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Alternative Transcripts of *Dclk*1 and *Dclk*2 and Their Expression in *Doublecortin* Knockout Mice

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Abstract
The *doublecortin* (*DCX*) gene, mutated in X-linked human lissencephaly, has 2 close paralogs, *doublecortin-like kinase* 1 and 2 (*Dclk*1 and 2). In this study we attempted to better understand the dramatic differences between human and mouse DCX/Dcx-deficient phenotypes, focusing on the *Dclk* genes which are likely to compensate for Dcx function in the mouse. Using sequence database screens, Northern blot analyses and in situ hybridization experiments, we characterized the developmental transcripts of *Dclk*1 and 2, questioning their conservation between mouse and human, and their similarity to *Dcx*. Like *Dcx*, *Dcx*-like transcripts of the *Dclk*1 gene are expressed in postmitotic neurons in the developing cortex. No changes of expression were observed at the RNA level for these transcripts in *Dcx* knockout mice. However, a minor change in expression at the protein level was detected. The *Dclk*2 gene is less well characterized than *Dclk1* and we show here that it is expressed both in proliferating cells and postmitotic neurons, with a notably strong expression in the ventral telencephalon. No major differences in *Dclk*2 expression at the RNA and protein levels were identified comparing *Dcx* knockout and wild-type brains. We also analyzed *Dclk*1 and 2 expression in the hippocampal CA3 region which, unlike the neocortex, is abnormal in *Dcx* knockout mice. Interestingly, each transcript was expressed in CA3 neurons, including in the heterotopic pyramidal layer of *Dcx* knockout animals, but is presumably not able to compensate for a lack of *Dcx*. These results, in addition to characterizing the transcript diversity of an important family of genes, should facilitate further studies of compensation in *Dcx*-deficient mice.

Introduction

The brains of humans and rodents show enormous differences in size and complexity, which must be explained by differences in their genomes. Evolutionary processes act at the level of gene expression [Pollard et al., 2006] and amino acid composition [Dorus et al., 2004] but also on the spatiotemporal regulation of gene expression [Lindsay and Copp, 2005] and on the production of alternative transcripts from a single gene [Black, 2000]. All these processes have undoubtedly contributed to present-day differences in brain development between human and mouse.

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The *doublecortin* (*DCX*) gene, mutated in X-linked lissencephaly [des Portes et al., 1998; Gleeson et al., 1998], represents an interesting gene in this respect. Expression studies have been performed both in mouse [Francis et al., 1999; Gleeson et al., 1999] and human [Meyer et al., 2002], showing a remarkably conserved expression pattern. During development, DCX/Dcx is expressed in postmitotic migrating neurons and young postmigratory neurons, where it is particularly enriched in neuronal processes. There is, however, an enormous contrast between the brain malformations observed in human lissencephaly [Harding, 1996] and the lack of cortical abnormalities found in *Dcx* mouse knockout (KO) models [Corbo et al., 2002; Kappeler et al., 2006]. Despite the fundamental differences between mouse and human brain development, these data might suggest quite different functions for DCX in the primate brain compared to the mouse. Interestingly though, human and mouse proteins differ by only 3 amino acids, which suggests a very strong conservation of function [des Portes et al., 1998]. It has been shown that in mouse KO models there is a functional compensation for Dcx by the paralogous doublecortin-like kinase 1 protein, Dclk1 [Deuel et al., 2006; Koizumi et al., 2006]. Indeed, Dclk1 [Berke et al., 1998; Omori et al., 1998; Burgess et al., 1999; Matsumoto et al., 1999; Mizuguchi et al., 1999; Silverman et al., 1999; Sossey-Alaoui and Srivastava, 1999; Burgess and Reiner, 2000; Lin et al., 2000; Vreugdenhil et al., 2001; Burgess and Reiner, 2002; Friocourt et al., 2003; Shang et al., 2003; Engels et al., 2004] and a second paralogous protein, Dclk2 [Edelman et al., 2005], are most probably expressed at similar developmental stages to Dcx, and double KOs involving *Dcx* and *Dclk*1 show a severe phenotype, more closely resembling the human disorder than *Dcx* KOs alone [Deuel et al., 2006; Koizumi et al., 2006]. In addition, RNAi experiments for *Dcx* alone produce a severe phenotype [Bai et al., 2003; Ramos et al., 2005], suggesting that such a compensation does not occur when the concentration of Dcx is diminished acutely by RNAi. It remains unclear, however, why a similar compensation cannot occur in humans.

In addition, no studies have yet tested for a changed expression of *Dclk*1 or 2 in *Dcx* KO mice. Another question of interest is why Dclk2, for which the pattern of expression has not previously been well defined, does not compensate for both Dcx and Dclk1 in the double KO. Finally, how has each of these proteins evolved since the divergence of the rodent and primate lineages? We set out to address some of these questions, focusing on the expression patterns of different isoforms of these interesting genes.

Material and Methods

Mouse Dclk*2 Gene Characterization*

A reverse transcription (RT)-PCR product corresponding to the *Dcx* gene was used to screen gridded mouse embryonic 9- and 12-day cDNA libraries (RZPD, Berlin) with low stringency and a *Dclk*2 clone was identified. This clone was sequenced using ABI dye terminator chemistry and shown to be incomplete at the 5′ end and with an insert size of 3.7 kb. Further Dclk2 clones were identified in the sequence databases. For RT-PCR experiments, first-strand cDNA was synthesized using random hexamer priming of total RNA prepared by extraction with guanidine isothiocyanate. RT was performed using Superscript II reverse transcriptase (Gibco BRL) following manufacturer instructions. Twenty-five-cycle PCRs were performed.

Cell and Tissue Extracts

Primary neuronal cultures were prepared from fetal mouse brain at E15 as previously described [Berwald-Netter et al., 1981]. Glial cultures containing >95% astrocytes and devoid of neurons were prepared according to Nowak et al. [1987] from newborn mouse cerebral hemispheres and used as secondary cultures. Cell and tissue extracts were separated on 9–11% SDS-PAGE. Immunodetection was performed with an ECL detection kit (Amersham).

Northern Blot Experiments

Probes for the different *Dclk*1 and *Dclk*2 transcripts were generated by PCR using the following primers [forward (F); reverse (R)]: mouse *DC* transcript, F 5′-atggtttgtgatgggatcaaggt-3′, R 5′-gtctcctacagaagtccctca-3′; mouse *Dclk* transcript, F 5′-ccacgtgtgtgactataaaacac-3′, R 5′-gttccttcatcaatgtggaggg-3′; human *DC* transcript, F 5′-aatgtatatggtttgtgattgggtg-3′, R 5′-cctctaaaaaccaagcaattca-3′; human *DCLK* transcript, F 5′-aacagtggaatgtgaccagtca-3′, R 5′-atgcaccacgtgtaagttagatga-3′. For mouse *Dclk*2 probes a 1.6-kb PCR product was initially amplified using F 5′-gcgacacttcagagtttcgttct-3′ and R 5′-agcccagcggacagacggacggacg-3′. This product was digested with *Xba*I and *Bgl*1 giving rise to an 800-bp probe (forward primer to *Xba*I site) corresponding to exon 16a 3′ UTR sequences and a 600-bp probe (*Bgl*1 to reverse primer) corresponding to part of exon 18. The human *DCLK*2 exon 16a PCR probe (947 bp) used primers: F 5-gaggcacctcagagtttccttc-3′ and R 5′-catggccttggacctagccaga-3′; the human *DCLK*2 exon 18 probe (796 bp) F 5′-aacacggctctagataaagagg-3′ and R 5′-ggaagagctgggggaaaccca-3′; human *DCLK*2 coding region probe (online suppl. fig. 3, www.karger.com/doi/10.1159/000109861) F 5′-ggacatcccgagcgctggctg-3′, R 5′-cctgctttccatccagggtgcagag-3′. Northern blots were purchased from Clontech.

Sequence Databases

Human and mouse gene structures were assessed using UCSC Genome Browser version 141 [http://genome.ucsc.edu, Kent et al., 2002]. Repeat-free PCR probes of 3′ UTR regions for Northern blot analyses and in situ hybridizations were searched with the Web-based BLAT function of Genome Browser and the Web-based blastn program at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using the nonredundant database, to verify their specificities. Mouse sequences of a particular transcript were searched against human genome and mRNA sequences in Genome Browser, the NCBI nonredundant database, and the NCBI est_human database using blastn.

In situ Hybridizations to Mouse Embryonic Sections

For all experiments sense and antisense RNA probes were generated in parallel by T3 and T7 in vitro transcription of DNA sequences cloned in the plasmid pBluescript, in order to assess the specificity of the signal. *Dclk*1: the probe for the *Dclk* transcript represents the expressed sequence tag sequence in the Genbank accession AA116787 and the probe for the *DC* transcript represents 350 bp of the 3′ UTR, from the 3′ *Pvu*II site to the polyA stretch (Genbank accession BC050903). *Dclk*2: the probe detecting both adult and developmental transcripts was generated from a 1-kb *Xba*I-*Sac*II fragment of the 3′ UTR of exon 18 and the probe detecting the *Dclk*2 developmental transcripts (exon 16a) was generated from a 675-bp PCR product (F 5′-gcgacacttcagagtttcgttct-3′ and R 5′-cctctggtatcctttgactaaca-3′). Mouse embryos were either fixed in 2–4% (w/v) paraformaldehyde, cryoprotected with 30% (w/v) sucrose in phosphate buffer and frozen with isopentane before sectioning, or not fixed and frozen directly in isopentane. Hybridization of sections (10–20 μm thick) with α35S-labeled RNA probes was carried out in a 50% (v/v) formamide solution at 60°C. Sections were washed in 50% (v/v) formamide, an RNAse (A and T1) treatment was performed for 30 min at 37°C, and then successively stringent SSC wash solutions were performed, with a final wash at 0.1× SSC at 60°C. In some cases (fig. 6A, F, G), digital autoradiograms were obtained using a Micro Imager [Crumeyrolle-Arias et al., 1996]. In this method detected ionized particle energy is converted into light spots, which are collected using a CCD camera. The counting surface of the Micro Imager was 9 × 13 mm. For the other experiments, both film autoradiography and emulsion (Kodak NTB2) autoradiography were performed. Sections were counterstained in Mayer's hemalum solution, mounted in Eukitt or Vectamount and examined using light microscopy. Images were compiled with Adobe Photoshop.

Mice

Dcx KO mice were generated using the Cre-loxP site-specific recombination system, as described elsewhere [Kappeler et al., 2006]. Dcx is present on the X chromosome and hence male hemizygote mutant mice have no functional Dcx protein [Kappeler et al., 2006]. Mice were analyzed on the C57BL/6N and Sv129Pas backgrounds [Banbury conference, 1997]. No major differences were observed between hemizygote males and homozygote females. Hemizygote males were generated in most cases with wild-type (WT) littermate controls by crossing heterozygote females with pure C57BL/6N or Sv129Pas males (Charles River, France). Mice were genotyped at embryonic stages by PCR following standard methods [Sambrook et al., 1989]. Experiments involving mice were performed by authorized investigators according to national ethical guidelines.

Results

Differences in Human and Mouse Isoforms of Dclk*1 during Development*

Four major *Dclk*1 transcripts have been described in rodents: a long version which contains both a doublecortin-like domain and a kinase domain (referred to here as *Dclk* transcript); a kinase domain only version; a doublecortin-like domain only transcript (referred to here as the *DC* transcript), which shows a remarkably conserved exon-intron structure with *Dcx*; and fourthly a short peptide transcript corresponding to certain exons in the middle of the gene (fig. 1A, UCSC Genome Browser). This latter transcript and the kinase only transcript are upregulated under certain conditions such as kainate treatment [Berke et al., 1998; Silverman et al., 1999; Vreugdenhil et al., 1999]. In addition to these major transcripts, alternative splicing leading to modifications of the kinase domain and the C terminus are known to occur in the kinase only and *Dclk* transcripts [Burgess et al., 1999; Burgess and Reiner, 2002; Vreugdenhil et al., 2001; Shang et al., 2003]. The *Dclk*, *DC* and short peptide transcripts are believed to be expressed both during development and in the adult brain, whereas the kinase only domain is thought to be expressed mainly in the adult [Silverman et al., 1999; Burgess and Reiner, 2002; Engels et al., 2004]. We focused here on the developmental transcripts, potentially expressed in the same cells as Dcx, but in addition shown to have wider temporal and spatial expression patterns [Mizuguchi et al., 1999; Lin et al., 2000; Friocourt et al., 2003; Vreugdenhil et al., 2007; Boekhoorn et al., submitted].

Northern blot analyses were performed using 3′ UTR probes, to allow the specific detection of *Dclk*1 gene transcripts and not the highly similar *Dcx* or *Dclk*2 genes. Probes targeting the common 3′ UTR of the *DC* and short peptide transcripts revealed a probable doublet at approximately 7 kb in mouse embryonic RNA samples (fig. 1B) and a single band of the same size in a human fetal brain sample (fig. 1C). In human sequence databases, 4 cDNAs were identified with the same 3′ UTR sequences, but in each case these clones corresponded to the short peptide variant of this gene and not the *DC* transcript. We were, however, able to amplify a human *DC* transcript by RT-PCR (data not shown). Using *Dclk* transcript 3′ UTR probes, 2 bands (8 and 5.8 kb) were detected in mouse embryonic samples, whereas at least 3 bands (8, 6.5, 5.8 kb) were detected in human fetal brain (fig. 1D, E). The strongest human band (6.5 kb) is thus absent in the mouse embryonic samples. Further examination of mouse and human cDNA sequences in the databases does not shed further light on this primate-specific transcript. Spliced expressed sequence tags corresponding to both α (without alternative exon 19) and β (with alternative exon 19) versions of the *Dclk* transcript [Burgess and Reiner, 2002; Vreugdenhil et al., 2001; Shang et al., 2003] exist in both mouse and human. Kinase only transcripts were not identified in human sequence data-

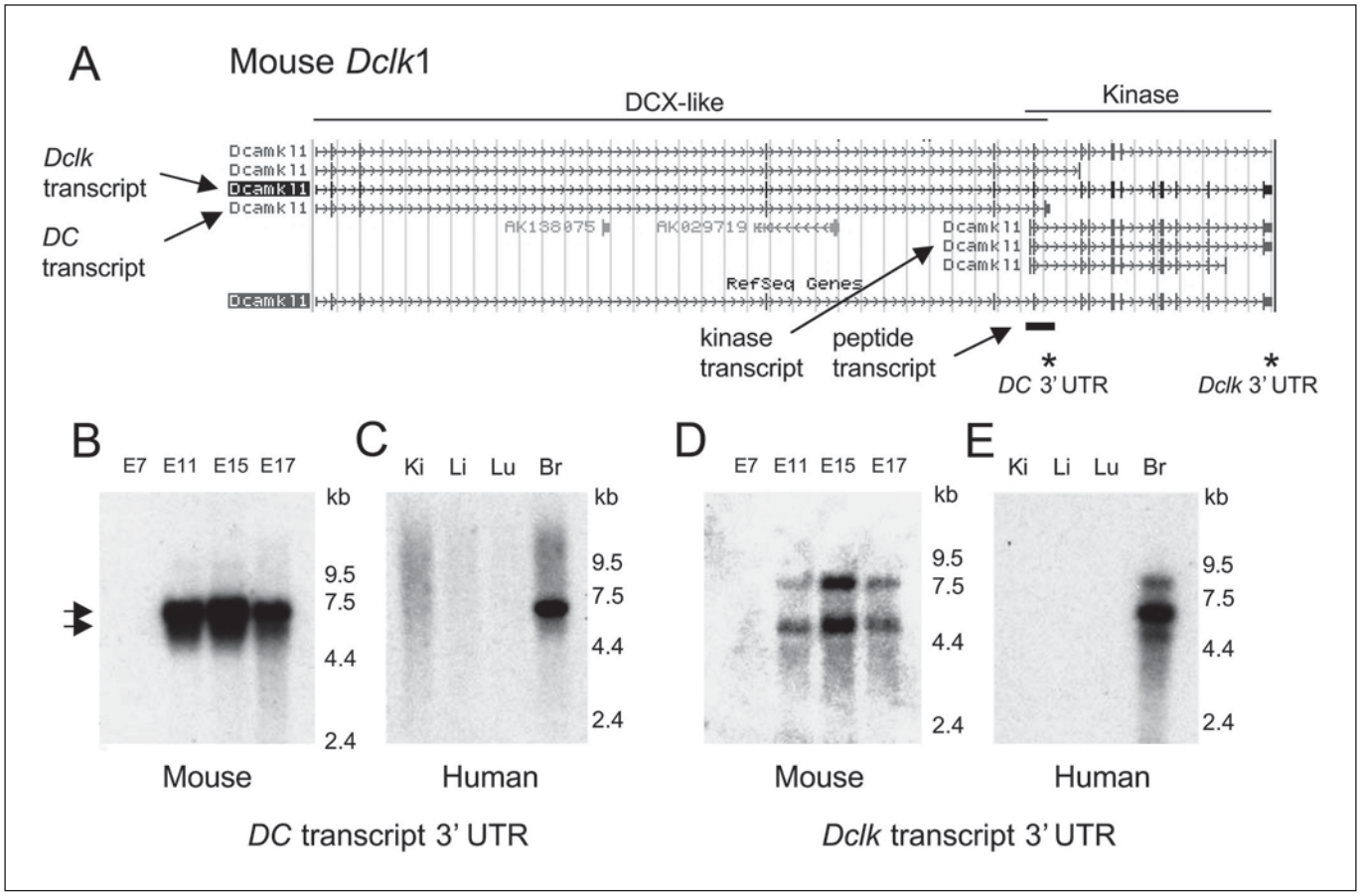


Fig. 1. Differences in human and mouse isoforms of *Dclk*1 during development. **A** Captured image from Genome Browser in the 3qC region of the mouse genome showing the exon-intron structures of the different *Dclk*1 transcripts (also referred to as *Dcamkl*1). Transcripts corresponding to the *Dclk*, *DC* and kinase only domain transcripts are shown, as well as an indication of the exons making up the 55-amino-acid peptide transcript. Asterisks show the position of the *DC* and *Dclk* 3′ UTR probes used in Northern blot experiments. **B–E** Northern blot results of the *Dclk*1 gene for whole mouse embryo and human fetal tissues. **B**, **C** The 3′ UTR probes corresponding to the *DC* and short peptide transcripts reveal 2 similarly sized bands (approximately 7 kb, indicated by arrows) in mouse embryonic RNA samples starting at embryonic day 11 and one 7-kb band in a human fetal brain sample (Br). Human fetal kidney (Ki), liver (Li) and lung (Lu) show no such band. **D**, **E** Two bands are detected in mouse embryonic samples starting at E11 using a *Dclk* transcript 3′ UTR probe, whereas at least 3 bands are detected in human fetal brain. The strongest human band, sized approximately 6.5 kb, seems to be absent in the mouse embryo. kb = Kilobase; E7, E11, E15, E17 = Embryonic day 7, 11, 15 and 17, respectively.

bases. These combined data show certain differences between human and mouse developmental transcripts of *DCLK*1/*Dclk*1, which could be functionally important. It is also noteworthy that all these transcripts begin to be expressed between E7 and E11, as is the case for *Dcx*.

Dclk*1 Expression in* Dcx *KO Mice*

In situ hybridization was performed to test these transcripts in mouse brain sections from *Dcx* KO and WT littermates. cRNA probes corresponding to the 3′ UTRs of the *Dclk*1 transcripts were hybridized to sections at E14.5, E16.5 and E17.5, 3 time points during the period of cortical neuronal migration. No major differences were observed in the KO sections compared to WT. Thus, for the *Dclk* transcript, the distribution and intensity of the signal in the neocortex was relatively equal between KO and WT brains (fig. 2). Similar to *Dcx* [Francis et al., 1999; Gleeson et al., 1999], the signal was strong in subplate and cortical plate (cp), lower in the intermediate zone (iz) and not distinct from the background in the ventricular zone (vz) (fig. 2A–F, J, K; online suppl. fig. 1A, B, www.karger.com/doi/10.1159/

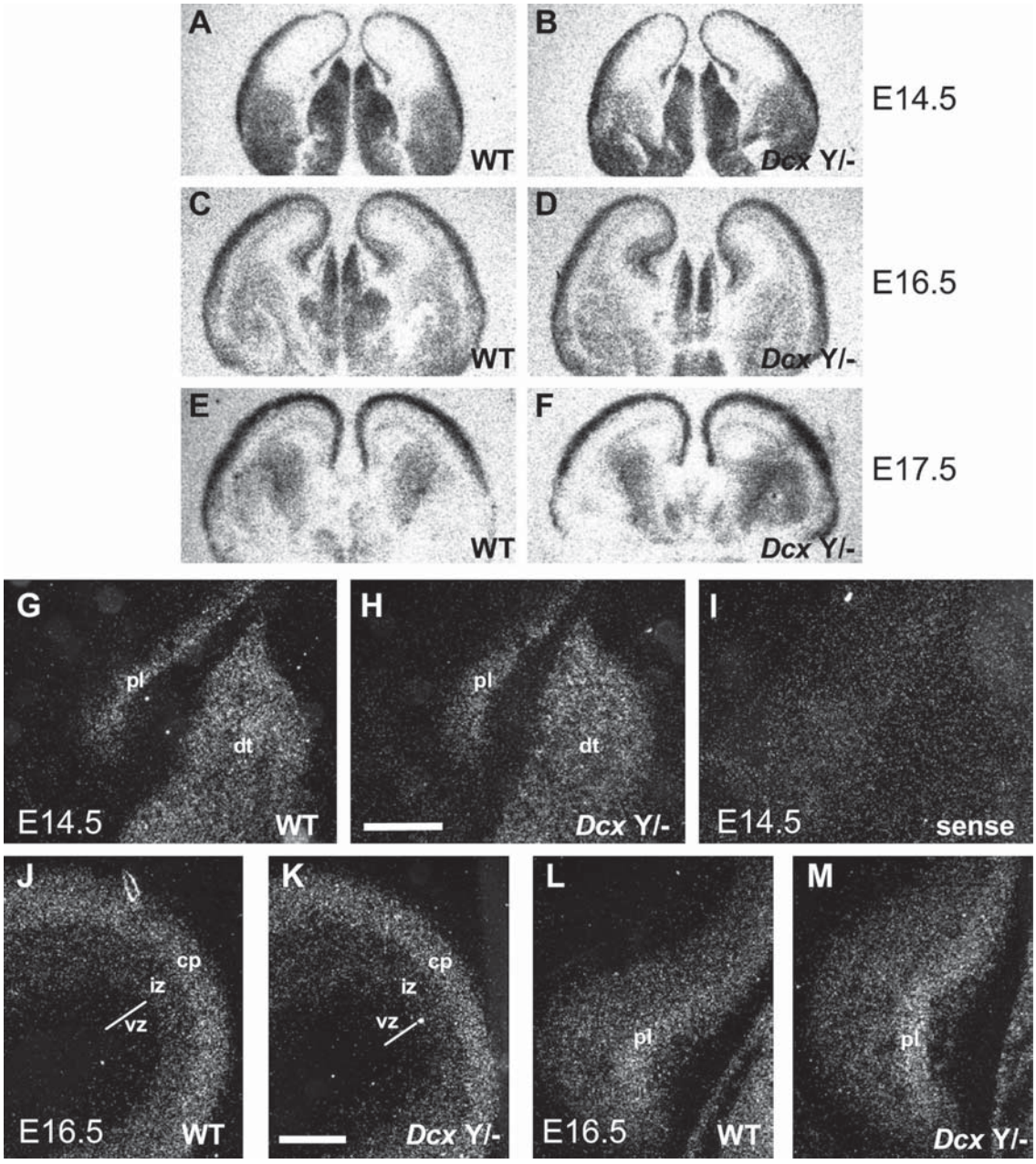


Fig. 2. Expression of the *Dclk*-transcript in *Dcx* KO mice. The *Dclk* transcript was assessed in coronal sections at E14.5, E16.5 and E17.5. No major differences were observed between WT and KO (*Dcx*Y/–). **A–F** Low magnification images after film autoradiography show a similar expression pattern in WT and KO brains. **G–I** Higher magnification images after emulsion development show similar signal intensities in the WT and KO E14.5 developing hippocampus and dorsal thalamus (dt). pl = Pyramidal layer. Very little background signal is observed with the sense probe (I). **J–M** Similarly, at E16.5 (and E17.5, see online suppl. fig 1A–D, www.karger.com/doi/10.1159/000109861), no major differences in signal intensity were found between the WT and KO in the developing cortex (**J**, **K**) and hippocampus (**L**, **M**). The *Dclk* transcript probe detects a strong signal in the cp and a less dense signal in the iz in fitting with a lowered cell density in this zone. Higher magnification shots (not shown), to assess deposited silver granules, showed similar densities in the WT and KO sections. Scale bars, shown in **H** for **G–I** and in **K** for **J–M**, represent 200 μm.

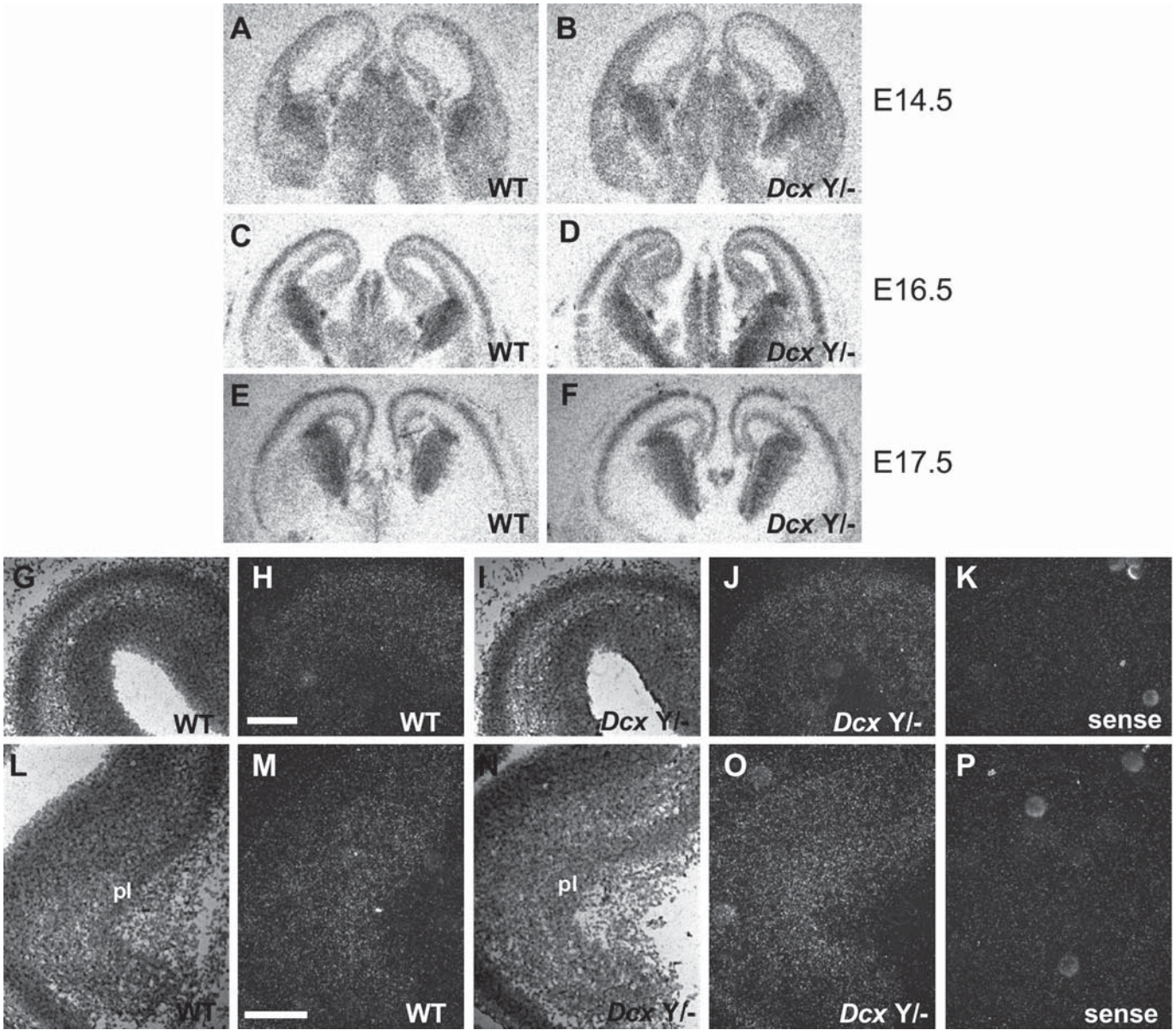


Fig. 3. Expression of the *DC* transcript in *Dcx* KO mice. The *DC* transcript was assessed in coronal sections at E14.5, E16.5 and E17.5. No major differences in pattern or intensity of expression were observed between WT and KO (*Dcx*Y/–). **A–F** Low magnification images of film autoradiography. After emulsion autoradiography, the dorsal cortex (**G–J**) and the developing hippocampus (**L–O**) appear similarly labeled at E16.5 in WT (**G**, **H**, **L**, **M**) and KO (**I**, **J**, **N**, **O**) brains. Light field images of the sections stained with Mayer's hemalum solution (**G**, **I**, **L**, **N**) and dark field images (**H**, **J**, **M**, **O**) are shown. An adjacent WT section hybridized with the sense probe is shown for the neocortex (**K**) and the hippocampus (**P**). Slightly elevated signal intensities were observed in the KO sections at this age, although this was not the case at either E14.5 (data not shown) or at E17.5, as judged by in situ hybridization (online suppl. fig. 1G–J, www.karger.com/doi/10.1159/000109861) and Northern blot results (data not shown). Scale bars, shown in **H** for **G–K** and in **M** for **L–P**, represent 200 μm.

000109861). Similarly, in the hippocampus, intense labeling was observed in the developing pyramidal cell layer, including the CA3 region in both WT and KO sections (fig. 2G–I, L, M; online suppl. fig. 1C, D, www.karger.com/doi/10.1159/000109861). In E17.5 KO sections, a heterotopic pyramidal layer can be seen in the CA3 region in addition to the normal pyramidal layer [Kappeler et al., 2007], and both of these are labeled with

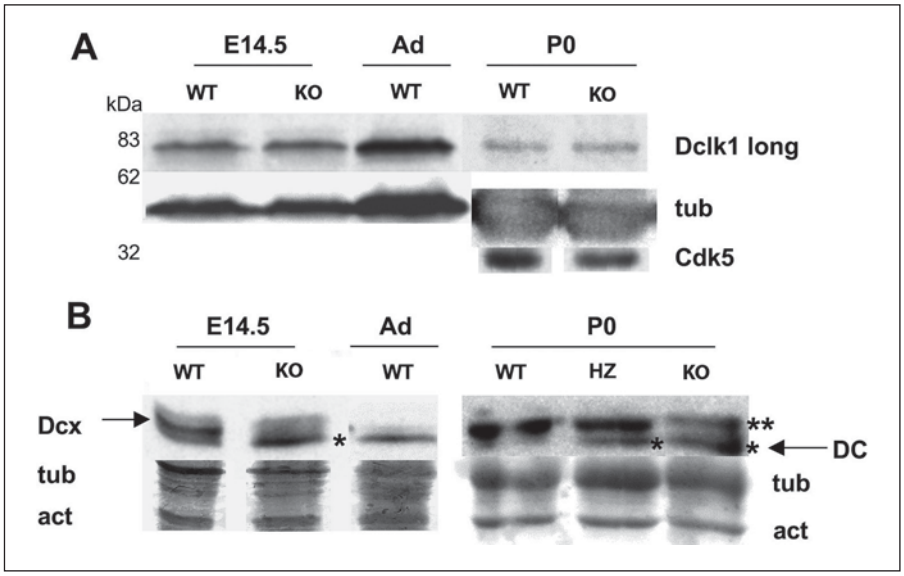


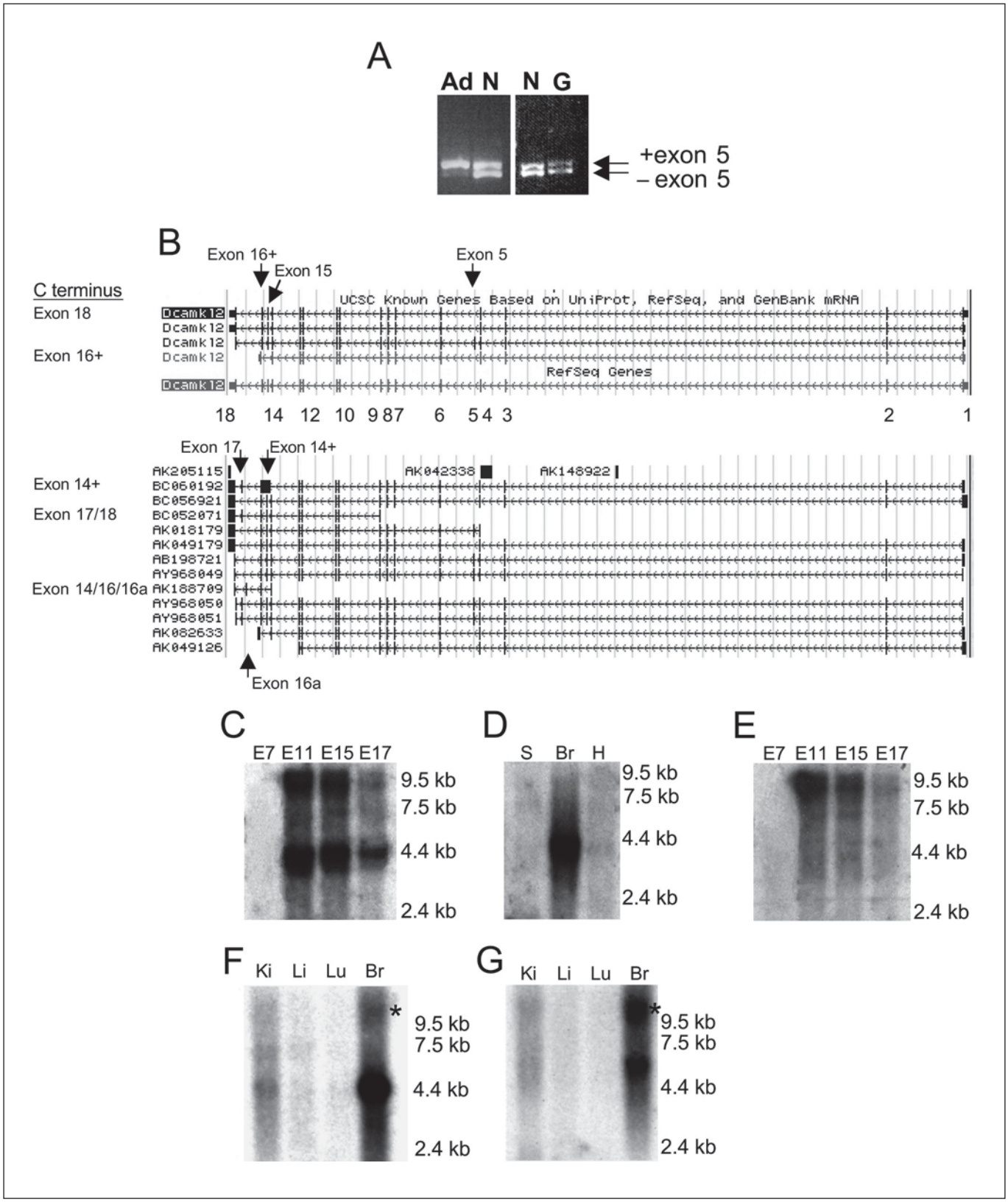
Fig. 4. Dclk1 protein expression in *Dcx* KO mice. Western blot experiments were performed using anti-Nter (**A**, left) and anti-CAMKLK1 antibodies (**A**, right and **B**, left and right) against E14.5 total embryo extracts and P0 neonatal brain extracts. **A** No major differences were detected in the amounts of the 80-kDa long Dclk isoform in WT versus KO mice. Extracts from WT adult mouse brain (Ad) are shown for comparison. Cdk5 and/or tubulin (tub) were used to control the quantities of protein loaded in each lane. **B** The 40-kDa DC isoform closely resembles Dcx but has a slightly lower molecular weight [Friocourt et al., 2003], as shown here by comparison with an adult brain extract (Ad), where Dcx, strictly developmentally regulated, is not detected. Thus, the prominent higher molecular weight band (45 kDa) observed in the WT (first lane) and absent in the KO (second lane) embryonic extracts probably represents cross-reacting Dcx. Alternatively, this upper band could be a phosphorylated form of the DC isoform, downregulated in KO mice. In contrast, the lower 40-kDa DC isoform (indicated by single asterisks) is increased in mutated animals, both at E14.5 and P0 stages. At P0 increased quantities of a second higher molecular weight band (indicated by a double asterisk), perhaps a phosphorylated version of the DC isoform, were also observed in heterozygote and KO extracts. Using anti-Nter Dclk1 antibodies, a similar, minor increase of the DC isoform occurred in KO extracts at E14.5 (online suppl. fig. 2, www.karger.com/doi/10.1159/000109861). These data could therefore be in fitting with a compensatory change of the DC isoform in *Dcx* KO mice.

the *Dclk* transcript (online suppl. fig. 1E, F, www.karger.com/doi/10.1159/000109861).

In WT sections, the *DC* transcript probe revealed a distinct expression pattern compared to the *Dclk* transcript (fig. 3). In the neocortex, a similar, although less intense signal was observed in the cp, however, the vz was also labeled with an intensity comparable to the cp (fig. 3A–F, G–K) [Boekhoorn et al., submitted]. In addition, labeling of the ganglionic eminences appeared relatively more intense compared to the neocortical labeling, whereas the inverse is true for the *Dclk* transcript probe. Indeed, the *DC* transcript signal becomes more regionalized during the course of embryonic development (fig. 3A–F). Comparing WT and KO sections at E14.5, E16.5 and E17.5, no major differences in distribution or intensity of the signal were observed (fig. 3; online suppl. fig. 1G–J, www.karger.com/doi/10.1159/000109861). In the hippocampus, both the vz and the pyramidal layer were labeled (fig. 3L–P) and in E17.5 KO sections, both CA3 pyramidal layers were labeled (online suppl. fig. 1K, L, www.karger.com/doi/10.1159/000109861).

Dclk1 Protein Expression in Dcx *KO Mice*

A number of antibodies exist for Dclk1, targeted against different regions of the protein, although in some cases it is difficult to distinguish between reactivity of Dclk1 and Dcx. We therefore tested 2 different antibodies in WT and KO extracts: anti-Nter Dclk1 antibodies directed against a fusion protein containing Dclk1 N-terminal amino acids 14–108 [Friocourt et al., 2003], expected to recognize Dclk1 isoforms containing the microtubule-bindingdomain;andanti-CaMKLKantibodies directed against a 55-amino-acid peptide corresponding to the short peptide transcript exons in the middle of the gene and expected to recognize all isoforms of Dclk1 [Kruidering et al., 2001]. In Western blot analyses, each



antibody was tested against E14.5 total embryo extracts and neonatal (P0) brain extracts.

No major quantitative differences were detected for the long Dclk isoform (80 kDa) in WT versus KO mice at either developmental age (fig. 4A). The DC isoform (40 kDa), which closely resembles Dcx (45 kDa), was more difficult to assess due to a cross-reaction between these 2 proteins by both antibodies, and the fact that both proteins are phosphorylated, giving rise to multiple bands [Francis et al., 1999; Friocourt et al., 2003]. The DC isoform could, however, be clearly recognized in both KO embryonic and WT adult brain extracts, where Dcx is obviously absent. In WT E14.5 extracts (fig. 4B, first lane), the upper 45-kDa band therefore probably represents cross-reacting Dcx. Interestingly, the 40-kDa DC isoform appears increased in KO E14.5 extracts (fig. 4B, first and second lanes; online suppl. fig. 2, www.karger.com/doi/10.1159/000109861). In P0 WT extracts, and to a lesser extent in heterozygote extracts, Dcx is still present. In heterozygote and KO extracts an increase in the 40-kDa DC isoform is again detectable along with an additional higher molecular weight band, which may represent a phosphorylated form of the DC isoform. These data are therefore in fitting with a compensatory translational or posttranslational change in expression of the DC isoform in *Dcx* KO mice.

Differential Expression of Dclk*2 Transcripts*

The *Dclk*2 gene is less well characterized than *Dclk*1, nevertheless transcripts have been identified containing both a microtubule-binding domain and a kinase domain, similar thus to *Dclk*1 [Edelman et al., 2005]. Previous Northern blot analyses principally revealed an approximately 4.5-kb murine transcript, present during mouse development and in the adult [Edelman et al., 2005]. The adult rat cDNA sequence of *Dclk*2 originally reported was composed of 18 exons, 2 of which were shown to be alternatively spliced [exons 5 and 17, Edelman et al., 2005]. The inclusion of exon 17 gives rise to a different C terminus. We were interested in further assessing possible developmentally regulated transcripts of this gene.

Edelman et al. [2005] suggested that exon-5-containing transcripts were more prevalent than exon-5-lacking transcripts in rat adult brain samples. To further test this, we performed RT-PCR experiments using cDNA derived from primary cultures of mouse neurons grown for 3 days in vitro (N, 3 DIV), therefore representing an enriched population of immature neurons compared to adult brain RNA. Using oligonucleotides in exons 4 and 6, we were able to predominantly amplify the exon-5-containing transcript from the adult brain sample, with only minor quantities of the exon-5-lacking transcript (fig. 5A), in accordance with the results obtained by Edelman et al. [2005]. On the other hand, approximately equal quantities of both exon-5-containing and exon-5-lacking transcripts could be amplified from the N, 3 DIV sample, and this was also the case in cDNA derived from proliferating glial cells in culture (fig. 5A). These combined data suggest that exon-5-lacking transcripts are present in proliferating as well as postmitotic cells and are more likely to be present in immature than mature neurons.

Fig. 5. Alternative splicing of mouse *Dclk*2. **A** RT-PCR bands are shown for the exon-5-containing and exon-5-lacking *Dclk*2 transcripts. Initially, mouse adult brain cDNA (Ad) was compared with cDNA derived from young developing neurons in culture (N, grown for 3 DIV). Only minor quantities of the exon-5-lacking transcript were observed in the adult brain cDNA sample. The neuronal cDNA (N) was also compared to glial cell cDNA (G) and no major differences were observed in the relative intensities of the exon-5-containing and exon-5-lacking products. **B** A captured image from Genome Browser shows the mouse 3qF1 region containing *Dclk*2. Alternative exons are indicated in the mRNA sequences (upper) or expressed sequence tag sequences (lower). **C–G** Northern blot analyses were performed for mouse and human tissues with probes derived from exon 18 (**C**, **D**, **F**) and exon 16a (**E**, **G**). Blots represent mouse embryonic (**C**, **E**) or adult (**D**) tissues and human fetal tissues (**F**, **G**). The mouse exon 18 probe showed a 4.4-kb band and 2 fainter, similarly-sized bands of approximately 9.5 kb in E11, E15 and E17 samples (**C**). No expression was observed at E7. Alternative splicing of exon 15 coding for 27 amino acids may explain the doublet at 9.5 kb. In the adult mouse multiple tissue Northern blot, only the 4.4-kb band was observed, most predominantly in brain, and also at a very low level in heart tissue (**D** and data not shown). **E** A mouse exon 16a probe identified only the 9.5-kb transcripts in E11, E15 and E17 samples and thus exon-16a-containing transcripts are predominantly developmentally regulated. **F** Using a human exon 18 probe, we similarly identified a strong 4.4-kb band and weaker >9.5-kb band (asterisk) predominantly in fetal brain and to a lesser extent in fetal kidney. This same probe identified a 4.4-kb band in adult heart (data not shown) and in different human adult brain structures (online suppl. fig. 3, www.karger.com/doi/10.1159/000109861). **G** A human exon 16a probe identified intense >9.5-kb (asterisk) and 6.1-kb bands in the human fetal brain sample, with faint bands of the same size observed in the human fetal kidney sample. kb = Kilobase; E7, E11, E15, E17 = embryonic day 7, 11, 15 and 17, respectively; Lu = lung; Li = liver; S = spleen; Br = brain; H = heart.

Exon 5 codes for 16 amino acids, including 4 serine and 1 threonine residue, which may be a target for phosphorylation, involved in the regulation of microtubule binding [Edelman et al., 2005].

We identified a mouse *Dclk*2 cDNA from an E12 library, not containing exon 5 and differing from the adult rat cDNA previously described, since it contained an additional long exon between exons 16 and 17, which we have termed exon 16a. This exon contained an in-frame stop codon, followed by intronic sequences and exons 17 and 18, both therefore comprising 3′ UTR sequences in this transcript. With such a spliced 3′ UTR, we believe that this transcript is likely to be subjected to nonsense-mediated mRNA decay [Nagy and Maquat, 1998]. Further analysis of mouse cDNAs and expressed sequence tags in UCSC Genome Browser (fig. 5B) showed 3 additional alternative transcripts: one derived from a mouse embryonic brain cDNA library with an extended version of exon 14 (exon 14+, BC060192), also likely to be subjected to nonsense-mediated mRNA decay; the second derived from a neonatal cerebellar cDNA library, with an extended version of the original exon 16 as its last exon (exon 16+, AK082633), likely to give rise to a 708-amino acid protein; and the third, an incomplete sequence encompassing exons 14, 16 and a short version of exon 16a (AK188709), suggesting that exon 15 may also be alternatively spliced in some cases. Thus, several different 3′ ends are known for this gene and certain of these variations may modulate the kinase function of this protein.

We further investigated *Dclk*2 transcripts by Northern blot analyses, using initially a PCR probe corresponding to part of mouse exon 18, present in a proportion of embryonic and adult cDNA sequences. At least 3 different mouse transcripts were identified, including a 4.4-kb band, in close agreement with the 4.5-kb transcript previously reported [Edelman et al., 2005], and 2 fainter bands of approximately 9.5 kb (fig. 5C, D). These 3 bands were observed at E11, E15 and E17 but not at E7 (fig. 5C). Thus, like *Dcx* and *Dclk*1 [Francis et al., 1999; Gleeson et al., 1999; Sossey-Alaoui and Srivastava, 1999], expression of *Dclk*2 begins between E7 and E11. In the adult multiple tissue Northern blot, only the 4.4-kb band was found, most predominantly in brain, with very low level expression also observed in heart tissue (fig. 5D). Thus, it appears that the 9.5-kb transcripts are developmentally regulated. We further hybridized a PCR product corresponding to exon 16a, which identified only the 9.5-kb transcripts specifically in the embryonic samples (E11, E15 and E17, fig. 5E and data not shown). These data therefore imply that exon-16a-containing transcripts are predominantly developmentally regulated.

DCLK*2 Expression in Human Brain*

In human sequence databases, we were only able to identify 2 of the transcripts observed in the mouse. These were the exon 18 long transcript described by Edelman et al. [2005] and transcripts containing a short version of exon 16a (online suppl. fig. 3A, www.karger.com/doi/10.1159/000109861). By Northern blot analyses using a human probe corresponding to exon 18, we identified a 4.4-kb band present predominantly in human fetal brain, to a much lesser extent in human fetal kidney (fig. 5F), and in adult heart and brain samples (data not shown). Further analyses of different brain structures showed the presence of this transcript throughout the brain (online suppl. fig. 3B, www.karger.com/doi/10.1159/000109861). A faint band of >9.5 kb was also detected in human fetal brain with this probe (fig. 5F). The human exon 16a probe identified intense bands of >9.5 kb and 6.1 kb in the human fetal brain sample with faint bands of the same size detectable in human fetal kidney (fig. 5G). These transcripts were not detected in an adult brain sample (data not shown), suggesting that they are also developmentally regulated. Thus, *Dclk*2, like *Dclk*1, shows certain differences in the use of transcripts between mouse and human, but on the whole, remarkably similar results are obtained in each organism.

Dclk*2 Expression in the Developing Brain of WT and* Dcx *KO Mice*

*Dclk*2 expression in the developing mouse brain has never previously been characterized. We therefore performed several preliminary experiments to assess the expression profile of this gene. Using a probe encompassing part of exon 18, similar to that used in Northern blot experiments, and expected therefore to detect developmental as well as adult forms, we initially checked expression patterns by in situ hybridization at E14.5 and newborn stages (fig. 6). At E14.5, a strong, predominant labeling of *Dclk*2 throughout the central nervous system was observed in medial sagittal sections (fig. 6A). This widespread labeling of the developing central nervous system is very reminiscent of both *Dcx* [Francis et al., 1999] and *Dclk*1 [Reiner et al., 2006] expression patterns. For *Dclk*2, the strongest labeling was observed in the ganglionic eminences, the olfactory lobe, the dorsal telencephalon, the thalamus and the roof of the midbrain (fig. 6A). Looking more closely in coronal brain sections at this age by emul-

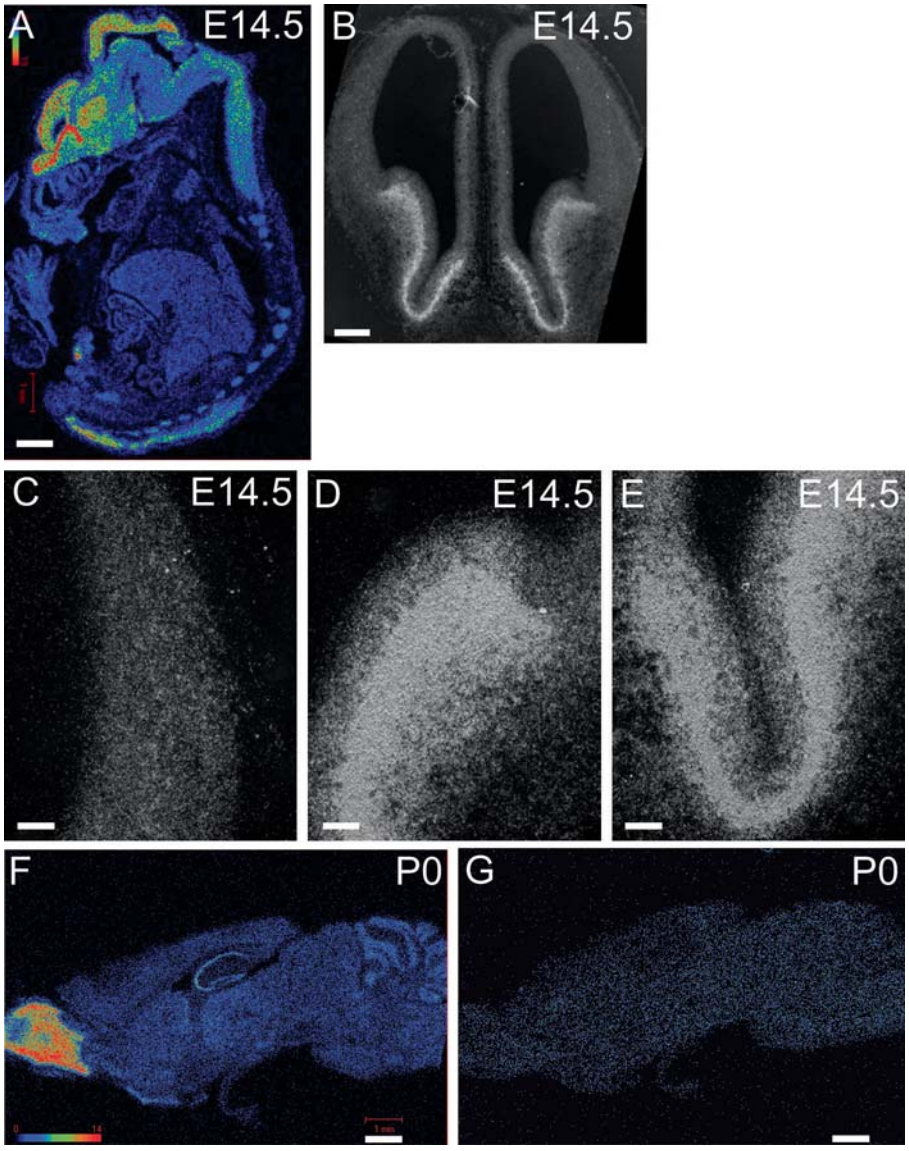


Fig. 6. Expression pattern of *Dclk*2 in the developing brain. A cRNA probe from exon 18 was hybridized to E14.5 and P0 mouse embryo or brain sections. Slides were vizualized using a Micro Imager [Crumeyrolle-Arias et al., 1996] (**A**, **F**, **G**) or, after emulsion development, by dark field exposure on a standard light microscope (**B–E**). **A** In a medial sagittal section at E14.5, labeling is observed throughout the developing nervous system, in particular in the dorsal telencephalon, the roof of the midbrain, the ganglionic eminences, the olfactory lobe and the thalamus. **B** An E14.5 coronal brain section shows an intense labeling in the ventral part of the telencephalon, including the ganglionic eminences, anterior entopeduncular region and the preoptic area. Labeling is also observed throughout the dorsal telencephalon. **C**, **D**, **E** Higher magnification shots of the section presented in **B** show the lateral wall of the developing cortex (**C**), the medial ganglionic eminence (**D**) and the anterior entopeduncular region (**E**) at E14.5. **C** At this stage, deposited silver grains are present homogeneously in the vz, iz and cp of the developing cortex. **D**, **E** Deposited silver grains are present throughout each structure, although with a higher density in the subventricular zone and iz. **F** Sagittal section from a neonatal mouse brain. A weak signal was detected throughout the brain, although the cerebellum, hippocampus and olfactory bulb show a higher intensity. **G** A sense probe was hybridized to an adjacent section, showing no background labeling with this probe. Scale bars: 1 mm (**A**, **F**, **G**), 300 μm (**B**), 100 μm (**C**, **D**, **E**).

sion autoradiography, homogeneous labeling was observed throughout the developing cortex in vz, iz, cp and marginal zone regions (fig. 6B, C). However, the strongest labeling occurred in the developing ganglionic eminences, and the anterior entopeduncular and anterior preoptic areas (fig. 6D, E). In these 3 regions there was a marked difference between the weak labeling in the vz, and the intense labeling in the subventricular zone and iz. In neonatal sagittal brain sections, the strongest labeling was observed in the olfactory bulb and optic chiasm, then in the hippocampal pyramidal layer and the dentate gyrus, and in the cerebellum (fig. 6F). A weak labeling was observed throughout the rest of the brain. These combined data suggest that *Dclk*2 transcripts display a wider expression pattern than *Dcx*, being detected in immature migrating and differentiating neurons during development, and also in non-*Dcx*-expressing cells of the proliferative cortical vz. Compared to *Dclk*1, and in particular to the similar long *Dclk*-transcript, the major difference also appears to be the expression of *Dclk*2 in the cortical vz.

We then tested the *Dclk*2 transcripts in *Dcx* KO mouse sections at 3 developmental ages, E14.5, E16.5 and E17.5. We used both exon 18 and 16a probes, which showed similar expression patterns, although a higher signal intensity was observed with the exon 18 probe (fig. 7;

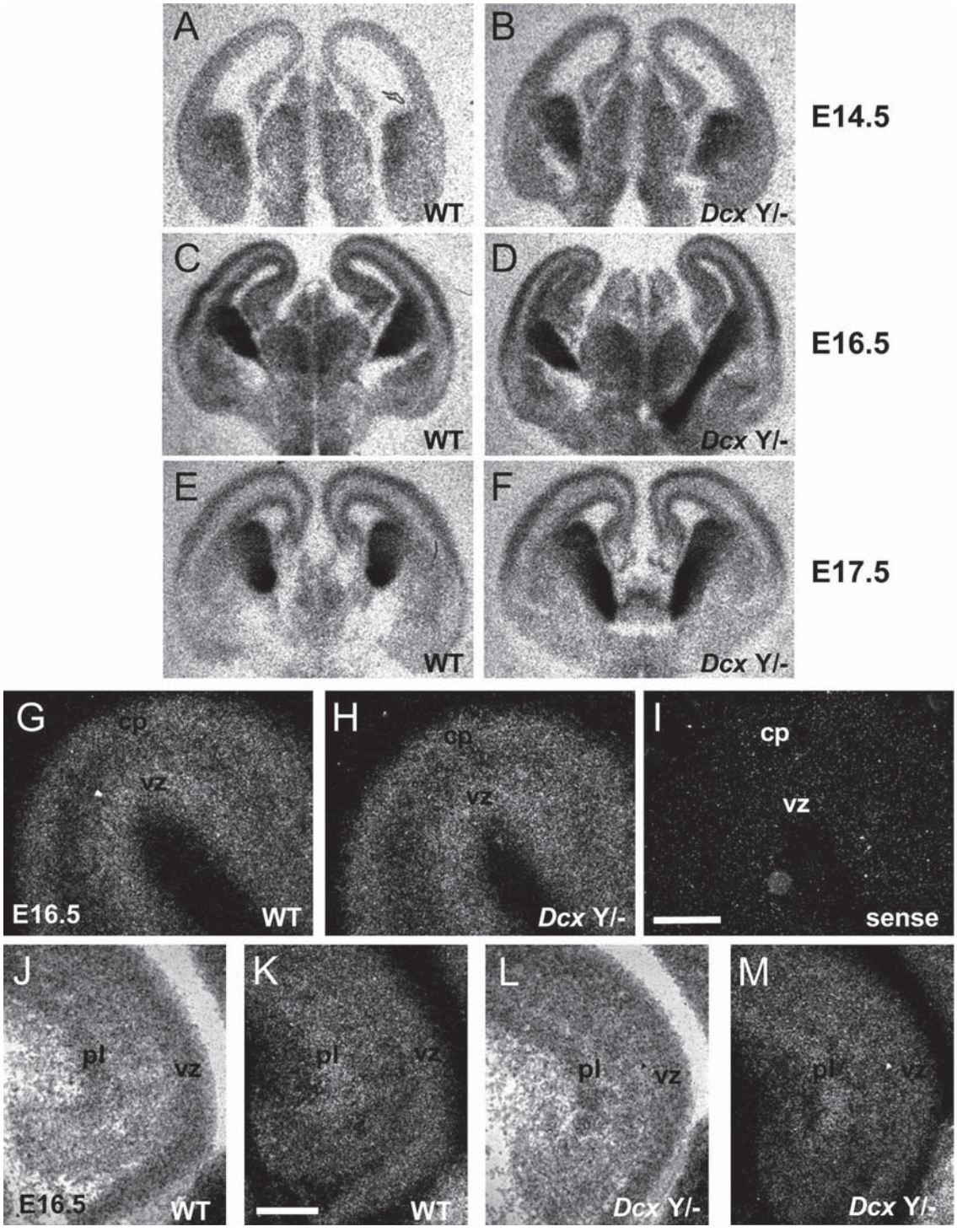


Fig. 7. Expression of the *Dclk2* exon 18 transcript in *Dcx* KO mice. A cRNA probe from exon 18 was hybridized to E14.5, E16.5 and E17.5 WT and KO (*Dcx* Y/–) embryos. **A–F** Film autoradiography showing a similar expression pattern between WT and KO brains. Differences in signal intensity were observed in E14.5 WT and KO sections, however, no similar differences occurred in the later embryonic stages tested, nor in E14.5 protein extracts (fig. 8), suggesting an artifact. The ventral parts of the sections shown in **D** and **F** differ from those shown in **C** and **E**. **G–I** Emulsion autoradiography images of the dorsal cortex are shown for E16.5 WT (**G**) and KO (**H**) brain sections (**I**, hybridization to the sense probe). Strong signal is observed in the vz and the cp in both WT and KO sections. Similar results are observed at E17.5 (online suppl. fig 5A–C, www.karger.com/doi/10.1159/000109861). **J–M** The devel-

online suppl. fig. 4, 5, www.karger.com/doi/10.1159/000109861). Comparing WT and *Dcx* KO sections with this latter probe, slight differences in signal intensity were observed in the E14.5 sections (fig. 7A, B), however, no major differences occurred at the protein level (see below) or at later stages (fig. 7C–M; online suppl. fig. 5A–C, www.karger.com/doi/10.1159/000109861), suggesting this to be an artifact. As for the *Dclk*1 transcripts, both *Dclk*2 probes showed a strong expression throughout the developing hippocampal pyramidal cell layer (fig. 7J–M), and in KO sections at E17.5, the heterotopic CA3 pyramidal layer was also labeled (online suppl. fig. 5D–G, www.karger.com/doi/10.1159/000109861).

Dclk2 Protein Expression in Dcx *KO Mice*

Anti-Dclk2 antibodies directed against the coding portion of exon 18 have been previously described [Edelman et al., 2005]. We used these antibodies to compare Dclk2 in E14.5 WT and KO mouse embryonic extracts. As shown in figure 8, no major differences were observed, a single band of approximately 80 kDa being detected with equal intensity in each lane. These combined results suggest that there are no convincing differences in *Dclk*2 expression at either the RNA or protein level in *Dcx* KO mice.

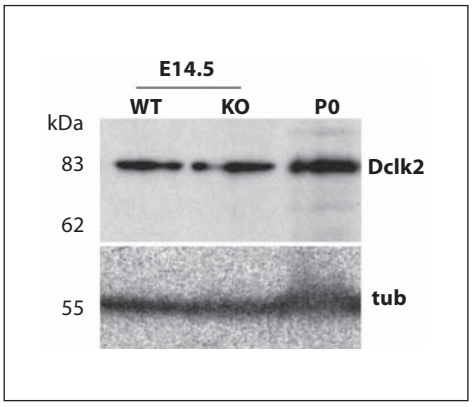


Fig. 8. Dclk2 protein expression in *Dcx* KO mice. Anti-Dclk2 antibodies were used in Western blot analyses against WT and KO E14.5 protein extracts. No major differences in band intensity were observed between WT and KO embryos. Anti-α tubulin (tub) was used as a control.

Discussion

In this study we have attempted to better characterize the murine *Dclk*1 and *Dclk*2 transcripts, especially those present during brain development, and to identify their human counterparts. The increasing redundancy of data in the sequence databases helps make such a study possible, in particular allowing a detailed comparison between predominant mouse and human transcripts. Combining these results with Northern blot analyses and in situ hybridization experiments using specific and equivalent 3′ UTR probes, we have been able to specifically distinguish expression patterns for the different *Dclk*1 and 2 transcripts. Interestingly, each gene has a wider expression pattern than *Dcx*, with certain transcripts expressed in the embryonic vz, in glial cells and in mature neurons in the adult. We searched for visible differences in the distribution or intensity of Dclk1 and 2 developmental isoforms in *Dcx* KO mice, although in most cases, the expression was equal. Potential differences at the protein level were, however, found for the DC isoform of Dclk1.

The *Dclk*1 gene displays a higher complexity of alternative splicing than the *Dclk*2 gene, since the major *Dclk*1 transcripts show dramatic differences in their protein domain composition. Thus, DC microtubule-binding domain only [Friocourt et al., 2003] and kinase domain only [Silverman et al., 1999; Burgess and Reiner, 2002] versions exist, which does not seem to be the case for Dclk2. Additional, less dramatic alternative splicing occurs for both genes, leading most probably to a modulation of microtubule-binding and/or kinase activities [Burgess and Reiner, 2002; Shang et al., 2003; Engels et al., 2004; Edelman et al., 2005]. Both *Dclk* 1 and 2 show more alternative splicing than *Dcx*, which always retains the same complement of coding exons [des Portes et al., 1998]. A point of interest for the *Dclk*1 gene is that different patterns of expression are observed for the different transcripts analyzed. Thus, the *DC* transcript probe shows as much expression in the vz as in the cp, whereas the *Dclk* transcript is not detectable in the vz. The *DC* transcript probe also shows higher levels of expression in the ganglionic eminences than the *Dclk* transcript probe. Thus, it is very

oping hippocampus is shown at E16.5 in WT (**J**, **K**) and KO (**L**, **M**) brains. Light field (**J**, **L**) and dark field (**K**, **M**) images of Mayer's hemalum-stained, autoradiographic emulsion-coated sections are presented. The labeling pattern in the hippocampus is similar to that of the neocortex, since a strong intensity is observed in the pyramidal cell layer (pl) as well as in the vz. No major differences were noted between WT and KO sections. Scale bars, shown in **I** for **G–I** and in **K** for **J–M**, represent 200 μm.

likely that different functions have evolved for these different *Dclk*1 alternative transcripts in the mouse genome.

For the *Dclk*2 gene, common human and mouse transcripts show alternative splicing of exons 5 and 16a. Exon-16a-containing transcripts seem to be developmentally regulated, since 9.5-kb Northern blot bands were observed in embryonic and fetal samples but no similar bands were identified in either mouse or human adult brain RNA. In addition, human exon 16a transcripts lack exon 5. The exon 16a mouse in situ hybridization pattern on the other hand closely resembles that of exon 18 and both transcripts therefore show a less restricted expression than the similar *Dclk*1 long *Dclk* transcript. The conservation of exon-16a-containing transcripts is intriguing, since this stop-codon-containing exon is followed by exon 18, implying that both human and mouse transcripts are likely candidates for nonsense-mediated mRNA decay. Only the continued study of these genes will shed light on such a conservation.

Our in situ hybridization studies confirmed a coexpression of all *Dclk*1 and 2 transcripts analyzed in the same cells as *Dcx*, including postmitotic neurons in the iz and cp regions. No changes at the RNA level were identified in the *Dcx* KO for these transcripts, neither in their distribution nor their quantity. Similarly, at the protein level no quantitative differences were observed for the long isoform of Dclk1, nor the exon-18-containing isoform of Dclk2. We attempted to generate antibodies in a different region of the Dclk2 protein in order to test alternative isoforms, however, these antibodies are likely to cross-react with additional kinase domain proteins, making the results difficult to interpret (data not shown). Similar problems of cross-reaction were encountered for the 2 anti-Dclk1 antibodies tested [Kruidering et al., 2001; Friocourt et al., 2003], when attempting to identify the Dclk1 DC isoform. Indeed, this protein migrates on SDS-PAGE at a similar level to Dcx, and both proteins have a highly similar conformation [Kim et al., 2003]. However, a comparison with mouse adult brain protein extracts, where Dcx is not highly expressed, allowed us to specifically identify the Dclk1 DC isoform and to show possible elevated quantities of this protein in KO extracts compared to WT. Comparisons with further antibodies in the DC domain are necessary to confirm the changed expression of this Dclk1 isoform.

It is, however, tempting to speculate that an increased quantity of the DC isoform is in part necessary for the compensation observed in the KO mouse models. On the other hand, it is unlikely that this would be the only compensatory mechanism. Indeed the 2 double KOs of *Dclk*1 and *Dcx* giving rise to a severe neocortical phenotype targeted different regions of the *Dclk*1 gene. Koizumi et al. [2006] targeted the DC domain, however, Deuel et al. [2006] targeted the kinase domain. In this latter case an approximately 40-kDa Dclk1 protein was still identified in *Dclk*1–/– KO mice, likely to correspond to an intact DC isoform. Thus, in the double mutant mice, this protein is apparently not enough to compensate for the lack of Dcx and the Dclk1 long isoform. Further work is therefore required to assess the cooperation between the *Dclk*1 gene products and to better understand the proposed calpain cleavage of the full-length Dclk1 protein, shown under certain conditions to give rise to an equivalent DC isoform by protease digestion [Burgess and Reiner, 2001; Deuel et al., 2006]. It also remains probable that, even in the absence of visible changes in expression at the RNA or protein level, a more subtle subcellular functional compensation by Dclk1 or indeed other proteins could anyway occur in *Dcx* KO neocortical cells. Further functional studies are required to confirm this.

We also analyzed the hippocampal expression of the *Dclk*1 and 2 transcripts, since severe radial migration abnormalities are observed in CA3 pyramidal cells in the *Dcx* KO [Kappeler et al., 2007]. We therefore suspected that certain of the transcripts may not be expressed in migrating CA3 pyramidal cells, preventing a compensation. However, all the transcripts analyzed in this study were expressed throughout the CA pyramidal layer. Furthermore, these transcripts were also expressed in the KO heterotopic CA3 pyramidal cell layer. Other studies have, however, revealed that certain *Dclk*1 transcripts are not apparently expressed in at least the adult hippocampal CA3 region [Vreugdenhil et al., 2001]. Specifically studying these transcripts during development may therefore shed further light on the *Dcx* mouse KO phenotype.

In this study we have therefore attempted to better understand the differences between human and mouse *DCX*/*Dcx*-deficient phenotypes, focusing on the expression of the *Dclk1 and 2* genes in comparison with *Dcx*. These studies contribute to our understanding of this important issue. However, the question still remains as to why no human protein can replace DCX in cases of type I lissencephaly. Further expression studies in humans may help answer this question.

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