Microtubule associated proteins and plasticity in the developing and diseased brain

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Publication date
2006

Citation for published version (APA):
Differential spatiotemporal expression of doublecortin-like (DCL) and doublecortin (DCX) in the early embryonic murine neocortex

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Abstract

During early corticogenesis, radial glia-derived neural progenitors divide and migrate along radial fibers to their designated positions within the cortical plate. The microtubule-associated proteins doublecortin (DCX) and doublecortin-like kinase (DCLK) are critically involved in migration and division, respectively, and may function in a partially redundant pathway during cortical development. Since little is known about the role of these proteins during early stages of corticogenesis, when neurogenesis is extensive, we performed a detailed analysis of the spatiotemporal expression of DCX and DCL from E9 onwards.

DCL and DCX were expressed in a clearly differential manner before E13. DCL was already expressed from E9 onwards with a strong overlap with the radial glia marker vimentin. DCX expression in contrast, started only modestly from E10/E11 onwards. DCL was found mainly in the inner layers of the developing cortex before E13, often in mitotic cells in the VZ and in pial oriented radial fibers emerging from them, whereas DCX was primarily expressed in fibers in the outer region of the preplate (PP). After E13, DCX was largely overlapping with DCL except for the VZ. As development continues, DCL levels were further reduced contrary to DCX that remained high until birth.

In conclusion, the present study demonstrates a differential spatiotemporal expression pattern for DCX and DCL during early corticogenesis, consistent with the different functional roles of these two proteins. Given the distribution and involvement in neuronal precursor mitosis as well as radial fiber stability of DCL, it appears to have a unique role particularly in the early neuroepithelium. This could be of considerable relevance for the formation of the initial precursor pool that eventually determines number and complexity of the adult neocortex.
Introduction

During cortical development, a complex multi-layered neocortex is formed through a carefully orchestrated series of cell divisions and migratory events (for recent reviews see (Aboitiz et al., 2001; Gupta et al., 2002; Nadarajah et al., 2002)).

As early as E8.5, the first precursors leave the ventricular zone (VZ) and migrate radially to establish the preplate (PP) around E10 which is covered by the pial surface (PS), and separated from the VZ by a thin axonal layer called the intermediate zone (IZ). A second wave of cells around E12 then splits the preplate into the marginal zone (MZ) and the subplate (SP). The neuronal layer formed in between is called the cortical plate (CP). The remaining outer layers of the adult cortex are established by sequential waves of new neurons crossing the IZ and the CP (Angevine and Sidman, 1961; Rakic, 1974), mainly between E14 and E18, and in an inside-out manner (Marin-Padilla, 1971).

After E12, new neurons depend heavily on radial glia fibers as a scaffold for migration by locomotion, whereas prior to E12, migration occurs primarily by means of nuclear translocation (Rakic, 1972; Schmechel and Rakic, 1979; Kriegstein and Noctor, 2004). Radial glia cells (RGCs) are not only important as scaffold, recent evidence even indicates that these cells divide and generate neurons themselves. As such, they are considered the predominant neuronal precursors during cortical development (Noctor et al., 2001; Anthony et al., 2004).

The proteins controlling precursor division and (guided) migration during these periods, are largely unknown. A key event in both migration and division is the controlled stabilization of microtubuli, the major cytoskeletal elements in neurons, by microtubule-associated proteins (MAPs). Recent genetic studies have identified a number of microtubule associated proteins, such as Lis1 and doublecortin (DCX) (des Portes et al., 1998) that are crucially implicated in cortical development. Mutations in the DCX gene cause the double cortex syndrome, characterized by subcortical band heterotopias. Functional studies have further shown that DCX is a MAP (Francis et al., 1999; Gleeson et al., 1999) essential for radially migrating neurons to cross the IZ and enter the CP (Bai et al., 2003). Soon after the discovery of DCX, a gene with high homology was described in rat and human, called doublecortin-like kinase (DCLK). We have recently reported on a novel splice variant of this gene, called doublecortin-like (DCL) that also promotes microtubule stability, but lacks the kinase C-terminus of the DCLK gene (Engels et al., 2004). DCL is critically involved in mitosis of neuroblasts and in radial process stability (Vreugdenhil, submitted), functions that complement and partly overlap with DCX and other DCLK variants recently shown to be involved in mitosis, migration, axonal outgrowth and microtubule-associated vesicle transport (Deuel et al., 2006; Koizumi et al., 2006; Shu et al., 2006).

Even though the functions of the related proteins DCX and DCL appear synergistic as both promote microtubule stability, little is known about their spatiotemporal distribution and possible differences therein which could point to a specific role of each protein during early neurodevelopment. We
therefore examined the spatiotemporal distributions of DCL and DCX from early embryonic development onwards. In view of its putative role as neuronal precursor we also investigated possible overlap with radial glia. Despite their high sequence homology and considerable functional overlap, we show that DCX and DCL have a distinct differential distribution during early corticogenesis, supporting their different functional roles.

Materials and Methods

Animals and tissue preparation

CD1 mouse embryos of embryonic day (E) 9, 10, 11, 13, 15, 17 were obtained by caesarean sections of pregnant females. Mouse embryos were washed in 0.1M phosphate buffered saline (PBS), fixed in methanol/acetone/water 40:40:20 (Franco et al., 2001) for four hours and stored in 70% ethanol. After dehydration in a graded ethanol series of 70, 80, 90, 96 and 100% ethanol of 90 min each, embryos were incubated in 1-butanol overnight and embedded in Paraplast Plus (Kendall, Tyco Healthcare) at 60 °C for two hours. Subsequently, sections of 6 μm thick were obtained using a Leica Wetzlar microtome. Every fourth section was mounted on Superfrost Plus slides (Menzel) and stained with 0.25% cresylviolet acetate for orientation.

Antibodies

The generation of the SN370 anti-DCL antibody used in this study has been described in detail before (Vreugdenhil et al., 2006)(Kruidering et al., 2001). This antibody recognizes the C-terminus of DCL, that shares 70% amino acid homology with human DCX (Vreugdenhil et al., 1999) and also recognizes other splice-variants of the DCLK gene including DCLK-short (also known as CaMLK (Kruidering et al., 2001)). During embryonic development, a single 40kD protein band is stained by anti-DCL (Vreugdenhil et al, submitted) which might indicate cross-reactivity with the 40 kD DCX. However, as numerous DCX+ cells are not stained by anti-DCL, this indicates that anti-DCL is specific for DCL and does not cross-react with DCX. In line with this notion, knock-down of the DCL gene by si-RNA ablated anti-DCLK immunostaining in in vitro studies (Vreugdenhil et al., submitted). For DCX, the C-18 antibody was used (Santa Cruz) that has been described and validated before in detail (Couillard-Despres et al., 2005; Boekhoorn et al., 2006; Boekhoorn et al., in press).

For immunohistochemistry, the biotinylated secondary antibodies anti-goat (1:1000; Jackson ImmunoResearch) for C-18, anti-rabbit (1:200; Vector Laboratories) for SN370 and anti-mouse (1:200; Amersham Biosciences, UK) for vimentin were used. The primary antibodies were used at the following dilutions: DCX C-18 at 1:200 for immunohistochemistry and at 1:100 for immunofluorescence; DCL SN370 at 1:100 for immunohistochemistry and at 1:50 for immunofluorescence and vimentin at 1: 50 for immunohistochemistry and at 1:150 for immunofluorescence (Sigma-Aldrich).

For immunofluorescence, secondary antibodies conjugated to AlexaFluor488 (1:400 anti-goat; Molecular Probes, Oregon, USA), Cy3 (1:400
anti-rabbit; Jackson ImmunoResearch) or AlexaFluor647 (1:400 anti-mouse; Molecular Probes, Oregon, USA) were used.

**Immunohistochemistry**

To study the early distributions of DCX, DCL and vimentin, sections of the developing mouse cortex were mounted on Superfrost Plus slides and dried overnight at 37°C. They were then dewaxed twice in xylene for 10 min and hydrated in a graded ethanol series for 5 min each. After rinsing in filtered water (Aquadest), sections were postfixed in Zamboni-mix (2% PFA / 0.1% Picric Acid / 0.025% gluteraldehyde / 0.1M phosphate buffer [pH 7.4]) for 5 minutes and washed in 0.1M tris buffered saline (TBS) [pH 7.6].

In order to retrieve masked antigen epitopes, sections to be immunostained for vimentin were pre-treated in a microwave by placing them in 0.1M citrate buffer [pH 3] and heated for 15 min initially at 800 W and then at 200 W to prevent air bubbles from causing tissue damage. After cooling to room temperature, endogenous peroxidase activity was blocked by 0.1% hydrogen peroxide treatment for 20 min. Primary antibodies were applied in 0.25% gelatin / 0.1% triton X-100 in 0.1M TBS [pH 7.6] (Supermix) for 1 hour at room temperature and then overnight at 4°C.

Subsequently, sections were washed with 0.1M TBS and incubated with the secondary antibody for 1.5 hour at room temperature. Signal amplification was done using Avidin-biotin complex (ABC) Elite kit (Vector Laboratories, Burlingame) for 1.5 hour (1:800), that was further amplified with biotinylated tyramide (1:500) and 0.01% peroxide for 30 min, followed by another 45 min incubation with ABC (1:800). Sections were washed between each step with 0.1M TBS [pH 7.6] and finally twice with 0.05 M tris buffer, pH 7.6. Chromogen was 0.05% diaminobenzidine (DAB) dissolved in 0.05 M TB [pH 7.6]. Sections were counterstained with 0.25% cresylvioletacetate and embedded in Entellan (Merck).

**Immunofluorescence**

Sections were prepared as described above, including MW pretreatment for vimentin. Primary antibody was added to 1% BSA/0.1% Triton X-100/3% donkey normal serum/TBS (TBS++) and incubated for one hr at room temperature and then overnight at 4°C.

Secondary antibody was dissolved in 0.1M TBS [pH 7.6], and added to the sections for two hours after washing with 0.1M TBS. Sections were mounted in Vectashield containing DAPI and examined using a Zeiss LSM510 confocal microscope. Immunofluorescence was visualized using the following filter settings: 633 nm, LP 650 filter; 543 nm, BP 560-615 filter; 364 nm and 351 nm, BP 385-470 filter; 488 nm, BP 505-530 filter. To allow comparison of relative expression-levels, each slide was investigated using the exact same laser and filter settings for a specific staining.

**Nomenclature**

For nomenclature we made use of the atlases of Schambra and Altman (Schambra et al., 1992; Altman and Bayer, 1995).
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Results

At E9, DCX immunoreactivity was completely absent (figure 1A, color figure), whereas expression of both DCL and vimentin were already substantial (figure 1B, C) and particularly high throughout the inner wall of the third ventricle, the neocortical neuroepithelium and the dorsal and ventral diencephalon, including the thalamus and hypothalamus. Generally lower levels were found in the walls of the optic vesicles (Schambra et al., 1992; Altman and Bayer, 1995). As shown in figure 1D and H, DCL and vimentin were expressed in the ventricular zone (VZ) as well as the preplate (PP) with some DCL+ fibers also in the intermediate zone (IZ) (figure 1D). The regional expression of DCL and vimentin was almost completely overlapping at this age in the VZ, except for that vimentin was expressed at higher levels in the PP.

Figure 1. DCL expression in the neuroepithelium at E9.

Midlevel coronal sections of the developing mouse neuroepithelium. DCX (A) is absent at E9, whereas DCL is already abundantly expressed particularly in the VZ (arrowheads) (B), as well as in the preplate (PP). In the intermediate zone (IZ), occasionally a DCL positive radial fiber (left arrowhead in D) is seen. The pattern of DCL closely resembles that of the radial glia marker vimentin (C and H). Higher magnifications reveal many mitotic cells in the VZ express DCL (E, F) while also radially migrating cells leaving the VZ (arrow in G) and short radial fibers are DCL+ (arrows in lower part of D). Apoptotic cells do not express DCL (arrowhead in I).

Abbreviations: V3: 3rd ventricle, H: hypothalamus, OV: optic vesicle; DD (T): dorsal diencephalon (thalamus). Scale bar in A is 100 μm. Scale bar in D is 10 μm.
Many of the DCL+ cells in the VZ clearly were in mitosis (1E, F and G) and never was DCL immunoreactivity observed in apoptotic cells (Fig 11). The morphology of most DCL+ cells in the VZ was that of a mitotic cell with a round or ovoid, condensed soma and a nucleus in obvious telophase or anaphase (Fig 1E, F). Frequently the chromatin was organized into individual pairs of chromosomes positioned opposite of each other with a cleavage plane visible, and of which both the polar and kinetochore microtubules were DCL+. Strikingly, most DCL+ mitotic cells had a DCL+ process that extended from the VZ cells in a radial manner and could often be traced to the PP or pial surface (Fig 1D, see also Fig 2 E, F and G).

At E10, some very weak DCX expression was seen only in the PP of the fronto-lateral part of the LV epithelium (L), whereas the rest of the cortex was devoid of DCX immunoreactivity (figure 2A, color figure, H; arrow in A). This was in contrast to the abundant expression of vimentin and DCL throughout the lateral (L) and medial parts (M) of the LV wall. Between individual cortical layers, DCX distribution (figure 2 A, H) differed from that of DCL (figure 2B, E, F and G) and vimentin (figure 2C, I and J). DCL and vimentin expression, quite comparable to E9, were high in the PP and prominent in the VZ with expression in radial fibers in the IZ (Fig 2A,B,C).

Also in the dorsal diencephalon DCX was observed in the VZ, in mitotic cells traversing their radial fibers into the IZ (Fig 3A and B, color figure). Mitotic cells were also observed in the IZ, in close contact with radial fibers (Fig 3C). In the lower spinal cord (Fig 3D), some DCX+ fibers were observed as well as in a few lower dorsal root ganglia, whereas DCL expression, similar to the distribution in the diencephalon, was prominent in the inner wall of the dorsal spinal cord neuroepithelium (4th ventricle) with many DCL+ radial fibers traversing to the DRG (figure 3E). Frequent mitotic cells that were DCL+ or closely associated to DCL+ fibers, were present in this neuroepithelium as well.
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Figure 2. DCL expression in the lateral ventricle wall at E10.

Coronal sections of the lateral ventricle (A-C). At E10, only a tiny rim of DCX expression in the PP is observed laterally (L arrow in A) and only in the dorsal region of the neuroepithelium surrounding the LV (H). In contrast, DCL is abundantly expressed at E10 (B, E, F, G), particularly in the thinner, medial parts (M) of the neuroepithelium (arrow in B) but also in the dorsal regions of the neocortex. High power microphotographs (E-G) show DCL expression in the PP and especially in the VZ, often in mitotic cells (E, F, G) frequently possessing radial fibers oriented towards the PP and pial surface (arrowheads in E). Vimentin expression (2 C) is largely comparable to DCL expression at this age, with radial fibers originating from the VZ, extending through the IZ (I) and towards the PP (J). Asterisks indicate the LV wall. M; denotes the medial side and L the lateral side as shown in more detail in figure 4. Scale bar in A is 100 μm. Scale bar in E is 10 μm.

Figure 3. DCL expression in the neuroepithelium and spinal cord at E10.

In the neuroepithelium of the ventral and dorsal diencephalon (coronal sections A-C), DCL is highly expressed in mitotic cells in the VZ and in many radial fibers (A-C). Also at short distances away from the VZ, mitotic cells are found in the IZ (C). Contrary to the diencephalon, in the caudal part of the spinal cord, both DCX and DCL expression occurs in dorsal root ganglia (DRG), though with a differential expression (D and E resp.). DCL is expressed in the ventricular wall in radial fibers oriented towards the DRG, as well as in the DRG, whereas DCX expression is confined to the DRG alone. Scale bars in B and D are 10 μm.
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At E11, DCX immunoreactivity was prominent in the remainder of the dorsal telencephalon, particularly in the PP where many DCX+ fibers were visible with a tangential orientation. DCX localization in the LV was, similar to E10, largely absent from the medial part of the LV (figure 4A, location of the area shown in fig 4A is indicated in fig 2A with M, color figure) wall and slightly increased in the fronto-lateral part of the LV wall (location of the area shown in fig 4G is indicated in fig 2A with L). In the spinal cord, abundant DCX immunoreactivity was present in DRGs in both middle and lower regions (Fig K and M).

DCX expression at E11 was weak in the fronto-lateral part of the LV wall, (figure 4H), whereas higher levels were present in in the medial part (figure 4B). In the VZ and IZ, both DCL and vimentin were expressed in an overlapping manner. Particularly at these ages E 10 and 11, many DCL+ mitotic cells were present (4C,D and F). Whereas many DCL+ cell bodies were in mitosis, only one occasional DCX+ mitotic cell was observed in the PP (figure 4E). In the spinal cord, lower DCL levels were observed (4L and N). Contrary to DCX, DCL was observed in rostrocaudally orientated fibers (Fig N). Vimentin expression was abundant in all parts of the LV wall (figure 4I).

To address possible overlap between vimentin, DCX and DCL expression at E11, regional differences were further investigated using double immuno-fluorescent labeling and confocal analysis (figures 5, color figure). DCX hardly showed any overlap with DCL and was primarily expressed in the PP and not in the VZ where DCL was prominently expressed (Fig 5A-C). DCL and vimentin expression patterns were comparable and were shown before (Vreugdenhil et al., submitted) to have a nearly complete overlap in the VZ. DCL+ cells displayed morphological characteristics of RGCs with an ovoid soma in the VZ and pial orientated DCL+ radial fibers crossing the IZ. In the spinal chord, double staining was present for DCX and DCL in the tangentially orientated fiber, whereas the dorso-ventrally orientated radial fibers were only DCL+ and not DCX+ (Figure 5D).
Figure A and B represent higher magnifications of the medial part of the cortex as shown in fig 2A (depicted as M), although at a different age, while G-I are taken from the lateral side of the cortex as shown in fig 2A (L). At the medial side of the neuroepithelium of the LV, no DCX expression is observed (A), in contrast to DCL (B), which is robustly expressed in pial oriented radial fibers (arrow) extending from the VZ towards the MZ and in mitotic cells (arrowhead in C, D, and F). Only in one occasion DCX expression was observed in what appears to be a mitotic cell in the CP (arrow in E). In the neuroepithelium at the lateral sides of the LV, DCX is only expressed in the PP (G) and DCL expression is very weak in the PP, MZ, IZ and ventricular zone (VZ), and largely overlapping with vimentin (I). In the spinal cord (sagittal sections K-N), DCX expression (K, M) becomes apparent in the DRG (right arrow in K) and is also seen in fiber bundles of the vagal nerve oriented towards the pericardial cavity (left arrow in K). DCL (L, N) is not only found in DRGs, albeit at generally lower levels, but also in rostrocaudally oriented fibers (N), but not in cellular profiles, nor in apoptotic cells. Scale bars in B, D and N are 10 μm. Scale bar in J is 20 μm and in L is 250 μm.
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Figure 5. Multiple immunofluorescent analysis of DCL and DCX expression in the neocortex and spinal cord at E11.

Fluorescent double labelings of the neuroepithelium (A) confirm the differential expression pattern of DCX and DCL: DCL is expressed at particularly high levels in the VZ (arrows) and at considerably lower levels in the PP. DCX is mainly and more robustly expressed in the PP. In the spinal cord (D), DCX and DCL partly overlap in what appears to be tangential fibers, whereas DCL is additionally, and selectively, expressed in radial fibers. Scale bars in C and D are 30 and 12 μm, respectively.

At E13, the distribution of DCX and DCL was strikingly similar in regions like the thalamus and hypothalamus (figure 6A, B, color figure). In general, immunoreactivity of DCL+ elements (Fig 6B) in the telencephalon and particularly the VZ was clearly reduced when compared to previous ages. DCX immunoreactivity was abundant in the upper parts of the spinal cord, the pons, tectum, thalamus and in the telencephalon (Fig 6A), where it was seen in tangential fibers in the IZ and at the CP (Fig 6D). DCX at this age was never seen in mitotic cells but only in fibers. In the telencephalon, overlap with DCL (Figure 6B and E) was high, which was no longer expressed in the VZ. There was little overlap with vimentin (figure 6C,F), the expression of which was high in the VZ. In the thalamus and hypothalamus, expression of vimentin was low. In the spinal cord, vimentin was expressed in the DRGs and in the lower lumbar regions of the spinal cord, where it was often found in cellular profiles. This was in contrast to vimentin in the telencephalon, cerebellum and striatum where expression was mainly in a radial pattern (data not shown). A remarkable finding was the DCL expression in the olfactory epithelium of the nasal cavity (Fig 6G, H) and the expression of DCX and of DCL in fibers entering the optic vesicle through the eye stalk (Fig6 K,L). In contrast to DCX, DCL was expressed at the basis of the developing retina, again in close association with many mitotic cells residing in that area (inset in L), although at low levels. In several parts of the midbrain both radially (arrows) and tangentially (arrowheads) orientated fibers were observed, immunopositive for both DCX and DCL (figure 6I and J).
Figure 6. DCL expression in neuronal tissues at E13.

Compared to E11 and DCX levels (Fig A and D), DCL expression in the VZ is reduced at E13 (Fig B and E). The overall expression pattern of DCL in other areas like the spinal cord DRGs, thalamus, pons and CP in the midbrain (B) is generally comparable to that of DCX at this age (A), but differs from vimentin expression at this age (C) that is strongly reduced e.g. in the thalamus and hypothalamus. Higher magnifications of the developing neuroepithelium surrounding the 3V show extensive DCX expression in the CP (D) and a strong reduction in DCL (E) expression in the CP. IZ. Strikingly, DCL expression was completely absent in the VZ. This contrasts with vimentin, that is expressed in CP and VZ (F).

Additional regions that showed considerable DCL expression were the olfactory epithelium (OE) (G and H) where high levels were found in the outer layer close to the nasal cavity (NC), as well as in radial fibers oriented towards the outer layer (H). DCX is not expressed in this region (not shown). In the developing pons (P in A) both DCX (I) and DCL (J) are expressed in tangentially (arrowheads) and radially (arrows) oriented fibers. Also in optic fibers (OF) of the developing eye (K, L), DCX (K) and DCL (L) expression was found, with lower levels for DCL. In the basal layer of the developing retina only DCL and not DCX was expressed, often in association with mitotic cells (arrows, higher magnification in inset in L).

Scale bar in A is 1 mm, Scale bar in E, J and L is 50 μm, Scale bar in G is 250 μm, Scale bar in H 10 μm.

Abbreviations: ctx: cortex; bg: basal ganglia; thal: thalamus; ht: hypothalamus; mb: midbrain; C: cerebellum; p: pons; nc: nasal cavity.
While DCX expression in the upper part of the spinal cord was high, DCL predominated in the lower half (Figure 7A and B, color figure). DCX+ fibers were often orientated dorso-ventrally (Fig 7C), whereas DCL+ fibers were generally oriented in a rostro-caudal manner in lower regions (figure 7D).

![Figure 7](image)

Figure 7. Differential expression of DCX and DCL in spinal cord and dorsal root ganglia at E13.

Sagittal section showing DCX to be primarily expressed in the upper DRGs in fibers with a dorso-ventral orientation (arrow in A, C), whereas DCL is abundant in more caudally located, rostro-caudally orientated fibers (B, D). Scalebar in B is 500 µm. Scalebar in D is 50 µm.

At E15, DCX expression was reminiscent of that at E13 with large numbers of DCX+ fibers throughout the IZ, CP, SP and MZ of the neocortex (Fig 8A, color figure). Also, many radial fibers were observed in the spinal cord (data not shown). Expression of DCL in the neocortex on the other hand had further declined (Fig 8B) although the developing tongue showed intense immunoreactivity at this age (Fig 8E) in contrast to the olfactory epithelia that were now devoid of signal (data not shown). The pattern of vimentin expression was not altered and mainly limited to the VZ (figure 8C).

At E17, DCX expression was mainly present in the neocortex and spinal cord (Fig 8F). A weak signal was still observed in the developing tongue, and very little in the striatum (data not shown). DCL expression in the tongue was considerable, while also in the intestine, occasionally sparse immunoreactivity was seen (not shown). Otherwise, no remarkable changes occurred, except for a further decline in overall DCL expression to very low levels (figure 8G). Figure 8H shows unaltered vimentin staining.
Whereas DCX expression in the neuroepithelium remains high in MZ/PP and IZ (A), DCL expression (B) in the lateral epithelium of the lateral ventricles has further declined. Vimentin (C) is weakly expressed throughout the neuroepithelium but especially high in the VZ. Although in the CNS, DCX expression is higher than expression of DCL. DCL expression exceeds that of DCX in the developing tongue. DCL and DCX positive cells reside at the tongue base in fibers oriented in different directions (D and E). At E17, DCL expression has further declined (G) and is nearly undetectable in the ventricular zone. DCX (F) is now also expressed in the MZ. Vimentin expression remains stable (H). Scale bar in C is 10 μm, scale bar in E indicates 100 μm. Scalebar in G is 50 μm.
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Figure 9 (color) summarizes the main findings in this study. An important switch occurs between E11 and E13. Whereas at E11 DCL is expressed in the VZ, IZ and PP, it is only expressed in the IZ and PP at E13. In contrast, DCX is only expressed in the PP at E11, but in the IZ as well as the PP at E13 (Fig 9A). Note that the spatial distribution of DCL coincides with the occurrence of the nuclear translocation mode of migration. Also the levels of expression of DCL and DCX changed in opposite directions (Fig 9B). Whereas DCL expression was abundant at E9, it decreased after E11. DCX expression started very modestly at E10-11 and increased rapidly thereafter. At E17, DCX was still present at high levels.

Discussion

The MAPs DCX and DCLK are critically involved in the orchestration of structural plasticity changes during cortical development. Previous studies have indicated the functional roles of DCX and the DCLK splice variant DCL to be synergistic, and largely overlapping during the later stages of corticogenesis. Whereas both proteins are highly homologous and promote microtubule stability, DCL has been additionally implicated in proliferation, mitosis and radial process stabilization ([Vreugdenhil, submitted]). The present study is the first to address that issue by studying the detailed spatiotemporal
mapping of DCL and DCX protein expression during early corticogenesis. We show that in contrast to later stages, DCL and DCX are differentially distributed between E9 and E13. Also, onset of DCL protein expression in the VZ started already from E9 onwards, thereby preceding the onset of DCX expression. DCL distribution is consistent with a possible role in nuclear translocation, the predominant mode of migration in that period. Secondly, after E13, DCL expression declined and largely disappeared from particularly the VZ. This suggests that DCL and DCX are active at different stages of cortical development and thus may subserve different functions.

As to the first finding, recent reports have highlighted the involvement of the DCLK gene (Shu et al., 2006) in corticogenesis and more specifically neurogenesis in the VZ. Yet, expression of these DCLK variants has so far only been studied after E13 (Burgess et al., 2000; Lin et al., 2000; Vreugdenhil et al., 2001; Burgess et al., 2002; Engels et al., 2004; Deuel et al., 2006; Koizumi et al., 2006; Shu et al., 2006) in the mouse even though the most robust neurogenesis occurs before that period. These DCLK variants were found in the outer layers of the developing cortex, but not the inner VZ, where DCL is expressed. One study reported high expression of DCLK in a.o. the VZ until E19 and also in mitotic cells at E13 (Shu et al., 2006), an age at which we found hardly any DCL expression in mitotic cells anymore. DCL expression in our study rather peaked at earlier ages like E9/10. Clearly, the occurrence, at E13, of DCLK but not DCL immunopositive mitotic cells in the study by Shu et al., indicates that DCL is not selective for mitotic cells per se.

In contrast to DCLK, DCL expression is considerably diminished at later ages, highlighting the selectivity of DCL downregulation. In addition to the outer cortical layers, the expression of DCLK-long occurred also in the developing retina and dorsal root ganglia. This pattern is clearly reminiscent of that of DCX, which we found to display a nearly complete overlap with DCL mainly after E13 (Francis et al., 1999; Gleeson et al., 1999).

Together, this indicates a strong synergy of DCL with DCX and other DCLK variants in the later stages of corticogenesis, whereas DCL appears to have a more selective role during earlier phases. DCL is expressed at high levels in the VZ and IZ, where it is seen in radial fibers and mitotic cells. Its expression in radial fibers likely reflects microtubule stabilization of the early pial oriented processes enabling neuroblast displacement over short distances. Interestingly, the period of DCL expression matches that of nuclear translocation, an early type of migration by which neuroblasts translocate their somata through radially oriented processes, typically restricted to E10-13. DCX during that period is either absent, or expressed in very low levels in a pattern that does not overlap with DCL, suggesting an exclusive role of DCL in this period. After the nucleus has reached its destination in the PP/CP, the trailing process detaches from the VZ, consistent with our observations that DCL is no longer expressed in the VZ after E12. A crucial role for DCL in radial fiber stability is further supported by our earlier studies in which DCL knockdown in utero ablated the radial fiber patterning in the embryonic cortex and strongly reduced embryo survival (Vreugdenhil, submitted).
Taking these data together, DCL may be selective for RGCs migrating by means of nuclear translocation.

The expression of DCL in mitotic cells was not exclusive for the early VZ or cortex alone, but also occurred in the developing retina, where its temporal pattern paralleled the period of neurogenesis from E10.5 to E13 (Hinds and Hinds, 1974; Brown et al., 1998; Rapaport et al., 2004; Lee et al., 2005). The decline in DCL expression after E13 parallels the gradual disappearance of the large numbers of mitotic cells from the VZ and is consistent with a role of DCL in mitotic spindle stability in addition to radial process stabilization. In our previous studies, endogenous expression of DCL could only be identified in neuroblastoma cell lines, but not in any other type of cell line investigated, suggesting that DCL, in addition to its role in radial fiber stability, is selective for cells with a general neuroblast phenotype (Vreudenhil, submitted), a function so far not attributed to DCX.

Additional support for this concept comes from our vimentin double labelings demonstrating DCL expression in a subpopulation of radial glia cells (RGCs). DCL is clearly not expressed in all RGCs since after E13 the patterns of vimentin and DCL expression diverge and are no longer overlapping in the VZ. As recently demonstrated, RGCs not only serve as a scaffold for migratory neurons but can also generate new neurons themselves and are therefore considered neuronal precursors in the early neuroepithelium (Noctor et al., 2001; Anthony et al., 2004). It is tempting to speculate that a subpopulation of early RGCs selectively utilize DCL for microtubule stabilization.

Embryonic days 9-12 are critical for cortical development as the initial precursor pool is formed and rapidly expanding during that period. Factors affecting these processes will affect the eventual size and complexity of the postmitotic cortical cell population. Indeed, mutations in genes associated with mitotic spindle stability like microcephalin and abnormal spindle in microcephaly (ASPM), are associated with microcephaly, a human condition characterized by a small cortex (Bond and Woods, 2006). In view of this, DCL is of great relevance for early corticogenesis. It is interesting in this respect that DCLK knockdown mice, that also lack DCL, but not DCX, show only a very mild phenotype, whereas only after DCLK/DCX double knockdown, a severe phenotype of hypoplastic or absent fiber tract formation developed (Koizumi et al., 2006), suggesting that DCX and DCLK cooperate in axonal tract development and mediate the migration and positioning of cortical neurons within the correct lamina. Alternatively, further genetic redundancy may be present with the DCK2 gene (Edelman et al., 2005).

In conclusion, the present study demonstrates a differential spatiotemporal expression pattern for DCX and DCL during early corticogenesis, consistent with different functional roles. Given its current distribution and involvement in neuronal precursor mitosis as well as radial fiber stability, DCL appears to have a selective and important role in the early neuroepithelium while it cooperates with DCX and shares functional redundant roles in later stages of corticogenesis.