. 2002; Alifragis et al. 2006; Fig. 4A--B', Supplementary Fig. 1B). Nonetheless, none of the DCX+ cells of the PC and NC expressed Lhx6 and only about 1--2%, were positive for DLL (guinea pig 17/984, mice 3/511, and rabbit 12/846; n = 2) confirming that the majority are not interneurons (Fig. 4A--B'). Notably, most of the few DLL+/DCX+ cells were faintly labeled for both antigens (Fig. 4A, Supplementary Fig. 1B). By contrast, nearly all the DCX+ cells of the PC, NC, and APA were positive for Tbr1, a marker of pallium derived neurons directly involved in glutamatergic differentiation (Fig. 4C,C',D'; Englund et al. 2005; Hevner et al. 2006). According to previous reports (Hevner et al. 2006), DCX+ elements of DG expressed Tbr-1 in all the analyzed mammalian species (Fig. 4D). In conclusion, resident DCX+/PSA-NCAM + cells are mostly associated with a marker of pallial origin expressed by glutamatergic pyramidal neurons.

Morphological Analysis

Great morphological variability was found in cortical DCX+ elements. Yet, none of these cells show features of classical pyramidal neurons, a cell type usually characterized by 2 dendritic systems organized in an apical tuft and a basal skirt directed vertically and horizontally, respectively.

Two main types of DCX+ cells, which we will refer to as type I and II, were found in both the PC and NC (Fig. 4E—G,E'--G'). Type I cells have small cell bodies and often a bipolar morphology. They have short processes mostly confined to layer II (arrow, Fig. 4E,E'). Conversely, type II cells have, generally, larger cell bodies and show a wide and well-developed dendritic arborization, organized as a classical apical tuft into layer I (Fig. 4E,F'). This apical tuft originates either from 1 (type IIa; Fig. 4G,G') or 2 (type IIb; Fig. 4F,F') primary dendrites ramifying at variable distances from the cell body. The basal dendritic system is made up of very thin processes mostly radially oriented toward the deeper part of layers II and III and never organize as a basal skirt (Fig. 4F,F',G,G'). Clear predominance of subpial dendrites over basal dendrites could place type II cells within the population of atypical pyramidal cells previously defined as “extraverted neurons” (Sanides F and Sanides C 1972; Nieuwenhuys et al. 1998).

In the APA, similar cell types were identified although the cell bodies were located in layer III and were often clustered (data not shown). Thus, no evident difference exists in type I and type II cell morphology in different pallial derivatives.

PSA/NCAM, DCX, and Tbr-1 Expression in the Telencephalon of the Lizard

In the previous paragraph, we have shown that the 3-layered PC and 6-layered NC as well as the APA of mammals share DCX+/PSA-NCAM+/Tbr-1+ cells, which include a population of extraverted neurons. Extraverted neurons have

been interpreted as old and conserved pallial cell types (Sanides F and Sanides D 1972), owing to their preferential distribution in allocortical structures as well as their peculiar morphological organization (i.e., lack of basal skirt). Reptiles and mammals are thought to have originated from the stem amniote, a common ancestor (Evans 2000). In order to explore whether the occurrence and distribution of cortical DCX+/PSA-NCAM+ neurons represent a primitive (plesiomorphic) character in mammals, DCX expression in Tbr-1+ pallial neurons of the lizard (*P. muralis*) has been investigated. In the telencephalon of *P. muralis*, the Tbr-1 antibody coherently labeled several cells in virtually all pallial derivatives, including the mitral-tufted layer of the main and accessory olfactory bulbs, the anterior olfactory nucleus, the anterior DVR (ADVR) and posterior DVR (PDVR), the nucleus sphericus (NS), as well as the medial, dorsal, and lateral cortices (Fig. 5B-D). In accordance with previous reports of adult neurogenesis in other reptiles (Lopez-Garcia et al. 1988; Font et al. 2001; Kaslin et al. 2008), the anti-DCX antibody labeled cells in the medial cortex, main and accessory olfactory bulbs (data not shown), and germinative regions surrounding the olfactory and lateral ventricles (Figs 5C and 6C). These periventricular cells were often organized

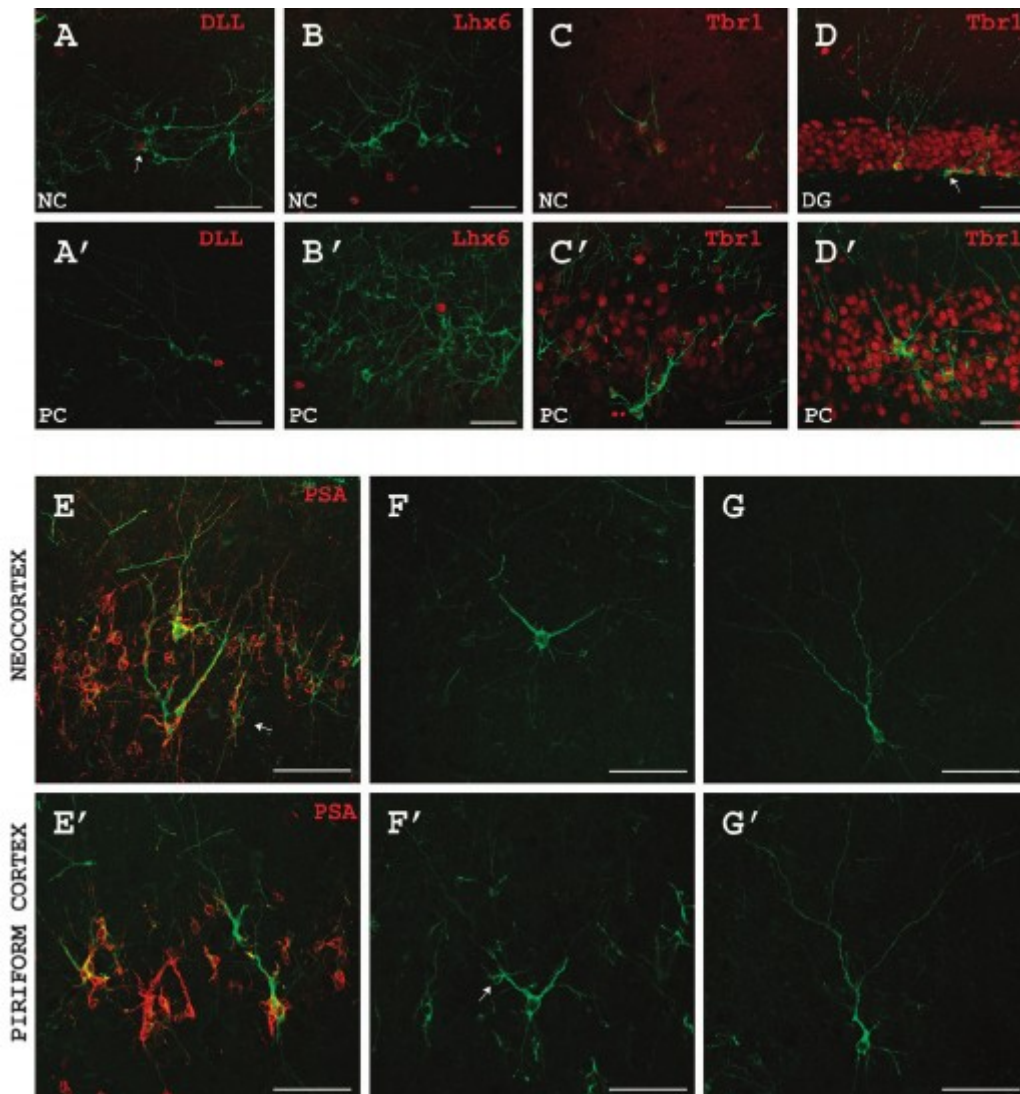


Figure 4. Phenotypic analysis of DCX+ cells in the NC (A--C, E--G), PC (A'--D', E'--G'), and DG (D) of the guinea pig (A, B, A', B'), rabbit (C, D, C', E--G'), and mouse (D, D'). In NC, PC, and DG, nearly all DCX immunoreactive cells are positive for Tbr1 (C--D, C'--D'), a specific pallial transcription factor, but not for DLL and Lhx6 (A--B, A'--B'), which identify subpallial-derived interneurons. A few faintly labeled DCX+ cells are also positive for DLL (arrow). In both the PC and NC, all DCX+ cells express PSA-NCAM (E, E'). Two main cells

types can be identified in layers II/III of NC and PC. Type I has a small cell body and its short processes are mostly restricted to layer II (arrows in E and F'). Type II is an extraverted neuron (see the text for details) with larger cell body located in layer II and a wide dendritic arborization developed into layer I (F, F, G, G'). The apical tuft originates from 2 (type IIb, F--F') or 1 (type IIb, G--G') primary dendrites. Only few thin basal dendrites are present. Both type I (arrow in D) and II cells can be observed in the DG. Noteworthy are the great similarities of DCX/PSA-NCAM+ cells in different pallial derivatives. Reference bars: 50 μ m.

as small chains and, along with the DCX+ cells of main and accessory olfactory bulb, were Tbr-1 negative. Scattered DCX+, Tbr-1 negative, cells were also detected in the dorsal and ventral striatum (Fig. 5D).

Numerous DCX/Tbr-1 double-labeled cells were found in the lateral cortex, ADVR, PDVR, and NS. Similarly to mammals, these elements are PSA-NCAM+ (data not shown) and show 2 main cell morphologies (type I and type IIa and IIb) consistent with those described above in mammals (Fig. 6A--F). These data suggest that DCX expression is shared by similar cell populations in reptilian and mammalian brains. A comprehensive view of the distribution of the DCX and Tbr-1 double-labeled cells in the lizard pallium is shown in a 3D model (Fig. 5A).

DCX+ Cells in Layer II of the Lateral Cortex

The reptilian lateral cortex is a 3-layered cortical domain lining the laterodorsal surface of the telencephalon and receiving a direct olfactory input (Haberly 1990). This structure is considered homologous to the PC of mammals (Haberly 1990; Puelles 2001). The DCX+ cells in the lateral cortex of the lizard are restricted to its ventral part, preferentially in layer II (Fig. 5A). Similarly to mammals, type II--like cells send diffuse dendritic arborizations into layer I (Fig. 6B,D,E). Along the anterior--posterior axis of the lateral cortex, DCX+ cells are not homogeneously distributed. No DCX+ cells are visible in the most anterior regions of the lateral cortex, whereas moving caudally, they increase and peak in the central region. DCX expression abruptly disappears caudally to the anterior commissure (Figs 5A and 6A). Such a distribution pattern is very similar to the one that we found in the cortical areas located underneath the rhinal sulcus in mammals, namely the PC and EC (see Fig. 2 for comparison). Indeed, in these areas, DCX expression increases from anterior to posterior PC and completely disappears in the more caudally located EC. Interestingly, the dorsal and caudal lateral cortex of reptiles have been proposed, owing to their connections with the medial cortex, as homologues to the mammalian EC (Martinez-Garcia et al. 1991, Hoogland and Vermeulen-Vanderzee 1995) and accordingly are mostly DCX negative.

DCX+ Cells in the ADVR, PDVR, and NS

Outside layer II of the lateral cortex, Tbr-1+/DCX+ cells are mostly distributed in the DVR (Fig. 5). This structure is located ventromedially to the lateral cortex, and its mammalian homologues are still debated (Reiner 2000; Aboitiz et al. 2002). In the lizard, the DVR shows a laminar organization pattern defined by 3 concentric areas which are the periventricular, cluster, and central zones (Nieuwenhuys et al. 1998). DCX+ cells are mostly located in the periventricular and cluster zones (Figs 5B and 6A,F). Type II--like cells of these regions show radially oriented dendrites directed toward the central zone (Figs 5B and 6A,F). As in the lateral cortex, DCX+ cells are not homogeneously distributed along the anterior--posterior axis of the DVR. In particular, very few DCX+ elements are observed in the anterior part of ADVR

while they are abundant in the posterior ADVR, PDVR, and NS (Fig. 5A). The latter regions are considered associative areas because they receive convergent connections originating in modality specific areas of the ADVR

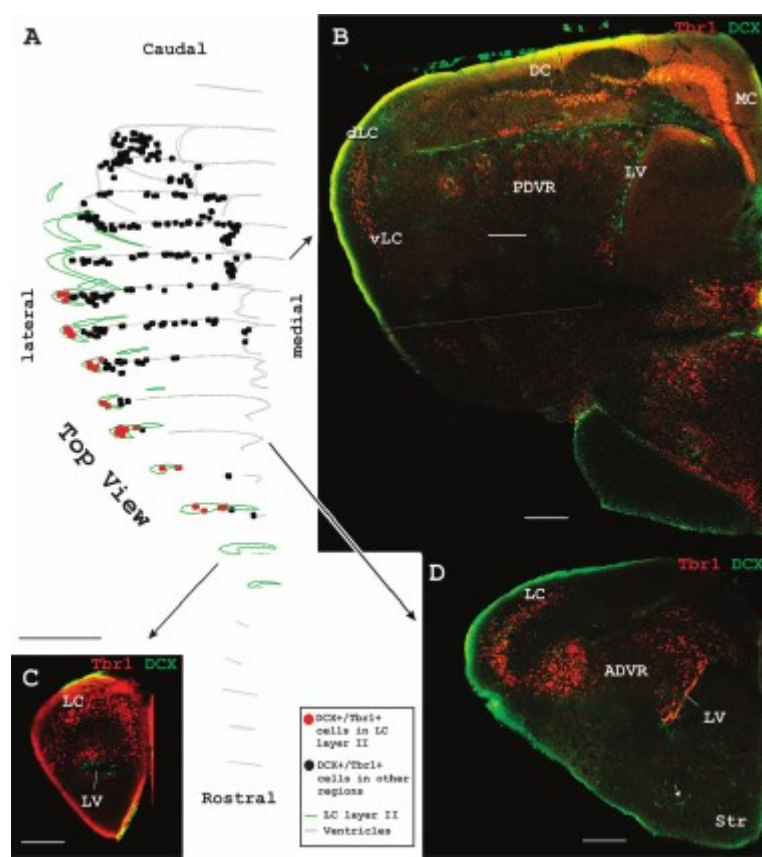


Figure 5. Distribution of DCX+ and Tbr-1+ neurons in the pallium of lizard. (A) The 3D model of the distribution of DCX+/Tbr1+ cells in the pallial derivatives of the lizard, top view. In the layer II of the lateral cortex (LC; green contours), DCX+/Tbr-1+ cells (red dots) are mostly restricted in the ventral LC (vLC; see also A). No DCX+/Tbr-1+ cells are visible in the more anterior regions of LC. DCX+/Tbr-1+ cells in

layer II of LC peak in the central region, then caudally to the anterior commissure, and abruptly disappear. DCX+/ Tbr-1+ cells outside the layer II of LC (black dots) are rare in the ADVL and numerous in the PDVL and NS (see the text for major details). The lateral and olfactory ventricles are in gray, DCX+/Tbr-1+ cells in medial cortex (MC) are not indicated. (B--D) DCX (green) and Tbr1 (red) immunoreactivity in 2 coronal sections at different anteroposterior levels.

As expected, Tbr-1+ cells are visible in all the main pallial derivatives. Strong expression of Tbr1 is visible in the medial and dorsal cortex (B). Tbr-1 immunoreactive cells are also present in the LC, PDVL (B), and ADVL (D). DCX immunostaining is evident at the level of the periventricular regions (B--D), in PDVL (B) and LC (D). LV, lateral ventricle; DC, dorsal cortex; dLC, dorsal LC; Str, striatum. Reference bars: 500 μ m in (A); 200 μ m in (B--D).

(Andreu et al. 1996; Lanuza et al. 1998). Thus, similar to the mammalian PC and NC, DCX+ cells in the DVR of reptiles are preferentially distributed into associative areas.

Interestingly, the dorsal cortex of the lizard, a dorsal pallial derivative that is a candidate homologous to the mammalian NC (Puelles 2001) does not contain any DCX+ cells. Thus, DCX+/Tbr-1+ cells show a well-conserved distribution in the lateral pallium derivatives, whereas they might represent an evolutionary novelty in the mammalian NC.

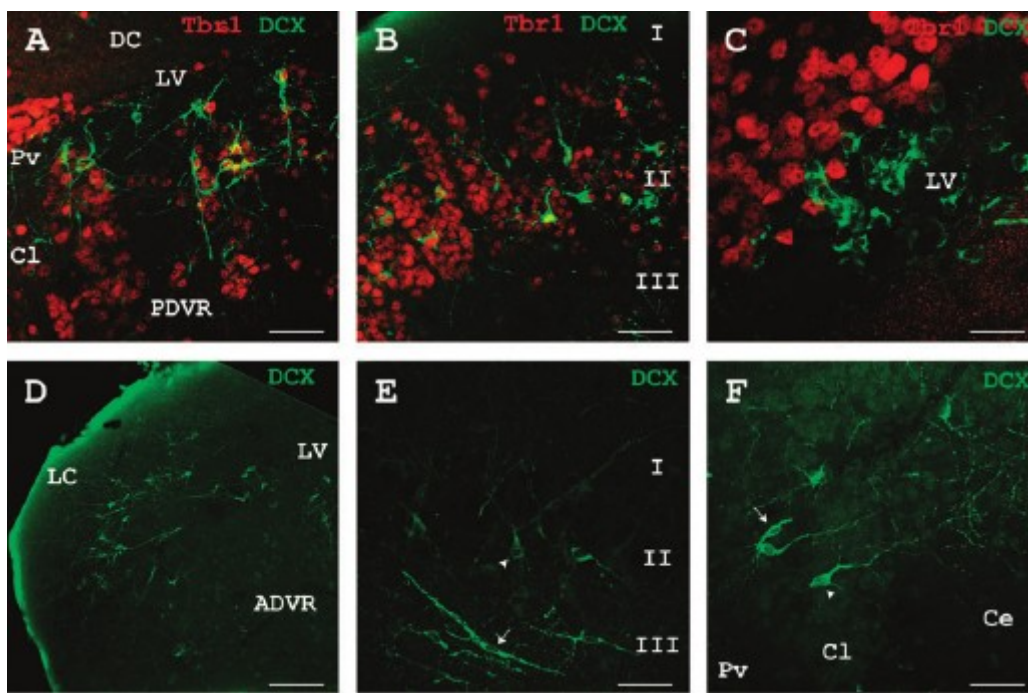


Figure 6. Phenotypic analysis of DCX+ cells in the pallium of the adult lizard. In both PDVR (A) and lateral cortex (LC) (B), DCX+ cells express Tbr-1. Note that a few DCX+ cells are Tbr-1 negative in the Pv of PDVR. In anterior regions of the lizard telencephalon (C), DCX+ cells associated with the periventricular region do not

colocalize with Tbr-1. Two main types, type I (arrow in E) and type II (arrowhead in E) of DCX+ cells are visible in layers II/III of the LC of lizard (D--E). Type I (arrow in E, F) and type II (arrowhead in E, F) DCX+ cells are also visible in the NS (F). LV, lateral ventricle; Pv, periventricular zone; Cl, cluster zone; Ce, central zone. Reference bars: 100 μ m in (D); 40 μ m in (A, B, E, F); 20 μ m in (C).

Discussion

In the adult mammalian brain, DCX and PSA-NCAM have been primarily related to the adult neurogenic regions (i.e., forebrain SVZ and DG of the mammalian hippocampus) where they are transiently coexpressed in newly formed cells during their migration and differentiation (Bonfanti and Theodosis 1994; Brown et al. 2003). Besides adult neurogenic regions, numerous DCX and PSA-NCAM expressing cells have been described in layer II of the PC and EC of mice and rats (Seki and Arai 1991; Bonfanti et al. 1992; Nacher et al. 2001, 2002; Gomez-Climent et al. 2008). In these species, a few DCX+ and PSA-NCAM+ cells have been also identified in the insular and perirhinal areas of the NC. In contrast to mice and rats, numerous DCX+ and/or PSA-NCAM+ cells have been described in the NC of primates (Bernier et al. 2002), cats (Nacher J, personal communication) and very recently in the guinea pig (Xiong et al. 2008). PSA-NCAM and DCX have also been reported in several pallial regions of reptiles and birds, respectively (Ramirez-Castillejo et al. 2002; Boseret et al. 2007). In the present study, we mostly focussed on the DCX+ cells which appeared as a subpopulation of the PSA-NCAM+ elements. These cells are specifically restricted to the adult germinative regions and to layers II/III of some pallial derivatives of both mammals and the lizard. In agreement with recent observations in the rat PC (Gomez-Climent et al. 2008), our BrdU time course analyses performed in mammals clearly indicate that apart from DG granule cells, pallial DCX+/PSA-NCAM+ cells are not newly generated but represent resident neurons. Because in the lizard some pallial regions including the lateral cortex, DVR, and NS have been proposed as sites of adult neurogenesis (Lopez-Garcia et al. 1988; Perez-Sanchez et al. 1989; Ramirez-Castillejo et al.

2002), we can not exclude that the DCX+/PSA-NCAM+ cells we found in these regions are newly generated.

Identity of DCX+/PSA-NCAM+ Cells

In line with our study and others (Gomez-Climent et al. 2008; Xiong et al. 2008), DCX+/PSA-NCAM+ cells of PC, NC, and APA do not express markers of mature pallial neurons, suggesting that they might represent immature elements. This interpretation is further supported by recent ultrastructural analyses in the PC of adult rats showing that the DCX+ cells with few and short processes do not receive synaptic contacts, whereas those with well-developed dendritic arborizations make a few synapses, mostly on their distal dendrites (Gomez-Climent et al. 2008). In addition, because the number of pallial DCX+ and PSA-NCAM+ cells progressively reduces with age in all the species considered (Abrous et al. 1997; Lazic et al. 2007; van der Borght and Brundin 2007; Varea et al. 2007; Xiong et al. 2008; Luzzati F, unpublished observation), it is tempting to speculate that these cells are early generated neurons undergoing delayed differentiation during postnatal life. A similar mechanism has been previously proposed for a subset of motor neurons of the spinal cord (Farel 2003). Nonetheless, the identity of the putative fully mature stage of cortical DCX+/ PSA-NCAM+ cells has not been clarified to date. To obtain insight on this issue, we have investigated the expression of early specification markers of pallial cells. In layer II/III of the mammalian PC, NC, and APA, as well as in the reptilian lateral cortex, DVR, and NS, nearly all DCX+/PSA-NCAM+ cells express Tbr-1, supporting a pallial origin and a possible glutamatergic phenotype (Englund et al. 2005; Hevner et al. 2006). In addition, a few faintly DCX immunolabeled cells coexpress DLL, a marker of subpallium-derived interneurons (Anderson et al. 1997; Stuhmer et al. 2002; Alifragis et al. 2006).

Accordingly, in the cerebral cortex of the guinea pig, a recent study indicated that a restricted population of cells faintly labeled for DCX expresses markers of nitrinergic interneurons.

The authors interpreted these elements as the final phase of the differentiation of the cortical DCX+ cells (Xiong et al. 2008). Nonetheless, if these DCX+ nitrinergic interneurons coincided with the DLL+/DCX+ cells which we identified, they would more likely belong to a different lineage than the main DCX+/PSA-NCAM+/Tbr1+ cell population.

Morphological Profiles of DCX+/PSA-NCAM+ Cells

The morphology of the pallial DCX+/PSA-NCAM+/Tbr-1+ cells of both mammals and the lizard was consistent with that described in previous studies in the rat (Seki and Arai 1991; Nacher et al. 2001; Gomez-Climent et al. 2008). Interestingly, these elements appear as a heterogeneous population of neurons shared by different pallial derivatives and animal species. Two main cell morphologies, we referred to as DCX+ type I and type II cells, can be identified. The DCX+ type I cells have a small cell body and neurites confined to layer II/III; the DCX+ type II cells have a larger cell body and send diffuse arborizations into the above layers. In the rat, the smaller DCX+ cells have been recently indicated as “tangled” cells (Gomez-Climent et al. 2008).

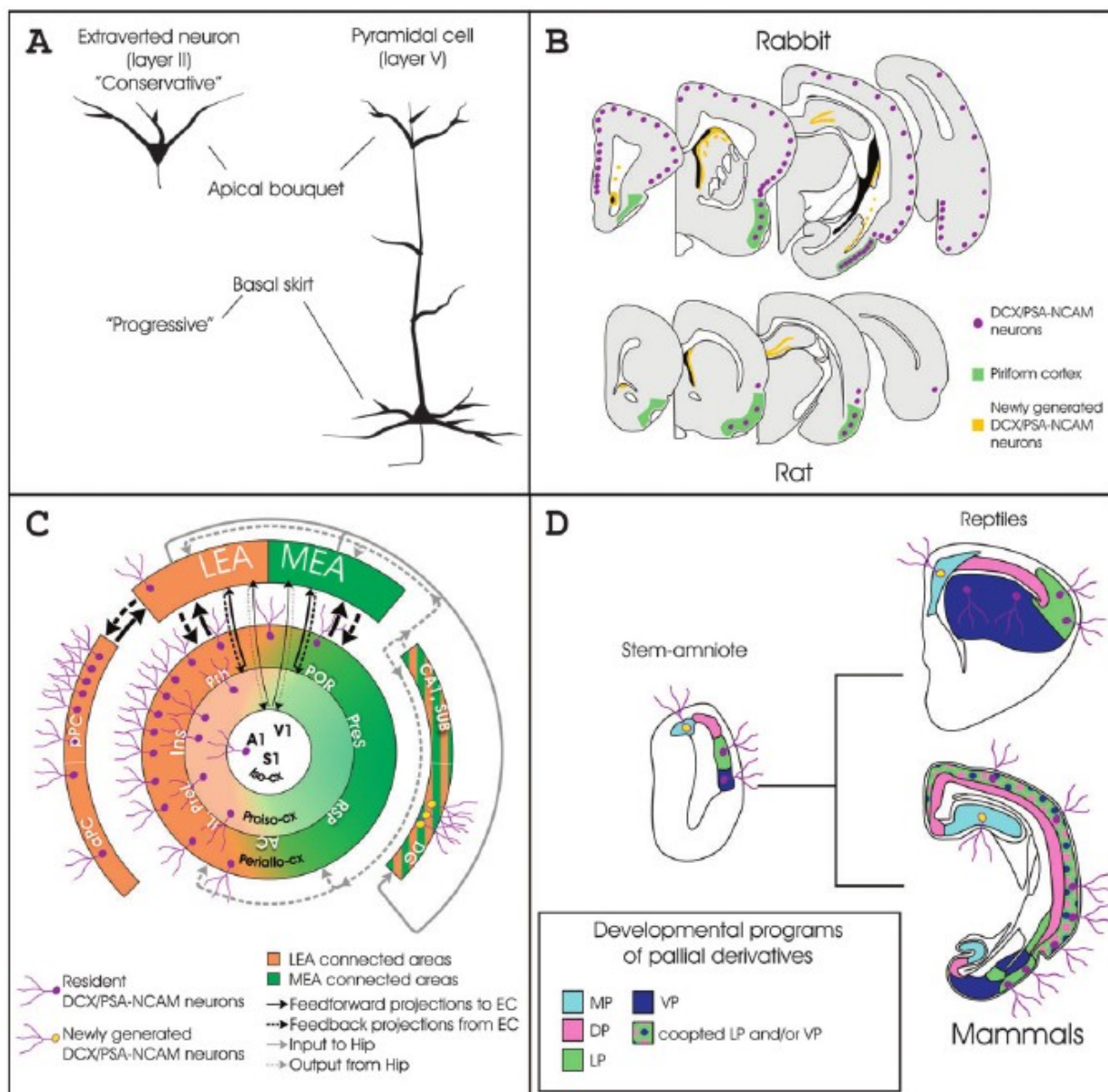


Figure 7. A) Representation of the main morphological features characterizing a cortical extrverted neuron of layer II (DCX+ type II cells) and a classic cortical pyramidal neuron of layer V. Extraverted morphology involves a clear predominance of subpial dendrites over basal dendrites, whereas that of a classic pyramidal cell shows a well-developed basal skirt. The lack of the basal skirt represents an ancient feature in the evolution of the pyramidal cell (Nieuwenhuys et al. 1998). (B) Distribution of PSA-NCAM+/DCX+ cells in coronal representative sections of the rabbit and rat telencephalon. PSA-NCAM+/DCX+ cells besides adult neurogenic regions (yellow) also occur in both allo- and neocortical domains. As compared with the rat in the rabbit NC, PSA-NCAM+/DCX+ cells are more numerous and widely distributed. (C) Schematic representation of DCX+/PSA-NCAM+ neurons in the PC, NC, and hippocampus of the guinea pig and rabbit. Positive cells are restricted to specific cortical areas. In the NC of the guinea pig and rabbit, DCX/PSA-NCAM density increases along 3 concentric rings of cytoarchitectonic organization defined iso-, proiso-, and periallocortex. Iso includes primary and secondary sensory areas; proiso and pAll includes cingulate, ventral temporal, insular, and parahippocampal cortices. In the NC and PC, DCX+/PSA-NCAM+ cells are specifically associated to LEA, and mostly absent in the MEA, connected regions (see the text for major details). (B) Contribution of LP and/or VP developmental programs to the evolution of NC. During the transition from the stem amniote to mammals, co-optation of developmental programs of the LP and/or VP by DP progenitors could have been used to originate NC upper layer-specific neurons (green/blue dots on pink background). Note that resident DCX+/PSA-NCAM+ elements are absent from the reptilian DP derivatives (in pink), whereas they are present in the VP (blue) and LP (green) derivatives. In mammals, they are located in LP and DP derivatives. A1, primary auditory cortex; AC,

anterior cingulate cortex; DP, dorsal pallium; IL, infralimbic cortex; Ins, insular cortex; LP, lateral pallium; MP, medial pallium; pPC posterior piriform cortex; aPC; anterior piriform cortex; PreL, prelimbic cortex; PreS, presubiculum; POR, postrhinal cortex; Prh, perirhinal cortex; RSP, retrosplenial cortex; SUB, subiculum.

We did not use this definition because it does not include the numerous uni/bipolar elements of this population. Type II cells have been previously described as semilunar or semilunar-pyramidal transitional neurons (Seki and Arai 1991; Nacher et al. 2001). As these definitions refer to PC specific neurons, we did not consider them appropriate to describe the same morphological cell type in other pallial derivatives of mammals and lizard. For their predominance of apical over basal dendrites, the DCX+ type II cells can be more generally referred to as “extraverted” neurons (Fig. 7A; Sanides F and Sanides D 1972).

An extraverted morphology characterizes several superficial projection neurons of the 3-layered allocortices of reptiles and mammals (i.e., granule cells of the DG, semilunar cells of PC, and bowl cells of the lateral cortex of reptiles; Sanides F and Sanides D 1972; Nieuwenhuys et al. 1998). In the NC, the number of extraverted neurons progressively decreases following 3 concentric rings defined according to their cytoarchitectonic organization: periallocortex, proisocortex, and isocortex (Sanides 1969; Sanides F and Sanides D 1972; Fig. 7C). The distribution of the DCX+ type II cells in different pallial derivatives of the analyzed vertebrate species are consistent with that described for the extraverted neurons. Nonetheless, DCX+ type II cells only represent a subpopulation of extraverted neurons, restricted to specific subsets of cortical areas and animal species. In addition, as the absence of the basal skirt represents an ancient feature in the evolution of the pyramidal cell (Nieuwenhuys et al. 1998), extraverted neurons were previously interpreted as old conserved cell types (Fig. 7A; Sanides F and Sanides D 1972). Our results give further support to this idea.

Pallial DCX+/PSA-NCAM+ Neurons Are Enriched in Areas Involved in Nonspatial Learning and Memory Networks

According to hodological and behavioral studies, 2 parallel cortical circuits preferentially connected with the medial entorhinal area (MEA) or lateral entorhinal area (LEA) and involved in the relay to the hippocampus of segregated inputs have been identified in different mammalian species (Insausti et al. 1997; Burwell and Amaral 1998a,b; Furtak et al. 2007; Jones and Witter 2007; Kerr et al. 2007). In the pallium of the rabbit and guinea pig, DCX+/PSA-NCAM+ neurons are strongly enriched in areas, including the PC and APA, connected with LEA (Fig. 7C). By contrast, the occurrence of these cells is strongly reduced in MEA converging areas (Fig. 7C). The nature of the cortical input involved in these segregated circuits suggests they represent parallel spatial (converging to MEA) and nonspatial (converging to LEA) learning and memory networks (Naber et al. 1999; Hargreaves et al. 2005; Jones and Witter 2007). Thus, such a specific pattern of distribution of DCX+/PSA-NCAM+ neurons suggests that they might play a role in the context of nonspatial networks. Notably, at the end of their differentiation process, newly generated PSA-NCAM+/ DCX+ neurons of the DG down regulate these molecules and integrate into spatial networks (Teixeira et al. 2006). An intriguing possibility is that a parallel differentiation process will lead DCX+/PSA-NCAM+ cells of PC, NC, and APA to integrate into nonspatial

networks.

DCX/PSA-NCAM Cells Are Preferentially Located in Higher Order Association Areas of Different Pallial Derivatives and Vertebrates Species

Within the DCX+/PSA-NCAM+ regions, the relative distribution of immunolabeled neurons is not homogeneous. In both mammals and lizard, these cells are reduced in areas receiving segregated sensory inputs from the thalamus, such as the primary sensory areas of the NC, and the ADVL (Karten 1969; Reiner 2000; Kaas and Collins 2001; Butler and Hoods 2005). By contrast, associative areas connected to the aforementioned regions, such as the perirhinal, cingulate, and insular cortex of mammals (Felleman and Van Essen 1991; Furtak et al. 2007), and PDVL/NS of lizard (Andreu et al. 1996; Lanuza et al. 1998; Novejarque et al. 2004), show the highest levels of DCX+/PSA-NCAM+ neurons. In the classical view of hierarchical sensory processing in the NC, information is relayed from the primary sensory areas to a sequence of association areas that encode increasingly complex features of external stimuli (Felleman and Van Essen 1991; Gilbert and Sigman 2007). The distribution gradient of DCX+/PSA-NCAM+ neurons in the NC parallels the hierarchical levels of sensory processing. Indeed, in the guinea pig and rabbit NC, the DCX labeling increases from primary to secondary sensory areas and reaches the highest level close to the rhinal sulcus, where DCX+ cells can also be observed in the mouse and rat (Fig. 7B). In the EC, that is the main relay station of sensory information to the hippocampus, DCX staining reduces abruptly in all species. A similar distribution pattern is visible in the PC, where the majority of DCX +/PSA-NCAM+ neurons are located in the posterior PC, an association cortex that has been proposed as a higher order station of olfactory processing (Kadoisha and Wilson 2006; Neville and Haberly 2004; Wilson et al. 2006; Gottfried et al. 2006). Similarly to mammals, in the lizard lateral cortex, the reptilian homologous to the mammalian PC (Haberly 1990; Puelles 2001), DCX+/PSA-NCAM+ neurons appear distributed following an anterior to posterior gradient which abruptly decreases in the region proposed as homologous to the EC (Martinez-Garcia et al. 1986; Hoogland and Vermeulen-Vanderzee 1995).

On the whole, these data strongly support that in the adult brain, the coexpression of DCX, PSA-NCAM, and Tbr-1 identifies a conserved population of neurons preferentially located in higher order association areas of different pallial derivatives and vertebrate species.

Interspecific Differences in the Occurrence of DCX+/PSA-NCAM+ Cells in the NC

As aforementioned, the occurrence of DCX +/PSA-NCAM+ elements strongly reduces with age in both the NC and PC of all the analyzed mammalian species. A time-limited expression of markers of structural plasticity occurs in many postnatal brain regions (Bonfanti 2006; Wagner et al. 2006). These time windows take part in area maturation and are referred to as critical or sensitive periods (Berardi et al. 2000; Hensch 2004).

In the NC, multiple evidence suggests a sequential maturation along the hierarchical levels, with higher order areas maintaining a prolonged capacity for structural plasticity after birth (Hensch 2004; Guillery 2005). The increased amount of DCX/ PSA-NCAM expression we found in higher order areas is consistent with this hypothesis. In addition, there is evidence that

critical periods protract proportionally to the expected life span of the species (Berardi et al. 2000). The reduced levels of DCX/PSA-NCAM expression in the rat and mouse compared with the rabbit and guinea pig (Fig. 7B) could be related to differences in brain-maturation time. The latter species have longer sexual maturation and by far a higher life expectancy; therefore, brain maturation potentially occurs on a longer timescale (see Lindsey and Tropepe 2006; Bonfanti and Ponti 2008). To establish whether the presence and distribution of resident PSA-NCAM + /DCX + /Tbr-1 + cells is related to brain maturation, further analyses in the species of different mammalian orders with diverse life span and brain size and less dominated by olfactory sensory input are required.

DCX/PSA-NCAM/Tbr-1 Pallial Neurons in the Mammalian NC: Ancient Cells into New Layers?

Our morphological and distributive analyses in 4 different mammalian species and lizard support the fact that DCX + /PSA- NCAM + /Tbr-1 + elements could represent a conserved cell population shared by different pallial derivatives, including the NC. This 6-layered cortical domain is exclusive of mammals and its evolutionary origin is still highly debated. Each of the 4 domains described in the pallial germinative regions of tetrapods contributes to specific derivatives in the different vertebrate classes (Fig. 7B; Puelles et al. 2000; Puelles 2001; Brox et al. 2004; Jarvis et al. 2005). In particular, the NC and PC of mammals and the dorsal and lateral cortices of reptiles have a common origin from the dorsal and lateral pallium, respectively (Puelles 2001). The distribution pattern of pallial DCX + / PSA-NCAM + /Tbr1 + cells in the analyzed lizard and mammals is particularly similar in the well-conserved lateral pallium derivatives. In lizard, these cells are also present in the ventral pallium derivatives (NS and part of the DVR) but not in the dorsal pallium, the proposed homologue to the NC (Puelles 2001). Thus, in the NC, although the morphology of DCX + /PSA-NCAM + /Tbr1 + cells and their distribution in associative areas can be considered a conserved feature, their occurrence in layers II/III of this dorsal pallium derivative might be an evolutionary novelty.

Further analyses of DCX and PSA-NCAM expression in the dorsal pallium derivatives of other mammalian species and reptiles at different developmental stages are required to make reliable hypotheses on the mammalian and reptile stem condition. Nonetheless, studies in primates (Bernier et al. 2002) and carnivores (in cat, Nacher J, personal communication) suggest the occurrence of PSA-NCAM + and/or DCX + cells in the NC represent a common feature in mammals. In addition, consistent with our results, it has been reported that the turtle dorsal cortex lacks most of the NC upper layer--specific cell types, as defined by connectivity and neurochemical markers (Reiner 1991, 1993). Following these considerations, Anton Reiner proposed that the upper layers of the NC might represent an evolutionary novelty (Reiner 1991, 1993; Medina and Reiner 2000). One plausible hypothesis which integrates our findings with Reiner's theories is that the upper layers of the NC originated through co-optation, by dorsal pallium progenitors, of preexistent lateral and/or VP developmental programs (Fig. 7B).

A detailed analysis of NC upper layer specific genes, such as *cux-2* or *svet-1* (Tarabykin et al. 2001; Nieto et al. 2004; Zimmer et al. 2004), in nonmammalian vertebrates will be needed to test this hypothesis. Notably,

homologies between specific cell types of the DVR of birds and the upper layers of the mammalian NC have been hypothesized (Karten 1969, 1997; Jarvis et al. 2005). These ideas have been criticized because DVR and NC originate from different pallial domains (Reiner 2000; Puelles 2001). However, our hypothesis could reconcile these opposing views. Finally, besides the DVR, our data support the involvement of the developmental program of the olfactory cortex in the evolution of the upper layers of the NC. This is intriguing because common features exist in sensory information processing in the PC and NC, including a tangential sequence of hierarchically organized areas converging on the EC (Haberly 1990, 2001; Wilson and Stevenson 2003; Leon and Johnson 2006).

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Supplementary Material

Supplementary material can be found at:
<http://www.cercor.oxfordjournals.org/>.

Notes

Conflict of Interest : None declared.

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