Microtubule associated proteins and plasticity in the developing and diseased brain

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Summary and General discussion
Summary

Neurons often have a complex dendritic tree and an extensive axonal organization, which requires specific properties from the neuronal cytoskeleton to enable structural maintenance and stability. In contrast to the rather rigid nature of postmitotic neurons, during various dynamic processes like neuronal birth, migration and growth, a flexible and adaptive cytoskeleton is required. These reorganizations of the cytoskeleton of immature neurons are regulated by binding of a.o. microtubule associated proteins (MAPs) to cytoskeletal elements. However, also plastic changes in mature neurons, like alterations in synaptic connectivity, might be regulated by MAPs.

MAPs are an important class of structural proteins characterized by the presence of one or more microtubule (MT) binding sequence(s). Upon MAP binding, MTs are stabilized and tubulin polymerization is enhanced. The affinity of a MAP for MTs is further regulated through alternative splicing and phosphorylation. Many MAPs are exclusively expressed in the nervous system where they are involved in growth as well as structural stability of neurons. Examples include tau, but also novel members, like the doublecortin (DCX) and doublecortin-like kinase (DCLK) gene products, which are important a.o., in brain development.

Aim: In the present thesis we have tried to establish a role for the MAPs tau and DCL in structural plasticity during development and adulthood, including mitosis and radial migration but also in synaptic plasticity, LTP and learning and memory.

In this thesis, we have focused on two MAPs: 1) the DCLK splice variant doublecortin-like (DCL) that was studied in relation to early cortical development and radial migration, and 2) on (mutant) tau in relation to structural and synaptic plasticity and memory function. Also, we studied structural plasticity changes in the human brain in presenile Alzheimer cases.

Doublecortin (DCX) is a recently discovered MAP that is abundantly expressed in a.o., the embryonic cortex. It is involved in the migration of newborn cells from the ventricular zone (VZ) to their final destination in the cortical plate. Mutations in the DCX gene severely disturb neuronal migration and induce a doublecortex syndrome in humans. Recently, a related gene called doublecortin-camkinase-like (DCLK) has been discovered. One of its splice variants doublecortin-like (DCL) was shown to be involved in mitosis and radial fiber stability of neuronal precursors. These latter studies are presented in the addendum of this thesis.

Although the DCX and DCLK genes share at least partly overlapping functions, specific differences exist as well, e.g. in their spatiotemporal expression patterns. DCX e.g., in contrast to DCLK, does not appear to be involved in cell birth. To further compare different aspects of DCX and DCL, we performed a detailed spatiotemporal analysis of the expression of both proteins throughout embryonic development. In Chapter 2 we show that DCL is already expressed from E9 onwards and decreases after E13. DCX expression on the other hand, starts modestly at E11 and is still present at E17.
Moreover, DCL was expressed in the VZ, the intermediate zone and CP, whereas DCX is not expressed in the VZ. Before E12, DCL and DCX expression do not overlap, indicating clearly different roles in the important early phase of cortical development when precursor expansion is extensive. Finally, DCL is found to be specifically associated with mitotic cells and with vimentin positive radial glia cells and radial fibers in the VZ. This suggests that DCL could be involved in early neurogenesis.

Of the classical MAPs, especially protein tau has been extensively studied because of its involvement in the tangle pathology in dementia. Upon alternative splicing six different tau isoforms can be formed, containing either 3 or 4 repeats (tau-3R or tau-4R). In rodents, tau-3R is primarily expressed during development, whereas expression switches to mainly tau-4R from the second postnatal week onwards. This period largely coincides with a phase of extensive neurogenesis during the formation of the hippocampal dentate gyrus (DG) that is completed around week 3, after which neurogenesis is strongly reduced.

In Chapter 3 we addressed the role of tau in DG development and more specifically focused on the 3R to 4R switch around the first 2 weeks of life, and on the possible functional consequences for hippocampal development. Therefore, the effect of tau deletion was tested (by others) in primary hippocampal cultures. Deletion of the tau gene increased cell birth, decreased differentiation and decreased neuritic outgrowth. All these effects could be reversed by expression of tau-4R, whereas tau-3R could only partially reverse neuritic outgrowth but failed to affect cell birth or differentiation. To test the relevance of these tau functions for hippocampal development, we subsequently made use of a transgenic mouse tau knock-out, human 4R tau knock-in (KOKI) mouse model. These mice lack all mouse tau isoforms, whereas tau-4R is expressed from the second postnatal week onwards at reduced levels in the hippocampus. These mice show a transient increase in cell birth from the second postnatal week. At two months of age, cell birth was again reduced to levels comparable to those in wild types. This increased cell birth was reflected by increased DG neurogenesis and eventually by an increased number and volume of the adult hippocampus. However, the size of the individual dendritic tree of DG granular neurons was significantly reduced at two months of age, suggesting a more immature population of cells. Only in the developing wildtype hippocampus, tau-4R has an inhibitory effect on neurogenesis, whereas in adult mice, tau-4R exerts stimulatory effects on neuritic outgrowth. We subsequently tested the consequences of these structural alterations for hippocampal functioning and found that memory function was significantly improved in KOKI mice in an object recognition task (ORT), but this was not associated with altered LTP.

MT affinity of tau can also be influenced by phosphorylation as this reduces MT binding. Phosphorylation is high during development, where it is associated with cell birth, but low in adulthood. However, in Alzheimer's disease (AD) and frontotemporal dementia (FTD), a familial form of dementia often caused by tau mutations, tau is again heavily phosphorylated.
Hyperphosphorylation of tau is thought to be relevant for memory impairments in AD and FTD.

To test this, we studied in Chapter 4 memory function in tau transgenic mice bearing the FTD mutation P301L. These mice recapitulate many of the features of FTD including axonopathy and memory impairments that are paralleled by tau hyperphosphorylation at later ages. We hypothesized that memory was not affected before the onset of hyperphosphorylation and therefore studied these mice at young adult ages of 2 months. Surprisingly, ORT performance was improved in P301L mice, which was associated with increased LTP. No changes were noted in any morphological parameter like neurogenesis or individual structure of the dendritic tree. Thus, in mice bearing the P301L tau mutation, hippocampal functioning is not impaired, but rather improved before the onset of tau phosphorylation. These results show that tau can directly affect learning and memory. They also indicate 1) that tau plays an important beneficial role in normal processes underlying hippocampal memory, clearly beyond “merely” the control of cellular morphology, and 2) that not tau mutations per se, but rather the ensuing hyperphosphorylation must be critical for the cognitive decline in tauopathy.

We showed in Chapter 3 that tau affects neurogenesis during development. Albeit at a much lower pace, neurogenesis continues to occur also in the adult DG. Since insults or damage to the hippocampus causes compensatory responses in neurogenesis in rodent models, neurogenesis might be affected by the pathological manifestations occurring in Alzheimer’s disease. Therefore, in Chapter 5, we studied proliferative changes in the AD affected hippocampus of a cohort of presenile cases. We showed that the expression of the proliferation marker Ki-67 was increased in AD, which was attributed to changes in glia-rich areas and cells associated with the vasculature but not to changes in neuron rich areas. Based on these results and on additional DCX, GFAP and VWF stainings, we conclude that increased proliferation in the AD hippocampus does not reflect neurogenesis but rather represent glial proliferation and vasculature-associated changes.

Previously, MAPs were always considered to solely act as microtubule stabilizers. Recent data including the results presented in this thesis now show that MAPs are involved in various different functions in the nervous system, including migration, mitosis, structural and synaptic plasticity as well as memory function. Although many of these MAP functions might be conserved amongst different proteins, differences in expression patterns, number of microtubule binding sites, projection domains and phosphorylation sites between MAPs and MAP isoforms are likely to represent functional differences. Indeed in this thesis we have shown a functional difference between tau-3R and tau-4R, matching the switch in their developmental expression. Furthermore, the differential spatiotemporal expression of DCX and DCL matches their different functions. Together, the different roles of these MAPs provide a better insight in their respective roles during early development.
II. General Discussion

From the mid eighties onwards, the expression of various, now classical MAPs has been described in the adult brain and during development (Bernhardt and Matus, 1982; Bernhardt et al., 1985; Calvert and Anderton, 1985; Black et al., 1986). Of these proteins, tau has been studied most extensively because of its role in AD and FTD. Consequently, the focus of tau research has been mainly on the adult and even aged brain. Thus, although their role in growth and neuronal maturation is well recognized, the majority of MAP research focussed on the adult and aged brain.

However, the recent discovery of a new family of proteins including DCX and DCL, has also stimulated a strong interest in the role of MAPs during development. The implementation of state-of-the-art technologies like spatiotemporal controlled transgene expression, in utero RNA silencing as well as imaging of migrating precursors has allowed the detailed study of MAP functioning during development and further established their important role in developmental disorders like e.g. lissencephaly (Edelman et al., 2005; Deuel et al., 2006; Koizumi et al., 2006; Shu et al., 2006).

So far, tau related dementia and DCX related developmental disorders have been studied separately. However, both fields share the common hypothesis that disturbed MAP expression leads to neuronal dysfunctioning. Therefore the underlying mechanisms could well be overlapping. Moreover, the important role of other MAPs during development and the developmentally regulated switch in tau isoform expression suggest that also tau might be of relevance for neurodevelopment. Thus both fields could benefit from an integration and synthesis of their separate developments. For example, phosphorylation of DCX is thought to have great impact on its functioning, yet only 3 kinases and 1 phosphatase have been described (Gdalyahu et al., 2004; LoTurco and 2, 2004; Reiner et al., 2004; Schaar et al., 2004; Tanaka et al., 2004; Shmueli et al., 2006). In the tau field, however, aberrant phosphorylation is critically implicated in the role of tau in neuropathology and many kinases and phosphatases are already known, while the effects of tau phosphorylation on microtubule dynamics have been extensively documented (Stooffhoff and Johnson, 2005; Avila, 2006). Since homology in the MT binding domains is high, knowledge about phosphorylation especially in these regions could be extrapolated from tau to other MAPs and add to our knowledge of the diversity in the functional roles of developmentally regulated MAPs. Probably, similar sites are phosphorylated by the same kinases, and the effect on MT binding is likely to be comparable.

One of the conclusions to be drawn from the studies focusing on MAPs during development has been that MAPs cannot be regarded "merely" as stabilizers of microtubules, which are involved in maintenance and stabilization of cellular shape. Rather, MAPs are regulators of highly dynamic processes in the cell such as growth and even cell division. With the general consensus that also the adult brain is highly plastic during e.g. learning processes, in diseased conditions, and even in "rest" when e.g. hippocampal neurogenesis is occurring, the novel field of cortical development might
reveal functions of MAPs that are also of relevance for adulthood and normal brain functioning. In this thesis for example, we show that the classical protein tau plays a subtle, yet important regulatory role in the development of the hippocampus and is involved in aspects of memory function in that area. Given the possible relevance for both fields, I will here try to integrate our experiments in relation to MAPs and cortical development with those relating to tau and dementia.

**MAPs and cortical development**

The formation of the neocortex heavily depends on the proper spatiotemporal orchestration of precursor mitosis, migration and neuronal differentiation. All these processes require tight regulation of microtubular cytoskeletal stability. Non surprisingly mutations in MAPs have serious consequences for the formation and viability of the neocortex and hippocampus.

Using DCX knock out strategies in mice, it was shown that DCX is essential for hippocampal lamination in the mouse (Corbo et al., 2002), by regulating migration of newly formed neurons. Using an in utero RNAi approach, it was subsequently shown that DCX is essential also for the laminating of the neocortex of rats. DCX knockdown at E14 resulted in a disruption of radial migration that led to the formation of sub cortical band heterotopias within the intermediate zone and later the white matter, resembling the doublecortex syndrome. Secondly, many neurons did migrate away from the VZ but ended up in the wrong cortical layer (Bai et al., 2003). Thus using a KO approach, a migration defect was only found in the hippocampus, whereas an in utero RNAi approach led to a severe defect in cortical lamination, similar to the human situation.

Recent experiments based upon the RNAi approach in both rats and mice, show that these differences are species related, and the sub cortical band heterotopias and laminar displacement occur only in rats but not in mice (Ramos et al., 2005). These findings indicate that in the mouse other proteins might carry out the function of DCX as well. As such, DCLK products are interesting candidates.

The DCLK gene, giving rise to both DCL and DCLK, shares a high homology with DCX. Whereas DCLK KO causes only a very mild phenotype, i.e. a selective axonal disruption in the corpus callosum (Deuel et al., 2006; Koizumi et al., 2006), deletion of both DCX and DCL resulted in a severe phenotype with a high (postnatal) lethality as well as disrupted cortical migration, abnormal dendritic morphology, axonal elongation, and defective trafficking of synaptic vesicles (Deuel et al., 2006; Koizumi et al., 2006). Strikingly, RNAi targeting of either DCX or DCLK resulted in a similar disruption of cortical migration. In this case, a possible species difference was not involved as all experiments were done in mice (Koizumi et al., 2006). As possible explanations compensatory mechanisms are more likely to occur in a KO strategy than in a quick RNAi approach. Another difference is that RNAi affects only a small population of cells, whereas in a KO approach all cells are affected. The authors argue that lack of DCLK in some cells might have a
larger impact than when all cells lack DCLK. Finally, the targeting of RNAi might not be completely selective, and also other RNAs might be affected. Clearly, the novel method of RNAi is elegant, provides a quick and location- and time-specific knockout situation. However, the different results obtained by KO and RNAi approaches need to be addressed in more detail in order to find out the exact functions of these genes. In the addendum of this thesis the DCLK splice variant DCL, is described. In early stages, RNAi mediated DCL knock down in utero was lethal. Later knock down of this gene at E14 led to severe disruptions in the extension of radial fibers and cortical migration reminiscent to the result obtained after DCLK and DCX RNAi. Similar to DCLK, DCL is expressed in mitotic cells in the VZ, and regulates spindle morphology. Thus, although their functions might be overlapping, DCX, DCL and DCLK all are critically involved in cortical development.

Obviously, also other, "classical" MAPs are important for cortical development. Double inactivation of both MAP1b and MAP2 disturbed cortical development, by disrupting migration and impaired axonal outgrowth (Teng et al., 2001). Also double inactivation of both the MAP1b and tau gene led to impaired migration and axonal outgrowth (Takei et al., 2000). As all these MAPs act through stabilization of microtubules, their functions have been regarded as highly redundant, which is probably why a single knock-out of either MAP2 or tau does not produce a phenotype, or mutations in the MAP1b gene alone cause a milder defect than MAP1b/Tau or MAP1b/MAP2 double mutants (Takei et al., 2000; Teng et al., 2001).

These synergistic roles for several MAPs reveal the relevance of MAP functions. The fact that different MAPs are distributed over different genes might provide the cell with a tool for the conservation of these essential protein functions, which could still be carried out should one of the genes become damaged or mutated, or its expression dysregulated. Although the functions of many MAPs might be overlapping, their differences might also be of relevance.

All MAPs are characterized by one or more microtubule binding repeat sequence(s) which share high homology but have a highly variable acidic tail (Bielas and Gleeson, 2004). The relevance of these microtubule-binding domains for MAP functioning is demonstrated by mutations in DCX in this domain which cause migration defects (reviewed by (Feng et al., 2001b)). Also the mutations in protein tau that cause dementia are often located within these microtubule binding domains (reviewed by (Spillantini et al., 1998; Tolnay et al., 1999)).

Although these basic microtubule-binding sequences of MAPs share high homology, the acidic tails, or projection domains, of these proteins vary considerably in size and composition. Over-expression of MAPs leads to excessive microtubule binding and greatly enhances stability and bundling of microtubules. The spacing of the microtubules within these bundles seems to be controlled by the length of their projection domains (Chen et al., 1992). Moreover, also interactions of mainly the MAP2 projection domain have been described between organelle membranes (Linden et al., 1989; Maas et
al., 2000; Farah et al., 2005), actin microfilaments (Sattilaro, 1986; Cunningham et al., 1997; Tsukada et al., 2005), neurofilaments (Leterrier et al., 1982; Hirokawa et al., 1988) and signalling molecules (Harada et al., 2002). Thus, whereas the MT binding sequences of the MAPs share a similar function and are of great relevance for MAP function, their highly variable projection domains probably determine the MAPs specific function that distinguishes them from each other. Since their shared functions have now been characterized quite well, the challenge for future MAP research lies within the characterization of these more subtle ways of (inter)action which make MAPs unique.

The strong individual roles of these proteins is not only demonstrated by their unique sequence but also by their spatiotemporal expression patterns that are variable and selective yet temporally restricted. Whereas MAP1b e.g. is only expressed during development, MAP2a expression is absent during that period but increases from day 20 after birth. Also spatial expression patterns of neuronal MAPs are also highly variable. MAP2 is barely expressed in the 24-month-old rat hippocampus and neocortex. On the other hand, MAP1a is abundantly present in these areas at this age ((Chambers et al., 2000) and references therein). Also at the sub cellular level there are marked differences. Whereas MAP2a, b and c are specific for dendrites, tau is enriched in axons (Mandell and Banker, 1995). Together, these differences reinforce specific functional roles for these different proteins.

Differential expression of DCL and DCX during cortical development

Although the expression of classical MAPs like tau is relatively well characterized, the expression of the recently discovered DCX and DCLK splice variants is so far poorly studied. Our spatiotemporally mapping of the expression of DCX and DCL (Chapter 2) revealed that the onset of DCL expression commences before that of DCX and DCLK, which may explain the strict impact of the lack of DCL expression on the survival of the animal. Clearly, the earlier changes in expression occur during development, the more cells are affected by it. Furthermore, the DCL pattern of expression both in time and localization, fitted well with the nuclear translocation mode of migration. By carefully analyzing differences in expression patterns, novel insight is provided about the overlap and differences between of DCL and DCX.

Although for the expression of other DCLK variants we have to rely on literature, the comparison of DCL with DCLK expression is of interest. Whereas DCLK is only expressed from E12 onwards (Deuel et al., 2006), DCL expression peaks before E13 (Chapter 2). Moreover, DCL expression declines after E13 and is no longer expressed in the VZ from that time onwards, whereas DCLK is present until birth and at least until E17 present in the VZ/SVZ (Shu et al., 2006). Consistent with its functional role in mitosis and neuroblasts, DCL appears only to be involved in early neurogenesis before E13, whereas DCLK is only expressed afterwards and likely to be involved in neurogenesis throughout all stages until at least E17. This difference in expression of DCL and DCLK might
also be related to the different modes of migration, which is mainly nuclear translocation before E12 and radial glia guided after E12. Contrary to DCX and DCLK, DCL expression coincides with nuclear translocation, suggesting a role for DCL in this process.

Recently, a few novel MAPs were discovered that contain DCX like MT binding domains as well. The first one, called DCDC2 was found to be associated with dyslexia and is expressed in the developing and adult brain. In utero DCDC2 RNAi reduces migration speed of neuronal precursors (Meng et al., 2005). Another MAP is called doublecortin-kinase 2 (DCK-2). Like DCLK it contains both MT binding sequences and a kinase domain. Upon autophosphorylation it loses its MT binding affinity. Interestingly, this MAP is also widely expressed in the adult brain, where it is localized in growth cones (Edelman et al., 2005). From a temporal perspective we propose that the first protein to be expressed is DCL, already present from E9 onwards. After E12 its functions are taken over by DCLK that is involved in mitosis, outgrowth and migration and by DCX, primarily involved in migration. DCK-2 and DCDC2 are also expressed in the adult brain. Although at this age mitosis and migration are of less relevance, it might play an important role in growth, as do the other MAPs.

**Conclusion 1:** The data in chapter 2 show that, although literature suggests their functions in cortical development might be partially overlapping, the early expression patterns of DCL and DCX are distinctively different.

**A developmental role for protein tau**

Interestingly, the isoform switch from DCL towards DCLK, resembles to some extent that of protein tau but at a different developmental stage. Also the expression of protein tau switches from one isoform, i.e. the 3R form that predominates before the second postnatal week, to mainly the 4R isoform after this time point. Also functionally the tau and DCLK genes are comparable as also protein tau also appears to be involved in both neurogenesis (Chapter 3), the elongation of neuritic extensions (Chapter 3) and migration (Takei et al., 2000). This suggests that neurogenesis, neuritic elongation and migration might be tightly linked processes since they can be influenced by the same genes such as DCLK or tau. Interestingly, whereas both DCLK and DCL are involved in neurogenesis, in the case of tau only the 4R splice variant appears to be involved in neurogenesis. Also the mechanism by which they are involved appears different. For DCL and DCLK the expression level is crucial, as both decreased or increased expression lead to mitotic arrest (Shu et al., 2006), absence of tau-4R clearly does not lead to reductions in neurogenesis (Chapter 3) but rather stimulates neurogenesis. Over-expression of a tau-4R mutant, as occurs in the P301L model (Chapter 4) does not have any effect on neurogenesis.
The mechanisms by which DCLK splice variants and tau-4R regulate neurogenesis might be quite different. If tau were to act in a similar manner as DCL it would be expressed in mitotic spindles, which at present has not been reported. In Chapter 3 we found that tau-4R was mainly expressed in axonal and dendritic compartments of the cell. The mechanism by which tau-4R acts on neurogenesis might thus be opposite to that of DCLK splice variants. Whereas tau-4R promotes neuritic elongation and cellular differentiation and thereby reduces possibilities for the cell to return to the cell cycle, DCL and DCLK are located within mitotic spindles and can alter spindle morphology, and thus actively engage in mitosis, thereby possibly determining migration and differentiation at later stages. It would be interesting to know if and how tau can directly affect neurogenesis in more detail.

Tau is well known to be subject to conformational alterations following (hyper)phosphorylation by many different kinases. Although this subject is mainly studied in relation to pathological processes, tau phosphorylation is likely to be a functional process like is the case for e.g. DCX phosphorylation. At present it is known that DCX can be phosphorylated by at least four different kinases, i.e. JNK, cdk5, MARK and/or PKA (Reiner et al., 2004). DCX can be dephosphorylated by protein phosphatase 1 (Shmueli et al., 2006) which is mediated by neurabin II (Tsukada et al., 2006). Phosphorylation by cdk5 detaches DCX from microtubules in vitro; in vivo it localizes DCX to fine perinuclear MTs but not large MT bundles in proximal processes, which led authors to argue that in cdk5 phosphorylated form DCX might be involved in nuclear translocation (Tanaka et al., 2004). Phosphorylation by MARK and PKA also reduced MT affinity, and is involved in growth and migration (Schaar et al., 2004). Moreover, phosphorylation by JNK was essential for the localization of DCX in growth cones. Indeed expression of constitutive phospho-mutants increased neuritic outgrowth (Gdalyahu et al., 2004). Thus these data show that selective phosphorylation can direct the MAP DCX to different sites within the cell, that are associated with different functions exerted at these specific sites. It would be interesting to study such processes during development also in relation to protein tau, which is highly phosphorylated only during development.

Conclusion 2: Similar to the novel MAPs DCL and DCLK, protein tau is also involved in neurogenesis. Whereas both DCL and DCLK appear directly related to neurogenesis, tau-4R but not tau-3R, inhibits neurogenesis.

MAPs in relation to learning and memory

The previous section has illustrated the relevance of MAPs in developmental processes like neurogenesis, migration and growth. Although obviously most abundant during development, these processes continue to occur into adulthood at strongly reduced frequencies and in only a few
brain areas. In e.g. the hippocampus, neurogenesis and synaptic plasticity have been implicated in learning processes.

**Microtubules and LTP**

As one of the possible substrates for learning and memory, LTP strongly depends on structural plasticity through reorganization of the dendritic tree and spines, as well as on MT mediated transport. This is exemplified by a group of proteins referred to as SCG10-related neuronal Growth Associated Proteins (nGAPs), including stathmin, SCG10 and RB3. These proteins are upregulated in response to cortical and hippocampal lesions. A common feature is that contrary to MAPs, nGAPs are potent microtubule destabilizing factors (Mori et al., 2002). Interestingly, several correlations are known between these proteins and LTP. RB3 is e.g. upregulated after seizure or LTP induction (Beilharz et al., 1998) while SCG10 is upregulated only after LTP induction in the hippocampus (Peng et al., 2003, 2004). Interestingly, deletion of stathmin, a protein normally expressed at high levels particularly in the amygdala, leads to reduced LTP in that area as well as reduced fear conditioning and other fear related behaviors. These data show that alterations in microtubule dynamics can disturb LTP and behavior (Shumyatsky et al., 2005). This implies that MT stabilizing factors such as MAPs can exert opposite effects on microtubule dynamics, and can interfere with LTP, learning and memory.

So far, only one MAP is reported to be responsive to LTP induction, namely MAP2. Upregulation of MAP2 after LTP induction in the CA1 and DG or after NMDA receptor activation has been regarded an adaptation to facilitate the production of new synaptic contacts (Johnston and Morris, 1994; Roberts et al., 1998; Alie and Morris, 2004). Conversely, hippocampus specific MAP1B knock out mice show reduced LTP (Zervas et al., 2005), confirming the involvement of MAPs in LTP. Not only microtubule-stabilizing factors like MAP2 but also -destabilizing factors are up regulated after LTP, as explained above. Probably, in response to the induction of LTP, appropriate morphological and/or synaptic adaptations are required for which microtubule growth needs to take place, either facilitated by MAP2 and/or MAP1B, but without loosing microtubule flexibility, which would explain why e.g. also nGAPs are up regulated. As elaborated upon in the introduction also during cell division MT polymerization needs to be enhanced without affecting MT stability. Thus, MAP related mechanisms leading to growth might be comparable to those mediating cell division.

We infer, based on chapter 3 of this thesis, that altered expression of tau can affect LTP. This implies an important role for tau in LTP and learning and memory. Whereas MAP2, which is expressed in dendrites, regulates postsynaptic cytoskeletal adaptations, the axonal protein tau might affect presynaptic adaptations of the cytoskeleton. Since upregulation of MAP2 is associated with LTP, we argue that increased tau expression in the P301L model might be responsible for the increased LTP. Another option is that the introduction of the P301L mutation itself is involved in this effect. One might argue that the mutation reduces MT affinity, and thus that MT plasticity is
enhanced in the P301L model, which would be beneficial for LTP. Even if this were the case overexpression of tau would suffice to overcome this effect. Thus over expression of tau-4R even though mutated would increase tau binding to MT. Therefore, we propose that increased tau binding rather than decreased tau binding is beneficial for LTP. In the section "concluding remarks and future directions" we elaborate upon possible research options to test this idea.

Interestingly, for MAP2 it was suggested that an increased expression after LTP induction is needed for microtubule growth in order to facilitate morphological changes preceding alterations in network properties (Johnston and Morris, 1994; Roberts et al., 1998; Allen and Morris, 2004). Although in tau-P301L mice no major changes occur in any of the morphological parameters studied (hippocampal size, dendritic size, number of spines and filopodia), subtle morphological alterations might still play a role in our tau P301L model. The morphology of granular neurons under normal conditions may be unaffected by increases in tau expression, as was shown in chapter 4, whereas after the induction of LTP, morphological adaptations such as dendritic outgrowth, spine and synapse formation may be promoted in tau-P301L animals compared to WT animals.

Remarkably, we find upregulation of DG LTP in the P301L model (Chapter 4) but we find no effect on DG LTP in the tau KOKI mice (Chapter 3). This could be related to the endogenous expression of tau-4R in the DG, which is very low in WT animals anyway. In the P301L mice model, an overexpression of tau-4R occurs, a change that can affect LTP. The reduction, on the other hand, of tau expression in the KOKI model may have an opposite effect. In view of the low tau level in the WT situation, an even further reduction in tau expression may not have important consequences for LTP. However, tau knockdown in the KOKI animals clearly affects neurogenesis and neuritic outgrowth showing that tau knockdown, even if expression in the WT is very low, can have robust effects on other cellular processes.

MAPs and learning and memory.

Tau has been studied extensively in relation to and learning and memory processes in relation to changes in its expression and phosphorylation in AD and related tauopathies. As discussed in Chapter 4, the role of tau in learning and memory in animal models has so traditionally been studied only when severe tauopathy, including tau hyperphosphorylation and tangle accumulation, were already prominent (Tatebayashi et al., 2002; Arendash et al., 2004; Pennanen et al., 2004; Ramsden et al., 2005). Generally speaking, most mouse models show memory impairments that increase with age, parallel to tau phosphorylation and the accumulation of tau. Since tau knock out animals failed to show a severe phenotype (Harada et al., 1994; Dawson et al., 2001), except for some minor impairments in fear conditioning and axonal outgrowth under in vitro conditions (Ikegami et al., 2000), it was concluded that tau is functionally redundant and most likely replaced by other MAPs like MAP1B (Takei et al., 2000; Dawson et al., 2001).
The role of tau in cognition in dementia and in AD models has been regarded as a gain of tau (dys)function under pathological conditions, leading to neurofibrillary tau accumulations and subsequent neurodegeneration, eventually causing memory deficits. In a recent inducible mouse model, tauopathy rapidly developed following the onset of mutant tau expression, which coincided with impairments in memory function. Surprisingly, when expression of the mutant tau was turned off again, memory improved while the tau accumulation continued to increase (Santacruz et al., 2005). These data suggest that tau can affect learning and memory independent of tau accumulation. A possible mechanism by which this might be caused was proposed by Mandelkow and colleagues who suggested that the increased tau expression might cause impaired anterograde vesicle transport, which might impair learning and memory directly (Mandelkow et al., 2003). However, more insightful data come from a recent study showing that hyperphosphorylated tau can reduce MT stability by actively binding normal tau but also MAP1 and 2. Once hyperphosphorylated tau is polymerized in paired helical filaments and tangles it loses this property (Del C. Alonso et al., 2005). Using constitutively active, and inducible phosphomutants it was shown that especially temporally regulated phosphorylation by GSK-3 in the absence of tau accumulation is enough for memory deficits to occur (Engel et al., 2006; Plattner et al., 2005).

These data contribute to the accumulating evidence showing MAPs contribute to learning and memory. Increased MAP expression is regarded as beneficial for the neuron in general and for learning and memory in particular, as it is supposed to facilitate morphological adaptations needed for LTP and memory. At least, it has been shown that LTP induction increases MAP2 expression (Johnston and Morris, 1994; Roberts et al., 1998; Alier and Morris, 2004) while after contextual learning, MAP2 expression is also increased (Wooff, 1998; Wooff et al., 1999). Conversely, MAP1B KO reduces LTP (Zervas et al., 2005). Although MAP1B deletion in mice does not lead to impaired spatial learning in the watermaze, these animals have impaired motor performance and altered retinal functioning, suggesting neuronal functioning is impaired in these mice (Pangratz-Fuehrer et al., 2005).

Although not completely comparable, overexpression of KIF17, a member of the kinesin super family of motor proteins responsible for transport along microtubules (Hirokawa and Takemura, 2004), is associated with enhanced spatial and working memory (Wong et al., 2002). It needs to be mentioned though, that these motor proteins have completely different function than the classical MAPs.

Considering these data, a role for MAPs in neurotransmission is emerging. Consistent with this we show in Chapter 4 that increased expression of protein tau itself rather than the mutated form of tau, that leads to improved LTP and memory performance in young animals. Moreover, they are in line with the general assumption that the deleterious effects of tau on memory and learning in P301L mice are explained by the subsequent tau pathology such as tau hyperphosphorylation.
Summary & Discussion

In chapter 4 increased expression of mutant protein tau is associated with improved ORT performance. In chapter 3, tau expression is decreased in the DG of the tau-KOKI model, which also appears to be related to an increase in ORT performance. These differential changes might be explained by the pleiotropic function of tau. In Chapter 3 a relative lack of tau-4R expression in the DG is related to early increases in proliferation that result in more neurogenesis and eventually in an enlarged hippocampus. At 2 months of age, proliferation is back to its normal levels, yet ORT performance remains increased at this age. We propose that this improvement in learning and memory is not directly linked to tau functioning per se, but rather relates to the increased hippocampal volume and cell number resulting from the developmental changes. The tau-P301L model described in chapter 4, by contrast, has no such early neurogenic or morphological changes, yet memory is improved also in these mice. We proposed therefore that this is related to other functional aspects of tau, probably axonal transport. Apparenty, tau knock-down has an effect on other tau functions (those related to development) than tau over-expression, mainly affecting tau functions related to synaptic plasticity.

It needs to be noted that the memory improvement in both tau-KOKI and tau-P301L mice are based on LTP and ORT studies. The ORT since is a simple test for memory associated to the hippocampus and prefrontal cortex and, Compared to the more widely used morris watermaze the ORT has several advantages. Firstly, it is one of the less stressfull memory tests available. Since stress can seriously hamper memory this is an important feature. Moreover, there are several indications that stress responses in tau-transgenic mice are altered (Tanemura et al., 2002; Pennanen et al., 2004). Secondly, the ORT involves less motor skills, an advantage relevant especially for the P301L mice, which develop severe impairments at later ages which are already present at 2 months of age, although less severe (Terwel et al., 2005), chapter 4. Finally, both tau-transgenic strains were generated on a FVB background. The FVB strain is characterized by mutations in the retinovision gene, which causes visual impairments (Pugh et al., 2004). Since the watermaze requires navigation relying primarily on spatial visual cues, this was not a good test for the FVB strain. The object recognition test relies not only on visual but also on tactile stimuli, which makes it a more suitable test for our current studies.

Although the ORT is a very reliable way to test memory in the present mouse models, this does not nescessarily predict that animals also perform better in other learning paradigms. It is tempting to speculate that especially in reversal paradigms as used in the watermaze and radial maze, the tau presented tau transgenic strains could perform less well, since improved long-term memory, and memory consolidation may have taken place at the expense of the learning of new information. To confirm the memory improvements in these tau-transgenic mice one could opt for additional studies using e.g. the wholeboard test or the radial maze. Both of these test require limited motor or visual skills, while also stress levels are relatively low.
Conclusion 3: Increased MAP expression could facilitate LTP and learning and memory through morphological alterations such as dendritic growth. Although LTP as well as memory are improved in young tau-4R over-expressing P301L mice (Chapter 4), no evidence was found that this is mediated through adaptations in dendritic morphology, indicative for tau functions beyond morphological maintenance and adaptations.

Neurogenesis and AD

So far, we have discussed the role of MAPs in neurogenesis and migration during development. Although at a more limited scale, neurogenesis continues to occur after birth, particularly in the adult hippocampal DG adult where it is thought to play an important role in learning and memory but also in response to injury (for a more detailed discussion of adult neurogenesis see Chapter 1). Given their role in structural plasticity MAPs are likely involved in neurogenesis. As AD is associated with aberrant expression of the MAP tau with hippocampal damage, known to stimulate neurogenesis, we will here discuss the role of neurogenesis in AD in relation to learning and memory.

For approximately a decade now, re-expression of various cell cycle markers is known to occur in mature cell profiles in the AD hippocampus (Smith and Lippa, 1995; Arendt et al., 1996; Kondratick and Vandre, 1996; McShea et al., 1997; Nagy et al., 1997; Vincent et al., 1997; Busser et al., 1998; Yang et al., 2003). Since mainly tangled neurons in both the DG and the CA expressed these markers, this has generally been regarded as a maladaptive process and a failing cellular response that does not lead to the birth of new cells. Re-expression of these markers was rather regarded as causing abortive exit hence causing pre-stage of apoptosis, or alternatively, as a longlasting cell cycle arrest, leading to cellular dysfunctioning.

With the parallel developments in the field of adult neurogenesis it became evident that relatively hippocampal specific damage such as that inflicted by hypoxia or epilepsy, induced increases in proliferation and neurogenesis, possibly as a compensatory response following cell loss (Parent et al., 1997; Covolan et al., 2000; Blumcke et al., 2001; Jiang et al., 2001; Jin et al., 2001). Subsequently, the hypothesis was put forward that the re-expression of cell cycle markers like e.g. PCNA and cyclins in AD could also be part of a regenerative process that may lead to the production of new neurons in the adult brain. So far, only one article recently reported increased expression of DCX and other immature neuronal markers in a cohort of senile AD cases, suggesting that neurogenesis may actually be increased in AD (Jin et al., 2004a).

As no information was available in the literature on younger, i.e. presenile patients, in which the disease is more severe and often runs a faster
Summary & Discussion

course, we chose in chapter 5 to address these questions in more detail. However, in a well-characterized cohort of presenile cases, we were unable to replicate the data by Jin et al. and rather found that the cell-cycle marker Ki-67 was increased in AD but only in non-neuronal compartments like glia cells and the vasculature.

Various reasons could have explained the discrepancies with the Jin et al., paper. One possibility is the difference in age of the cohort; Jin et al focused on a senile group of patients, a condition generally associated with a slow deterioration over time. As it is assumed that the cognitive decline generally follows the onset and occurrence of the neuropathology, a key difference with the presenile cases is that in this group, the presenile form runs a much faster course and the pathology is often much more severe and widespread. As such, any reaction to hippocampal injury is expected to be more prominent in this group, particularly since also responses in structural plasticity are expected to be larger in younger individuals. Despite this, no indications were found for increases in neurogenesis in presenile AD.

AD is a very heterogeneous disease with a wide range in age of onset, and disease duration. Neuropathologically, a profound overlap between controls and AD cases exists in the number and extent of the plaque- and tangle pathology. Although AD etiology is still poorly understood, it is tempting to speculate that these large clinical and pathological differences might actually evolve from different etiologies. Hence, one possibility is that neurogenesis is increased in senile but not in presenile AD, since these types of AD have different characteristics and possibly different underlying causes. Clearly, initial expectation that changes in neurogenesis might be more obvious in presenile AD compared to senile AD does not hold true. However, the present data in chapter 5 and the data by Jin et al. are not enough to conclude that senile AD affects neurogenesis but not pre-senile AD, because both studies were performed using a completely different approach and also methodological issues may have lead to the contradictory data.

One of the markers used in the Jin et al., study was DCX. DCX is expressed in the adult brain in those areas where neurogenesis continues to occur, i.e. the SVZ and the DG. Previous studies have shown DCX-expressing young neurons to accurately reflect modulations in adult neurogenesis rate (Brown et al., 2003; Rao and Shetty, 2004; Couillard-Despres et al., 2005). Importantly, unlike BrdU, DCX does not require previous injections in live subjects. As such, it held considerable promise for application in postmortem human brain tissue. However, we showed that DCX, like many other MAPs (Swaab and Uylings, 1988) is very sensitive to degradation during post-mortem delay. Already after 1 h of PMD, a completely different pattern of immunoreactivity appeared in our rat PMD series, while the dendritic staining pattern disappeared rapidly. DCX IR remained only in a very disperse granular pattern, clearly not resembling normal neuronal morphology. We concluded that for human tissue with variable PMDs, as used by Jin et al., DCX is unreliable for the detection of quantitative differences in neurogenesis. Moreover, these authors used western-blots of hippocampal
homogenates of only a small selection of their patients (n=3) to quantify their data and no morphological information or quantification was available about the nature, localization or the condition of the cells immunocytochemically expressing early neuronal markers.

**Neurogenesis and AD models: APP processing**

As also described above, the use of human "end stage" brain material for the study of neurogenesis is hampered by methodological pitfalls. Not only is the large variation in PMD a serious concern, there is also a large variation in the course of the disease, treatment and background of each individual. Moreover, commonly used immunohistochemical procedures for studying neurogenesis like timed BrdU injections and subsequent stereological quantification are cumbersome and technically very difficult to perform in humans. Neurogenesis has recently also been studied in various mouse models of AD in an attempt to better link changes in neurogenesis to specific aspects of the disease such as tauopathy or amyloid pathology. We will first discuss APP and PS1 mutant or deletion studies.

Some authors have shown that hippocampal neurogenesis is decreased in mice over expressing the APPsw (Tg2576) mutation (Dong et al., 2004) and mice in which the PS1 gene is replaced by a mutated PS1 variant (M146V) (Wang et al., 2004). Other authors point to the specific stage of the amyloid pathology occurring in these animals. They show that neurogenesis is unaffected as long as Aβ pathology is absent but that it is decreased as soon as plaque pathology becomes evident in either an Aβ peptide injection model or in a PD APP mutant mouse model resp. (Haughey et al., 2002; Donovan et al., 2006). Together, these studies suggest that Aβ pathology decreases rather than increases neurogenesis (Yang et al., 2006).

By contrast, it has been shown that in vitro, aggregated Aβ 42 but not the soluble and smaller forms of Aβ, increase neurogenesis (Lopez-Toledano and Shelanski, 2004). Also in the SVZ, infusions of APP increased neurogenesis (Caille et al., 2004). It has further been suggested that neurogenesis in the SVZ might be regulated by astroglial expression of APP (Yasuoka et al., 2004). Conversely, in PS1 knock out mice, a reduced amyloid production is found, which was associated with a reduction in the hippocampal increase in neurogenesis after environmental enrichment. Also this effect was mainly on proliferation and not so much on survival of the newborn cells (Feng et al., 2001a). These results appear rather contradictory to those showing that Aβ pathology reduces neurogenesis (Haughey et al., 2002; Dong et al., 2004; Wang et al., 2004; Donovan et al., 2006). It is important to note however, that except for the in vitro data (Lopez-Toledano and Shelanski, 2004), these studies have been performed in the absence of pathology (Caille et al., 2004; Yasuoka et al., 2004; Feng et al., 2001), thus these results are not conflicting with the hypothesis that neurogenesis decreases after amyloid pathology. Considering the important role of both APP and PS1 in embryonic development as regulators of Wnt signaling (Caricasole et al., 2003; Chevallier et al., 2005; Wines-Samuelson and Shen, 2005; Chen and Tang,
2006), both genes may be important regulators of neurogenesis during development. Therefore, the results presented in this section showing increased neurogenesis could reflect a developmental role of APP rather than a pathological one.

Whereas Aβ or APP might promote neurogenesis in the young and developing brain and in adult brains in which the pathological forms of Aβ are still absent, changes in neurogenesis might be rather different or even contradictory, once pathology occurs. Indeed the number of neurons is increased in the neocortex of APP23 mice at 8 months of age when no signs of pathology were present yet, whereas at 27 months of age these mice had developed a considerable plaque load, which was negatively correlated to the number of neurons (Bondolfi et al., 2002). Mice overexpressing mutated PS1 (A2546E) showed an increase in neurogenesis compared to mice that overexpress WT PS1. Importantly, also this was an effect only seen in earlier stages of development and not at later ages, thus supporting the hypothesis that APP dysregulation can increase neurogenesis but only in a development dependent manner. Mice overexpressing the PS1 A246E mutation developed no Aβ pathology, thus it is indeed not surprising that they did not show decreased neurogenesis in later stages (Chevallier et al., 2005). Therefore, also this study points to the relevance of the developmental or pathological stage for the effect of amyloid on neurogenesis. This assumption is further supported by studies of Wen et al who first showed increased neurogenesis in the hippocampus in a cohort overexpressing WT PS1 but not in a group of animals over expressing mutated (P117L) PS1 (Wen et al., 2002). In a later study these same authors found decreased neurogenesis in mutant PS1 overexpression and no effect of WT over-expression (Wen et al., 2004). The only difference between their studies was the age of the animals, which was higher in the latter study. These studies show that the effect of WT PS1 on neurogenesis is either positive or neutral depending on the age of animals whereas mutated PS1 had a neutral or negative effect on neurogenesis depending on the age of the animals.

Concluding, the effect of increased or mutated Aβ production on neurogenesis appears to be dependent on two factors: the developmental stage of the animal and the presence or absence of pathology. Obviously, these two parameters are not independent of each other since most PS1 or APP mutated mice show increasing pathology with age. Altered APP or PS1 expression can increase neurogenesis in younger animals in the absence of Aβ pathology, but it decreases neurogenesis in later stages, when Aβ pathology is present.

It needs to be noted that one animal study does not fit within this hypothesis. Namely, Jin et al. showed that neurogenesis is increased in the hippocampus of PDAPP mice that bear both the Swedish and Indiana mutations. These effects were found both at 3 months of age, still in the absence of plaques, and at 1 year of age, at which time many plaques were present in the hippocampus. Within the SVZ the difference of this study with others is even more striking since there is no change in cell birth at 3
months of age whereas there is an increase at 1 year of age (Jin et al., 2004b). These data in fact suggest quite the opposite, namely that amyloid pathology increases neurogenesis.

Interestingly, various cell-cycle events were found to be increased in several strains of APPswe mutant mice (Yang et al., 2006), resembling the situation in human AD in which ectopic expression of these markers has repeatedly been reported. The cells expressing these markers were post-mitotic neurons. Therefore, the authors in this paper disagree with the conclusion of Jin et al. on increased neurogenesis in AD, but suggest that rather than producing new neurons, these cells have an apoptotic fate. A second conclusion that can be drawn is that these studies indicate that amyloid affects cell-birth rather than survival of neurons, a conclusion also supported by Jin et al (Jin et al., 2004b), although this might be different old under in vitro conditions (Lopez-Toledano and Shelanski, 2004).

If we try to translate the above data to the human AD hippocampus this would predict that neurogenesis is decreased rather than increased. However, neurogenesis in this situation is increased according to Jin et al. (Jin et al., 2004a) or unaffected according to our own data. One obvious explanation could be that human AD is of course much more than altered APP expression alone. For example also changes in tau expression could play an important role in this, although our own data show that changes in tau expression might only affect neurogenesis in a specific developmental period (chapter 3). Moreover, although APP pathology might not directly stimulate neurogenesis, the resulting damage and cell loss could increase cell birth, as it does e.g. in ischemia.

**Protein tau and neurogenesis**

Considering the wealth of data on neurogenesis in relation to amyloid expression, it is remarkable that so far very few data have been reported on the possible link between protein tau and neurogenesis. This is especially striking since literature on cultured neurons suggests an important role for tau, and for other MAPs as well for that matter, in structural plasticity changes during development that include cytokinesis, neuronal maturation and neuritic outgrowth (Caceres and Kosik, 1990; Andersen, 2000; Gonzalez-Billault et al., 2002; Shu et al., 2006); all processes tightly linked to neurogenesis. Furthermore, tau phosphorylation occurring in AD closely resembles tau phosphorylation during mitosis (Cross et al., 1996; Preuss and Mandelkow, 1998; Delobel et al., 2002b). Moreover, many of the cell cycle alterations in AD are found in, or in close association with, the tangle pathology. Together, this indicates a role for tau in mitosis and neurogenesis. However, the question remains if and how tau affects cell birth and whether it acts as an inhibiting or a stimulating factor.

Selective tau isoforms were found to act as cell cycle inhibitors. Tau-4R, but not tau-3R, and most of the tau mutations studied, were e.g. shown to inhibit Xenopus oocyte maturation (Delobel et al., 2002a), observations consistent with our own data showing that expression of tau-4R but not tau-3R inhibits neurogenesis both in vivo and in vitro (Chapter 3).
It is thought that for mitosis to occur fast microtubule growth needs to take place without the microtubules losing their flexibility or the ability to undergo catastrophe, an event in which rapid shrinkage of MT length occurs. As such the search and capture model is still a leading hypothesis: According to this model MTs grow randomly out of the centrosome. When they meet a chromosome they bind and MTs are stabilized; if not, they undergo catastrophe and shrink back to the centrosome (Mitchison and Kirschner, 1984a, b). Whereas the recently discovered MAP XMAP215 e.g. facilitates both growth and catastrophe, wild type tau itself facilitates growth but makes microtubules very rigid, hence preventing the occurrence of catastrophe (Noetzel et al., 2005). Therefore, also these data suggest that if tau plays a role in mitosis it is likely an inhibitory role. Also in vivo the tau mutation P301S was found to be associated with overexpression of the cell cycle dependent kinase inhibitors p21Cip1 and p27Kip1 (Delobel et al., 2006) that are known to inhibit mitosis. Moreover, the tau P301L mutation leads to a changed regulation of cyclins, inducing cell cycle arrest in the G2 and M phase (Zhao et al., 2003).

The above data suggest that overexpression of wild type tau or mutated tau is unlikely to promote neurogenesis. This conclusion is in line with our own data showing that overexpression of mutated P301L tau in the absence of hyperphosphorylation does not affect proliferation nor newborn cell survival in the hippocampus (chapter 4). Also in the KOKI model, reduced tau expression is associated with increased neurogenesis in a specific period during postnatal maturation, again consistent with the inhibitory role of tau in mitosis as suggested by several authors (Zhao et al., 2003; Noetzel et al., 2005; Delobel et al., 2006) (Chapter 3).

The conclusion that tau can inhibit neurogenesis does not imply that neurogenesis is inhibited in AD because of tau overexpression. In AD, a large proportion of tau is expected to hyperphosphorylated which may lead to reduced MT binding, and could result in reduced tau functioning. Possibly, this could lead to increased neurogenesis.

Thus for AD this would mean that whereas altered APP processing is likely to decrease neurogenesis, increased tau phosphorylation may actually result in the opposite effect. To test these hypotheses it would be interesting to study the effect of tau phosphorylation on mitosis in e.g. hippocampal cultures or in vivo mouse models.

**Conclusion 4:** Although both APP processing and tau might be related to neurogenetic changes during development, no evidence was found for changes in neurogenesis in presenile AD (chapter 5). The increased proliferation in presenile AD rather reflects changes in glia and the vasculature.
Concluding remarks and future directions

In previous studies, MAPs have often been regarded "solely" as important proteins in the stability of MT, allowing MTs to grow more quickly. When MAPs detach from the cytoskeleton, e.g. upon phosphorylation, growth is slowed down and MTs become more plastic. Contributing to this view was the observation that even though the MAPs form a large family and many new ones continue to be discovered MAP functions were shown to be highly redundant. Consequently, many have regarded the role of MAPs primarily as simple regulators of cellular morphology. As shown and reviewed in the present thesis, this view is currently changing. MAPs are not only involved in structural stability and morphology, but also in cellular processes like mitosis, radial migration and differentiation during early cortical development. Moreover, as shown in chapter 4, tau also appears to be directly implicated in structural plasticity in adulthood, affecting learning and memory processes. These functions are unique for individual MAPs, although members of the same family might share functions, like DCX and DCLX share their role in migration.

Interestingly, several MAP interacting organelles have been described, including the RER, mitochondria and the plasma membrane (Linden et al., 1989; Maas et al., 2000; Farah et al., 2005), cytoskeletal elements and proteins involved in signalling cascades. This suggests that additional novel functions of tau exist that go beyond regulation of MT stability and might be associated to e.g. transport of proteins to organelles. These specific MAP functions are mainly attributed to their projection domains, thus not to the MT binding sequences, which are highly diverse amongst MAPs. In my view one of the challenges of future MAP research lies within these domains that make each MAP unique, and might associate them with specific functions.

In chapter 4 we show over-expression of mutated tau to have a positive effect on memory and learning, notably in the absence of any major cellular, neurogenic or pathological changes. Obviously, it would be of interest to link these effects to either over-expression of tau-4R or the presence of the P301L mutations. Although in theory repeating these experiments in non-mutated tau-4R over-expressing mice would answer the question, this would be practically very difficult as the mice develop a severe axonopathy including motor defects already at early ages (Terwel et al., 2005).

Obviously, also subtle morphological changes, or alterations in the presynaptic compartment could have a large impact on the changes in cognition in the P301L mice. Other interactions with e.g. the cytoplasm membrane, axonal transport or the trafficking of vesicles could well be involved in the tau-mediated changes that evoke increased LTP and memory. Clearly, the full mechanism has not been unraveled yet.

Earlier I mentioned that it would be of mutual interest to integrate MAP studies from the developmental field with studies focusing on dementia. Indeed, at least my personal understanding of the role of tau during hippocampal development has benefited greatly from the studies on DCX
and DCLK. Moreover, for reasons explained below, the same type of research as in the DCX field might aid us in designing new experiments to increase our understanding of the physiological role of tau. Firstly, the novel field of corticogenesis has demonstrated their great importance in plastic processes such as neurogenesis, migration and growth. Also, protein tau is known to be involved in migration and growth, and the isoform switch during the second postnatal week indicates it plays an important role in early development. However, the latter role has, perhaps also because of the lack of proper tools, received little attention so far.

We show in chapter 3 of this thesis for the first time that protein tau is involved in development of the hippocampus by specifically regulating neurogenesis and neuronal differentiation. As such the role of tau appears quite comparable to e.g. that of DCL in cortical development. However, the most prominent role of LIS1, DCX and DCL is in migration. In the early postnatal as well as in the adult hippocampus, proper migration of newborn cells is important too. Regarding the present increase in expression of DCX in tau-KOKI mice it would be conceivable that protein tau also plays a role in this process. Direct imaging of live migration e.g. using virally labeled newborn cells as e.g. Noctor et al did in the developing cortex may provide a means to study such questions in the tau-KOKI model (Noctor et al., 2001). Although redundancy remains a theoretical problem, this is unlikely considering the clear effect of tau knock down on both neurogenesis and differentiation (Chapter 3).

In chapter 3 we have not focused on the role of tau phosphorylation in development. It is not unlikely that phosphorylation plays a prominent role here, in analogy to the different functions of DCL, which can e.g. be exerted through phosphorylation directed translocation, as explained in section II. Therefore, it would be challenging to study the influence of phosphorylation directed mutations on different possible functions like mitosis, migration or growth, or even just on intracellular localization of tau. Although in vivo this would be difficult, these types of experiments should be possible in culture systems.

In conclusion, in this thesis we show that MAPs, important regulators of MT stability, are involved in much more processes than “merely” maintenance of cellular morphology. Rather, they are mediators of dynamic processes like cell division, growth, migration and plasticity. In the future it will be of great relevance to pinpoint and further characterize these functions more specifically and to specify the signaling cascades involved.
ADDENDUM

Doublecortin-Like, a Microtubule Associated Protein Expressed in Radial Glia is Crucial for Neuronal Precursor Division and Radial Process Stability

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Abstract.

During corticogenesis, progenitors divide within the ventricular zone and rely on radial process extension enabling them to migrate along these radial glia cell (RGC) scaffolds to the proper layers of the cerebral cortex. Although the microtubule-associated proteins doublecortin and doublecortin-like kinase (DCLK) are critically involved in dynamic rearrangement of the cytoskeletal machinery thereby allowing migration, little is known about their role early in corticogenesis. Here we show that a splice-variant of DCLK, doublecortin-like (DCL), is expressed from ED8 onwards throughout the early neuroepithelium. It is localized in mitotic cells, RGCs and radial processes. DCL knockdown using RNAi in vitro induces spindle collapse in dividing neuroblastoma cells, whereas overexpression results in elongated and asymmetrical mitotic spindles. In vivo knockdown of DCL significantly reduces cell number in the inner proliferative zones and dramatically disrupts most radial processes. Our data emphasize the pivotal role of DCL in mitotic spindle integrity during early neurogenesis and demonstrate that DCL is essential for RGC proliferation and their radial process stability.

Introduction

Early cortical development relies on the precisely orchestrated synchronization of precursor pool expansion through cell division and migration along the extending fibers of radial glia cells (RGCs) to their designed positions within the cortical plate (Noctor et al., 2001; Gupta et al., 2002; Kriegstein and Noctor, 2004; Noctor et al., 2004). A key event in migration is controlled rearrangement of the neuronal cytoskeleton, consisting of actin, microtubules and microtubule-associated proteins (MAPs) (Feng and Walsh, 2001). During corticogenesis this complex cytoskeletal network undergoes drastic changes during different developmental stages (Feng and Walsh, 2001; Haydar et al., 2003b; Xie et al., 2003; Bielas and Gleeson, 2004; Solecki et al., 2004). While the factors involved in migration are becoming increasingly clear, little is known about the dynamic regulation of microtubular and cytoskeletal elements of earlier processes, like nuclear translocation, mitotic spindle assembly and neuronal proliferation. Recent evidence indicates that RGCs not only guide the leading processes of migratory neurons, but can also undergo cell divisions and generate neurons themselves. As such, they represent an intermediate stage in the stem-cell lineage of the CNS (Alvarez-Buylla et al., 2001) (Malatesta et al., 2000; Noctor et al., 2001; Gotz et al., 2002; Malatesta et al., 2003; Anthony et al., 2004; Haubensak et al., 2004).

Many human cortical developmental disorders such as lissencephaly and doublecortex syndrome are associated with genetic defects in MAP-related neuronal migration (Taylor et al., 2000; Feng and Walsh, 2001; Bielas and Gleeson, 2004). For example, doublecortin (DCX) induces phosphorylation-dependent microtubule stabilization and is mainly important for neuronal migration and correct cortical layering (des Portes et al., 1998; Gleeson et al., 1998; Francis et al., 1999; Gleeson et al., 1999; Horesh et al., 1999).
Addendum

1999; Taylor et al., 2000; Bai et al., 2003; Gdalyahu et al., 2004; Schaar et al., 2004; Tanaka et al., 2004). Whereas DCX mutations cause migratory and cortical positioning defects in humans (des Portes et al., 1998; Gleeson et al., 1998), DCX null mice, paradoxically, show no cortical malformations (Corbo et al., 2002). This suggests the presence of related, complementary genes. Indeed, in the human and rodent genome doublecortin-like kinase (DCLK) is present, that has substantial sequence identity similar to DCX. The complex human DCLK gene is subject to extensive alternative splicing, generating at least nine different proteins (Matsumoto et al., 1999; Burgess et al., 2002; Engels et al., 2004). Although the DCLK gene exerts important roles in calcium-dependent neuroplasticity and apoptosis in adulthood (Burgess et al., 2001; Kruidering et al., 2001) little is known about its role in CNS development. One transcript, DCLK-long, is expressed during development from ED12 onwards (Omori et al., 1998; Mizuguchi et al., 1999; Lin et al., 2000) and is, similar to DCX, capable of microtubule polymerization (Omori et al., 1998; Mizuguchi et al., 1999; Burgess et al., 2000; Lin et al., 2000). Interestingly, the analogue of DCLK in C. elegans, zyg-8, is involved in mitotic spindle positioning (Gonczy, 2001), suggesting a role for DCLK in cell division. Additionally, the DCLK gene has recently been implicated in mitotic spindle formation (Shu et al., 2006), migration (Koizumi et al., 2006) and microtubule-associated vesicle transport (Deuel et al., 2006). Given this, the DCLK and DCX genes may function in a partially redundant pathway during cortical development (Koizumi et al., 2006).

The recent suggestion that distinct domains of DCLK have different developmental functions (Burgess et al., 2002; Koizumi et al., 2006; Shu et al., 2006; Weimer and Anton, 2006) raises the question as to whether DCLK-derived proteins can have an exclusive role during early corticogenesis when DCX is absent. This period (E8-E12) is poorly characterized for DCLK, but is of great interest because specific factors controlling division and migration at these early ages will directly impact the development of the progenitor pool. As a result, it may affect layer formation and cortical cell number.

Moreover, the main mode of migration between E9-12 is through somal or nuclear translocation. The migration shifts to a different mode after E12, when migratory neuronal precursors begin to rely heavily on RGC scaffold guidance (Schmechel and Rakic, 1979; Gupta et al., 2002; Marin and Rubenstein, 2003; Kriegstein and Noctor, 2004). Here, we demonstrate that DCL, a poorly characterized splice-variant of the DCLK gene, plays a major role in early corticogenesis regulation, and is not only important for RGC or neuronal precursor division but also for radial fiber stability.

Materials and methods.

Cloning of the Murine DCL

We have cloned a novel CaMK-related peptide (CARP) (Vreugdenhil et al., 1999). As the predicted C-terminal amino acids of CARP are highly similar to the C-terminal 17 amino acids of DCX and located on a single exon (exon 8 in Figure 1A), we suspected an alternative splice product of the
DCLK functions in early cortical development

DCLK gene, which could combine this exon with upstream located DCX-like exons. To test this, we have developed an antisense primer 1A: CTGGAATTCTTACACTGAGTCTCCTGAG (EcoR1 site underlined) corresponding to the stop-codon region of the CARP-specific exon and a sense primer 2S: GCAGGTCTCAGCATTACCC corresponding to exon 3 of the murine DCLK gene. As predicted, in 30 cycles of PCR, we amplified a 457 bp fragment using mouse embryonic cDNA as a template and polymerase PfU (Stratagene).

DNA sequence analysis confirmed the DNA sequence was DCLK specific. Subsequently, a DCL cDNA encoding the complete DCL protein was amplified using CCAGGATCCACCATGTCGTTCGGCAGAGATAG (BamH1 site underlined) as a sense and 1A as an antisense primer. It was cut with BamH1 and EcoR1 and subcloned in the expression plasmid pCDNA 3.1 (Invitrogen, Groningen, The Netherlands). A DCL-EGFP construct was generated by subcloning a KpnI/EcoRV DCL fragment from pCDNA3.1.DCL in the Smal/KpnI site of pEGFP-C1 (Clontech; see also Figure 1).

In situ hybridization

DCL mRNA includes exon 8 (Figure 1), which is absent in most other DCLK transcripts, except for CARP. Because CARP is expressed at very low levels during embryonic development (see RT-PCR experiments) we have developed a 40-mer antisense oligonucleotide 5'-TTTGCTGTTAGATGCTTGCTAGGAAATGGGAAACCTTGGA-3', complementary to position 280-241 of AF045469 and specific to exon 8. Therefore, the ISH signals obtained with these probes represents DCL and not DCLK-long. As a negative control we have used 5'-TTTGATGTTATATGCTTGATTAGGACATGGGACACCTGGA-3', which contains 6 mismatches (underlined). Both oligonucleotides were end-labelled with α-33P dATP (NEN Life Science Products, Hoofddorp, The Netherlands, 2000Ci/mmol, 10 mCi/ml) using terminal transferase, according to the manufacturers instructions (Roche Molecular Biochemicals, Almere, The Netherlands). In situ hybridization and visualization of the signals were performed as described before (Vreugdenhil, 2001; Engels et al., 2004).

Antibodies

The anti-DCLK-antibody that specifically recognizes DCL at these ages (see Figure 4), has been described previously (Kruidering et al., 2001). Mouse monoclonal anti-a-tubulin was obtained from Sigma. Goat polyclonal anti-doublecortin (C-18) antibody, rhodamine-conjugated secondary antibodies and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc.

Cell culture and treatments

All cell culture chemicals were obtained from Life Science Technologies, Inc. unless otherwise stated. All cells were maintained at 37°C, 5% CO2. COS-1 cells were cultured in Dulbecco's modified Eagles medium (DMEM), supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin,
and 10% Fetal Bovine Serum. NG108-15 and N115 cells were cultured in DMEM without sodium pyruvate, supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, hybridoma (HAT) mix, and 10% Fetal Bovine Serum. For transient transfection experiments, cells were cultured on plates or coverslips coated with poly-L-lysine.

siRNA experiments
For siRNA experiments, the mouse neuroblastoma cell-line NIE-115 (ATCC number CRL-2263) was used because it was found to endogenously expresses DCL (data not shown). Three different synthetic RNA oligo's, 5'-GCTCACTCCTCGAGCAGGT-3' and 5'-CCTGCTCGAAGGAGTGAGCTT-3' (annealed siDCL-1), 5'-'CAAGAGAGCGGCUCACUCCTT-3' and 5'-GGAGUGAGCGUCUUCUGTT-3' (annealed siDCL-2) and 5'-'GAAAAGCCAGAGUUCGATT-3' and 5'-TCGACCUCUUGGCUUCTT-3' (annealed siDCL-3) in which the 3' thymidines are deoxynucleotides, were obtained from Eurogentec. For gene silencing, 60 pmol siRNA duplex was dissolved in 50 ml opti-MEM (Life Technologies) and mixed by pipetting with 3 ml oligofectamine reagent (Invitrogen) and dissolved in 12 ml opti-MEM. After 20 minutes of incubation at room temperature, the volume was increased with 32 ml opti-MEM and the total mixture (100 ml) was added to the cells (500 ml). After 48 hours, gene silencing was tested by Western blot analysis and immunofluorescence.

Immunocytochemistry
Cells were transfected as described above. At the indicated times, cultured cells were fixed with 80% aceton in water onto coverslips. Following a washing and block with 5% Normal Goat Serum (NGS, Sigma) in PBS, primary antibody incubation was followed by rhodamine-conjugated secondary antibodies. Nuclei were stained with 0.2mg/ml Hoechst 33258 and images were obtained with an Olympus AX70 fluorescent microscope coupled to a Sony 3CCD color video camera operated by AnalysisR software (Soft Imaging System, Corp.). To obtain information on its in vivo role, DCL protein distribution was mapped in CD 1 mouse embryos of ED 9, 10 and 11 that were dissected, shortly washed in PBS and then fixed for 4 h in methanol/acetone/water (40:40:20)(MAW). They were then stored for 2 weeks in ethanol 70% and embedded in Paraplast Plus (Kendall, Tyco Healthcare, Mansfield, MA 02048, USA). 6 um thick sections were cut. After clearing and rehydration, aspecific binding was reduced using 1% milkpowder solution (Campina, The Netherlands) in PBS. The primary DCL antibody was applied at a 1:50 dilution in 0.25 % gelatin/0.5% triton X-100 in TBS (Supermix). The secondary antibody was biotinylated anti-rabbit (Amersharm Life Sciences, 1:200) that was amplified two times with avidin-biotin (ABC) Elite (Vector Laboratories, Burlingame) and biotinylated tyramide (1:500) with 0.01% peroxide. Chromogen was visualized with diaminobenzidine (DAB), and sections were counterstained with cresyl violet.
DCL functions in early cortical development

For comparison, DCX protein distribution was studied in adjacent sections using the C-18 Doublecortin antibody (Santa Cruz Biotechnology, South Cruz CA, USA) at a 1:75 dilution. The same protocol was used as above, except for omission of the blocking step and the use of a biotinylated anti-goat secondary antibody.

Immunofluorescence

Sections were mounted on Superfrost Plus slides (Menzel-Gläser), dewaxed, hydrated and incubated in Zamboni-mix (2% PFA/0.1% Picric Acid/0.025% glutaraldehyde/0.1M phosphate buffer [pH 7.4]). To retrieve masked antigen epitopes, microwave pretreatment was performed in 0.1M citrate buffer [pH 3] for 15 min. Primary antibodies were C-18 (used at 1:100), 370 (used at 1:50) and vimentin (used at a 1:150 dilution in 1% BSA/0.1% Triton X-100/3% donkey normal serum/TBS (TBS++)). Secondary antibody was conjugated to Alexa Fluor488 (1:400 anti-goat; Molecular Probes, Oregon, USA), Cy3 (1:400 anti-rabbit; Jackson Immunoresearch) or Alexa Fluor647 (1:400 anti-mouse; Molecular Probes, Oregon, USA). Sections were mounted in Vectashield containing DAPI or Hoechst and examined using a Zeiss LSM510 confocal microscope.

Protein extraction and Western blotting

Mouse tissue and cells were solubilized with lysis buffer (20mM triethanolamine pH 7.5, 140mM NaCl, 0.05% deoxycholate, 0.05% dodecyl sodium sulfate, 0.05% triton X-100), supplemented with CompleteTM EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals) and centrifuged at 16,000xg for 30 minutes. Supernatant was collected and the protein concentration was determined using the Pierce method. Equal amounts of protein were separated by SDS-PAGE, transferred to immobilon-P PVDF membranes (Millipore). Blots were blocked in Tris-buffered saline, 0.2% Tween 20 (TBST), with 5% milk and incubated with primary and horseradish peroxidase-conjugated secondary antibodies. Antibody binding was detected by ECL (Amersham Pharmacia Biotech).

In utero electroporation

All experiments with embryos including the IUE, were approved by the animal experimental committee of the University of Leiden. All animal use and care during IUE were in accordance with institutional guidelines, University of Utrecht Experimental Animal Committee protocol # 05.08.076. The IUE experiments using DCL siRNA were performed in C57Bl/6JolaHsD mice purchased from Harlan Laboratories (Horst, The Netherlands). Euthanization was performed by cervical dislocation. The day of the vaginal plug was considered embryonic day 0.5 (E0.5) and the day of birth as postnatal day 0 (P0).

Cells lining the ventricle of the developing cortex were transfected with DCL siRNA in vivo by means of in utero electroporation (IUE). Timed, pregnant C57Bl/6JolaHsD mice were anaesthetised with a cocktail of Ketamine/Xylazine (100mg/kg and 10mg/kg, respectively). Following
laparotomy, plasmid DNA (2 mg/ml for the control plasmids pCMV-YFP and 2 mg/ml for pSuper-DCL183 and pSuper-DCL183mismatch in Tris-buffered 0.02 % Fast Green) was injected through the uterine wall into one of the lateral ventricles of E14.5 embryos using calibrated pulled glass capillaries (WPI, Sarasota) in a survival surgery set-up. Double-stranded small hairpin DNA was subcloned in pSuper (Brummelkamp et al., 2002) with DNA sequence for pSuper-DCL183: CCAAGCTAAGATGTCTTTA and for pSuper-DCL mismatch: CCACGCTAAGCTGTCTTTA. One hemisphere was used for the mismatch or the DCL siRNA, whereas the contralateral hemisphere served as an internal control. A series of five unipolar squarewave current pulses (33 V max) were delivered over the embryo’s head using ’Tweezertrodes’ (BTX, Harvard Apparatus). Embryos were placed back into the dam and gestation was allowed to proceed. Embryos were harvested one day later and processed for immunocytochemistry. Only embryos showing effective electroporation within the target area were included in the analyses.

Pictures were taken using either a Zeiss Axioplan epifluorescence microscope or a Leica TCS NT confocal microscope. Alexa488 was excited at 488 nm, Alexa555 at 568 nm and emissions were detected at 500-540 nm and 580-650 respectively. Excitation of the two fluorophores was performed sequentially.

Data analysis
At least 3 embryos per group (control versus DCLI) were analysed per timepoint. Only animals in which the cortex was broadly transfected were included in the analysis. Optical fields of 3-7 sections per animal, at least 80 μm apart, and at the same neuroanatomical level in each group, were captured with MCID software. A 10X objective of a Zeiss Axioplan was used and a bin of 0.2 mm wide was placed in the center of the transfected area. The length of each embryonic zone (proliferative/ventricular zone (PZ), intermediate zone (IZ) and cortical plate (CP)) was measured per bin in each optical field. YFP-positive cells were quantified per zone in each optical field. Data were normalized to total number/mm², averaged for each embryo, pooled and tested for significance by one-way analysis of variance (ANOVA; α=5%) and expressed as mean ± SEM.

Results
DCL, a microtubule-associated protein, is abundantly expressed during the earliest stages of neocortical development and precedes the onset of DCX expression

DNA sequence analysis of a DCL cDNA clone revealed an open reading frame of 362 amino acids with a predicted molecular mass of 40 kDa (Figure 1B) that also showed a 73% amino acid identity (81% similarity) with mouse DCX over the entire length (see Figure 1). We have shown that, similar to DCX, DCL is a microtubule associated protein (MAP) that can stabilize microtubules (Figure 2, color figure).
DCL functions in early cortical development

(A) Genomic organization of the DCLK gene and the cloning strategy of the DCL cDNA. Only the exon-intron structure of the DCL part is indicated including the recently identified exon 8 encoding the common 3' end of CARP as well as DCL [Vreugdenhil, 2001]. Exons are represented by rectangles and indicated by arabic numbers; introns are solid lines. The DCL transcript derived from this part of the DCLK gene is indicated below (DCL) the genomic structure. The ORF is represented by a rectangle, non-translated sequences by lines. The part of the protein that overlaps with the unique synthetic peptide sequence used to generate the DCLK antibody is indicated in (non-hatched) grey, and involves the combined spliced sequence of exon 7 and 8. The synthetic oligonucleotide, used in the ISH experiments and labeled by 33P, is indicated below the DCL mRNA. It is complementary to the 3'-untranslated region that is derived from exon 8. This exon is incorporated in the DCL mRNA but not in that of DCLK-long. The location of the primers used to clone DCL is indicated by arrows. The position of siDCL-1, siDCL-2 and -3 used to silence expression of DCL are indicated by bars.

(B) Alignment of the mouse DCL protein with DCX. Identical residues are dark grey and conserved substitutions are light grey. The two DCX domains and the SP-rich domain are indicated by arrows.

Figure 1. Genomic organization of DCL and alignment with DCX.
To confirm DCL is a MAP, three experiments were conducted. Firstly, expression of DCL in transfected COS-1 cells resulted in a fibrillar somal staining pattern overlapping the microtubular distribution (Panel I A-C). Overexpression of DCL lead to clear bundling of microtubule filaments (see also Fig. 6). Secondly, exposure to 10 mg colchicine, a compound that depolymerizes and disrupts tubulin microtubules, revealed around 90% of the DCL transfected cells to be resistant to 1-hr colchicine treatment (Panel I D+F). Thirdly, recombinant DCL polymerizes microtubules in a dose-dependent manner in an in vitro polymerization assay as described before [13] (Panel II). This shows that DCL, like DCLK-long and DCX, can directly polymerize and stabilize microtubules.

Legend: Green represents DCL; red represents α-tubulin and yellow indicates co-localization. Blue represents (nuclear) DNA. Arrows in F indicate DCL-associated microtubule bundles, which are resistant to colchicine treatment. Also note the clear association of DCL with a centrosome in A. Scale bar is 10 mm.
DCL functions in early cortical development

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CARP

II

A

n.g.

n.e.

ED8

B

me

mv

rh

di

tv

te

n.e.(o.n.t)

ED10

C

mt

me

ED12

D

iv

lv

cmp

nc

mo

di

Control
To study a possible role for DCL in early neurogenesis, we first analyzed the spatio-temporal expression pattern of DCL mRNA during early embryonic development. RT-PCR with DCL specific primers clearly showed mRNA expression in embryo heads of ED8 and ED10, with the highest expression found at ED12 and ED14, (Figure 3I). The expression slowly declined after ED14. In contrast to DCL, expression of CARP, a closely related but different splice variant (see methods and [Vreugdenhil et al., 1999]) was nearly absent during embryonic development (Figure 3I).

Using in situ hybridization with an oligonucleotide probe recognizing the 3’UTR of the DCL transcript but not the DCLK-long transcript (see methods and Figure 1A) we observed DCL mRNA expression from ED 8 onwards, along the length of the early neuroepithelium, a major site of neurogenesis (Noctor et al., 2001; Haubensak et al., 2004) (Figure 3IIA). At ED10, when massive precursor division occurs, substantial DCL expression was found in the early diencephalon, telencephalon, mesencephalon and neural tube (Figure 3IIB). Consistent with our immunocytochemical data (see below), DCL expression at ED12 was greatly elevated throughout the main neurogenic regions of the developing CNS, relative to ED8/10 (Figure 3IIC). Mismatch controls yielded no signal (Figure 3IID).

Western blot analysis of embryonic brain homogenates probed with an antibody that selectively recognizes DCL during development (Figure 4) and (Kruidering et al., 2001), revealed a 40 kDa protein and no other DCLK-related immunoreactive bands (e.g. the 80kd DCLK-long (Burgess et al., 2002) or 80kd DCK2 protein (Edelman et al., 2005); Figure 5). DCL protein on blot was already expressed from ED10 onwards with the highest levels of DCL protein found at ED12 and ED14 after which expression levels gradually declined. In contrast to DCL, DCX protein expression was not detected until ED 12, not even after prolonged exposure (Figure 5B).
DCL functions in early cortical development

The generation of the anti-DCLK antibody used in this study has been described in detail before (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 CaMK) (Kruidering et al., 2001) that are, however, only expressed in adult brain and not during development. Specificity of anti-DCL was analyzed by overexpressing DCX and DCL in COS-1 cells and assessing possible cross-reactivity by Western blot. Anti-DCLK strongly recognized DCL (lane 3-5) whereas only some weak cross-reactivity was observed with DCX (lane 2). On the other hand, the DCX antibody raised against the C-terminal 17 amino acid of DCX, strongly recognized DCX (lane 2) and not DCL (lane 3-5). Thus, anti-DCLK strongly recognizes DCL whereas the DCX antibody is specific for DCX alone and not for DCL.

The weak cross-reactivity of anti-DCLK with DCX is not relevant in vivo as in our confocal studies DCX-positive fibers failed to stain with anti-DCLK (Fig. 3 III), in addition, knock down of DCL by si-RNA lead to a complete disappearance of anti-DCLK immunostaining (Fig.4) indicating a high specificity of the anti-DCLK antibody for DCL. Lane 1: COS-1 cell lysate transfected with empty vector.

We next performed immunocytochemistry to map the spatio-temporal distribution of DCL protein at ED 8, 9, 10, and 11. DCL was expressed in the main neuroepithelia from ED 9 onwards, an age at which DCX is still absent (Figure 6, compare B with C, color figure). At ED 10 and 11, DCL was strongly and selectively expressed in the telencephalon, diencephalon, dorsal root and sympathetic ganglia e.g., whereas non-neuronal tissues were DCL negative (Figure 6A). DCL was expressed in many radial processes originating from the neuroepithelia and extending radially towards the pial surface. Transversal cross-sections illustrated a differential, and only partly overlapping distribution pattern of DCX and DCL. DCX

Figure 4. Characterization of the antibody recognizing DCL.

The generation of the anti-DCLK antibody used in this study has been described in detail before (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 CaMK) (Kruidering et al., 2001) that are, however, only expressed in adult brain and not during development. Specificity of anti-DCL was analyzed by overexpressing DCX and DCL in COS-1 cells and assessing possible cross-reactivity by Western blot. Anti-DCLK strongly recognized DCL (lane 3-5) whereas only some weak cross-reactivity was observed with DCX (lane 2). On the other hand, the DCX antibody raised against the C-terminal 17 amino acid of DCX, strongly recognized DCX (lane 2) and not DCL (lane 3-5). Thus, anti-DCLK strongly recognizes DCL whereas the DCX antibody is specific for DCX alone and not for DCL.

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Figure 5. Western blot analysis of DCL and DCX in embryonic lysates obtained at ED8 to ED18.

Note that the 40 kd DCL protein is already expressed at ED10, without any other bands visible. DCX protein expression is observed from ED12 onwards.

We next performed immunocytochemistry to map the spatio-temporal distribution of DCL protein at ED 8, 9, 10, and 11. DCL was expressed in the main neuroepithelia from ED 9 onwards, an age at which DCX is still absent (Figure 6, compare B with C, color figure). At ED 10 and 11, DCL was strongly and selectively expressed in the telencephalon, diencephalon, dorsal root and sympathetic ganglia e.g., whereas non-neuronal tissues were DCL negative (Figure 6A). DCL was expressed in many radial processes originating from the neuroepithelia and extending radially towards the pial surface. Transversal cross-sections illustrated a differential, and only partly overlapping distribution pattern of DCX and DCL. DCX
expression commenced around E11, primarily in tangentially-orientated processes in the early cortical plate/marginal zone (Figure 6H), whereas DCL was prominently expressed throughout the ependymal zone from ED 9 onwards (Figure 6C,D,F,I and J) that was DCX negative at that age. Striking DCL immunoreactivity was found in mitotic cells throughout the VZ, the neocortex and neural tube as well as in blast-like cells engaged in cell cycle (Figure 6E, F, K-O). In these mitotic cells, DCL was also found associated with centrosome-like structures (Figure 6M), kinetochore microtubules (Figure 6K) and polar microtubules (Figure 6J).

To confirm the role for DCL as a neuronal precursor marker and to address its possible overlap with DCX, we performed triple immunofluorescent labeling for DCL, DCX, and the intermediate filament protein vimentin which identifies neurogenic radial glia (Noctor et al., 2001; Gotz et al., 2002; Malatesta et al., 2003; Anthony et al., 2004) (Figure 7, color figure). Triple labeling revealed a differential distribution pattern of DCL and DCX in early development with a nearly complete overlap of DCL expression with vimentin-positive cells at E12 (Figure 7G-J) mainly in the VZ, but also in individual mitotic cells (Figure 7K-N). Vimentin+, DCL+, DCX+ triple labeled cells were only seen in the IZ (white cells in Figure 7N), consistent with the presence of migratory neuronal precursors undergoing secondary divisions in these regions (Kriegstein and Noctor, 2004). In contrast, DCX (Figure 7L) did not overlap with vimentin (Figure 7K,N and P) in other regions of the neocortex or spinal cord. Many long DCL+ radial processes were devoid of DCX signal (Figure 7O). DCL+ mitotic cells in the VZ displayed vertical as well as horizontal cleavage plane orientations (Figure 7D, C, K, L and M). Taken together, DCL mRNA and protein expression are abundant in the main neurogenic regions during early cortical development, where it occurs in radial processes and mitotic cells. The spatiotemporal distribution of DCL is different from that of DCX and the onset of expression of DCL precedes that of DCX.
DCL functions in early cortical development
Addendum

Figure 6. Immunocytochemical analysis of DCL and DCX protein expression in the embryonic brain.

A: At ED 11, DCL protein (sagittal section) is found throughout the telencephalon and diencephalon (left and right, respectively). DCL is found in the outer layers close to the pia as well as in the inner VZ (arrowheads; see also below). Non-neuronal tissue like the mandibular component of the first branchial arch (M) e.g., is devoid of any signal. IV: fourth ventricle. Bar represents 150 μm.

B + C: Adjacent transversal (coronal) sections from the early neuroepithelium at ED 9 immunostained for DCX (B) and DCL (C). DCX is absent at this age (arrowheads in B), whereas DCL is abundantly expressed in the inner ependymal layer (upper 2 arrows in C) and outer, early preplate/MZ region (lower arrow pointing leftward in C). Bar represents 25 μm.

D: Sagittal section of the neuroepithelium of the neural tube at ED11, showing abundant expression at the luminal border (arrowheads), while in the developing neuronal tissue, various mitotic cells express DCL (arrows). Bar represents 70 μm.

E: Detail of a DCL+ mitotic cell in the neocortical VZ. Arrowhead points to the chromosomes oriented in the midline cleavage plane. Bar represents 5 μm.

F: DCL expression in the neuroepithelium and VZ (arrowhead on the left) of the early telencephalon at ED10. Many DCL+ processes extend radially towards the pial surface (arrowheads in upper left corner). In the preplate (arrowhead on the right), DCL is also found in tangentially orientated processes. DCL+ mitotic cells in the IZ are also visible (arrows). Bar represents 25 μm.

G: Immunostaining for the intermediate filament protein vimentin that identifies neurogenic radial glia, shows immunopositive cellbodies (arrow) in the lumenal surface (asterisks) and radial processes running through the VZ and SVZ (ED11).

H+I: Transversal cross-sections illustrating the differential, yet partly overlapping distribution of DCX and DCL. DCX is expressed at ED11 (H) in the upper preplate and CP/MZ (arrow). In contrast, DCL is already strongly expressed at ED9 (I) in the VZ/ependymal layer (arrowhead to the left), with DCL+ radial processes extending towards the IZ (arrowhead on the right). The ventricular layer (asterisks in H) is devoid of DCX, but not of DCL signal. Bar represents 5 μm.

J: Detail of the VZ at ED9 showing DCL expression in processes radially extending into the SVZ and IZ (middle arrowhead). Also a mitotic cell lacking DCL expression between the chromosomes (right arrowhead) is visible. Bar represents 12 μm.

K: Detail of the V2 showing DCL+ dividing cells, that appear to be in telophase (left) and anaphase (middle), with a vertical cleavage plane. The cell on the left is likely in telophase or may represent two cells in interphase. A DCL+ cell on the right is visible with DCL expression between the chromosomes, with a horizontal cleavage orientation (arrowheads). Bar represents 8 μm.

L: DCL immunoreactivity in the V2 at ED11, in prophase and telophase cells (arrowheads) and in a blast-like precursor cell in metaphase/anaphase (arrow). Bar represents 10 mm.

M: Two DCL+ mitotic cells in the neuroepithelium displaying expression between chromosomes (upper arrow) as well as in structures resembling centrosomes (lower arrows). Bar represents 1.5 μm.

N+O: DCL immunoreactivity in dividing cells in anaphase II / telophase II (N) and in metaphase/anaphase I, with the chromosomes clearly visible (arrow in O), and in a radially extending process (arrowheads). Bars represent 1 μm.
Figure 7. Confocal analysis of DCL and DCX protein expression in the embryonic brain.

A-D: DCL expression in the VZ [ED10] (D) and in mitotic cells at the ventricular surface (arrow in D). DCL, but not DCX (fig C), is associated with kinetochore microtubules (A; arrow) as well as polar microtubules (arrow in B). Blue stain: Hoechst identified chromatin. Mitotic cells are DCX negative (inset C, see E.F.M.N&O), indicating a function of DCL different from DCX at these ages. Bars represent 5 μm (A+B) and 12 μm (D).

E-F: At E11, many DCL+ precursor cells (arrows) are found in the VZ and SVZ (arrows in F). These blast cells resemble those shown in Figure 5D, E and F, and are DCX negative. DCX+ processes are in close apposition to, but not overlapping with, the mitotic DCL positive blasts (inset E). Bars represent 20 (F) and 5 μm (E).

G-J: Triple labeling of the neuroepithelial layer of an E13 mouse reveals expression of DCL in vimentin+ radial glial/neural precursors (merged image in J). A mitotic cell oriented close to the ventricular surface is visible (H; arrow). Bar represents 12 μm.

K-N: Triple labeling (E11) showing DCL expression in vimentin+ RG C throughout the neuroepithelium and VZ (upper arrowhead in K and M), and in neuroblasts in the SV2/SV3 (arrow on the left in K and M). This distribution pattern is distinctly different from DCX (shown in L), with weak expression in o.o. tangentially oriented migratory fibers in the VZ/SV2, and expression in the early preplate/primitive plexiform zone (PPZ) (H). Only in the second proliferative layer between (SV2 and PPZ, where migrating precursors undergo M phase, are vimentin+, DCX+ and DCL+ triple labeled cells found (white; lower right arrow and cell on the left in N). Precursors in the VZ/lower SVZ are only double positive for vimentin and DCL (pink cells; upper right arrow). P: pial surface. Bar represents 35 μm.

O: DCX and DCL colabeling of the spinal cord (E11) reveals DCL expression at upper and lower border regions (arrows) with long processes (arrowheads) extending radially, amidst of extensive (non-overlapping) DCX expression.

P: Unlike DCL, DCX expression is not expressed in vimentin+ RG C (2 lower arrows) in the neural tube at E11. Only very rarely is colabeling seen (upper arrow), indicating neuronal migration under guidance of RG C. Bar in O and P represents 18 μm.

To address whether DCL is associated with a neuroblast phenotype, we screened a large number of different cell lines and found that DCL is expressed endogenously only in neuroblastoma cell lines (Figure 8).

Figure 8. DCL is a phosphoprotein, that is endogenously expressed in neuroblastoma, but not in other cell lines.

To study DCL subcellular functions, various cell lines were screened for endogeneous DCL expression, which was found in neuroblastoma lines, and not in any other cell line, including PC12 cells (data not shown). In the neuroblastoma cell line N1E-115, a 40 kDa immunoreactive doublet co-migrated with the doublet resulting from overexpressing DCL (Panel B). RT-PCR experiments and Western blot analysis failed to detect any DCX signal using DCX-specific primers and antibodies (data not shown) showing that this 40 kDa band was not due to DCX. The doublet disappeared after incubation with phosphatase, demonstrating that DCL, similarly to DCX, is a phosphoprotein.

A: Screening by Western blot analysis of various cell lines. Lane 1: COS-1 cells, lane 2: HeLa cells, lane 3: NG108-15 cells, lane 4: NS20Y cells, lane 5: N1E-115 cells, lane 6: molecular weight marker, lane 7: SHSY5 cells. Three more non-neuroblastoma cell lines were screened including PC12 cells, that also failed to reveal any endogenous DCL expression (data not shown).

B: DCL is a phosphoprotein, NG108-15 lysates stained with anti-DCL. Lane 1: untreated lysate, lane 2: lysate incubated at 37 °C without phosphatase, lane 3: lysate incubated at 37 °C with phosphatase. Lane 4-6 are similar as 1-3 but after DCL overexpression. Note that endogenous DCL comigrates with overexpressed DCL in lane 4-6.
DCL functions in early cortical development

Figure 9. DCL knock-down leads to deformation of mitotic spindles.

I: Effectiveness of RNA interference to knock-down DCL.
Western blot analysis of DCL expression in N1E-115 cells with (1 to 3) and without (4) siRNA treatment performed in duplo. Three different siRNA molecules targeting DCL were used: siDCL-1 (lanes 1), siDCL-2 (lanes 2) and siDCL-3 (lanes 3). siDCL-2 and 3 induce an effective knock-down (80% and 90% respectively) while siDCL-1 failed to do so, and was subsequently used as control for the siRNA procedure. As a reference, the same membrane was re-stained with alpha-tubulin.
II: Confocal analysis of mitotic neuroblastoma cells treated with DCL siRNA. In non-treated mitotic cells (A-C), DCL (A) colocalizes with alpha-tubulin (B). The merged image (C) indicates DCL association with polar and kinetochore microtubuli (arrow). Transfection with siDCL-1 (D-F) failed to induce DCL knockdown (D), and left the mitotic spindle intact (E). Effective DCL knockdown by siDCL-2 (G-I) or siDCL-3 (J-L) not only caused all immunoreactive DCL signal to disappear (G, J), but also induced collapse and deformation of the mitotic spindles (H, K). Green=DCL, Red=alpha-tubulin, Yellow=merged. Scale bar: 10 μm.
Suppression of DCL function by siRNA affects microtubule architecture and disrupts the mitotic spindle in neuroblastoma cells.

Functional aspects of DCL were addressed using three different siRNA molecules against DCL (see Figure 1A). Western blot analysis indicated that siDCL-1 failed to knock-down DCL protein (data not shown), which might relate to the lack of TT dinucleotides at the 3'-end in this antisense strand. SiDCL-1 was subsequently used as a negative control for possible off-target effects. Compared to non-treated and siDCL-1, transfection with siDCL-2 and siDCL-3 induced a knock-down of respectively 80% and >90% (data not shown).

Our immunohistochemical data suggested a role for DCL in proliferative division of neural precursors. Therefore, we have studied dividing N1E-115 cells after DCL knock-down by siRNA technology. Strong DCL immunoreactivity was observed in all dividing N1E-115 cells during metaphase or early anaphase (see Figure 9A and D, color). α-tubulin colabeling confirmed that DCL was expressed in association with the mitotic spindles and near the centrosome, suggesting a role for DCL in mitotic spindle formation. Indeed, DCL knockdown by siDCL-2 and siDCL-3 caused a dramatic collapse of the mitotic spindle (Figure 9G-L) in approximately 40% of all dividing cells (10 out of 24 cells in metaphase in two independent experiments) for siDCL-2 and in all dividing cells (30 out of 30, in two independent experiments) transfected with siDCL-3. Inefficient knockdown by siDCL-1 left DCL colocalization as well as the mitotic spindle unchanged (Figure 9D-F), while also the centrosome appeared normal when DCL was knocked down. Clearly, DCL is involved in mitotic spindle stability in neuroblastoma cells. Combined with DCL’s in vivo distribution, this indicates a role for DCL in neuronal precursor division.

DCL overexpression induces elongated and aberrant mitotic spindles.

Next, we tested the effect of DCL-overexpression on mitotic spindle formation in dividing COS-1 cells, a cell line that does not express DCL endogenously. Consistent with our earlier observations, DCL colabeled mitotic spindles in transfected COS-1 cells (see Figure 10, color). DCL gain-of-function in these cells induced two different phenotypes: the first, observed in 20% (n=126) of the dividing COS-1 cells analyzed, was characterized by DCL colabeling with α-tubulin. This phenotype is similar to the endogeneous pattern in dividing N1E-115 cells (Figure 10D-F). The second phenotype was present in 80% of the cells in M phase and was characterized by abnormally oriented (Figure 10B), elongated, asymmetrical, monopolar mitotic spindles. This phenotype was also characterized by an aberrant segregation of the chromosomes (blue stain in Figure 10C). The elongated, asymmetrical spindles of cells overexpressing DCL were clearly different from the stereotypical bipolar spindles in control cells (‘ref’ in inset in Figure 10B). This indicates that DCL can regulate mitotic spindle stability and formation.
DCL functions in early cortical development

Figure 10. DCL overexpression in dividing COS-1 cells.

A-C: A normally dividing COS-1 cell stained with alpha-tubulin is shown as reference ("ref" in inset). Overexpression of DCL (Green, A) leads to often unilateral, elongation of the mitotic spindle microtubules (B). The mitotic spindle length, indicated by arrows, of transfected cells was enhanced compared to non-transfected cells (ref: reference length). DNA is stained with DAPI (blue).

D-I: DCL overexpression in COS-1 cells during cell division revealed two phenotypes: one very similar (D-F) to wildtype COS-1 cells, in which DCL (D) largely colocalizes with a-tubulin (E). Similarly to the dividing N1E-115 cells, DCL (in green) is also associated with kinetochore microtubull and overlaps with mitotic spindles (alpha-tubulin in red). The predominant other phenotype revealed a profound elongation and monopolar orientation of the mitotic spindles (G-I). Green=DCL (A, D, G), Red=alpha-tubulin (B,E,H), Yellow=merged (C, F, I). Scale bar is 10 mm.

RNA interference in utero demonstrates an in vivo role for DCL in radial process stability and progenitor division.

To study the in vivo role of the DCLK gene in RGC proliferation and in stability of the radial processes, we performed in utero electroporation (IUE) using RNA-interference in cells lining the ventricle of the developing cortex. Plasmids encoding effective short-hairpin RNA molecules were selected for DCL knock down by transfection of neuroblastoma cells and monitoring the endogenous DCL protein levels by Western Blot analysis. As negative controls, we used mismatch short hairpin siRNA molecules and empty vector. The most effective plasmid, pSuper-DCL183 and its mismatch control (pSuper-DCL183m), were used for IUE. To visualize transfected cells, plasmids were co-transfected with plasmids encoding Yellow Fluorescent Protein (YFP) and immunocytochemically detected using an antibody against GFP/YFP (1:3000; Molecular Probes). IUE with pSuper-DCL183 performed at E13.5.
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yielded a 0% (n=16) survival rate compared to 40% (n=20) survival for the control plasmids pCMV-YFP. This indicates a specific lethal effect of DCL knock down at this particular time of development. Therefore, we applied IUE at E14.5 yielding 92% survival in 70% of the embryos and also showed YFP expression for the control plasmids. This is compared to 84% survival of which 73% expressed. YFP+ cells containing the siRNA for DCL were found coextensive with spots of reduced or absent DCL immunoreactivity, indicating the effectiveness of RNAi of DCL. This is comparable to the in vitro findings (Figure 11C, color).

Embryos transfected with empty vector or mismatch controls at E 14.5 and collected 24 hours later revealed proper cortical development and large numbers of YFP-expressing almond-shaped progenitors in the proliferative zone (PZ), whereas animals collected after longer survival times did not survive (not shown). Radial fibers spanning all embryonic cortical zones with tight endfeet at the pial surface were present. One day after transfection with pSuper-DCL183, cell number in the PZ was significantly reduced, and occasionally, YFP+ cells accumulated in the upper region of the SVZ/VZ (Figure 11F). Cortical thickness of the PZ, IZ and CP did not differ between the groups (data not shown). When expressed as percentage of all cells in PZ and IZ, no differences were observed between numbers of cells (data not shown). This lack of difference indicates no changes in migration or positional mismatch, thus implying that all cells are affected in a similar manner and their position has not changed as a result of the knockdown.

Structural organization of the dividing precursors was aberrant and many of the remaining cells had an enlarged, multipolar morphology, and lacked, or only had a very short, leading and/or trailing process (Figure 11C, F, K, M), typical of radial migrating neurons. Quantification revealed a significant reduction in the number of YFP+ cells between the control and DCL RNAi group (Figure 11O) in the PZ (control, 3752.3 ± 400.9; pSuper-DCL183, 2180.7 ± 115.64; P < 0.05*). Thus, the decrease in number of cells in the PZ may reflect a decrease in progenitor proliferation at the PZ/IZ boundary.
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A: Control plasmids pCMV-YFP delivered into E14.5 embryonic brain labeled many neural progenitors in SVZ/VZ (asterisk) and an extensive network of radial processes spanning the entire thickness of the cortex with a dense rim of pial endfeet (arrow, detail in J). Mismatch control plasmids revealed identical results (details in H).

B: Knockdown of DCL after pSuper-DCL183 delivery induced a profound reduction in SVZ/VZ precursor number and an almost complete ablation of the radial processes, with small cells in the IZ (arrowhead) and very few pial endfeet remaining (arrow).

C: DCL knockdown reduces DCL immunoreactivity (red). At the top right end border of a pSuper-DCL183 transfected zone, DCL is abundant, compared to the inner layers in the transfected zone on the left (asterisk), that are DCL negative, YFP+ cells are frequently accompanied by “dark holes”, devoid of DCL signal (arrow in C, and in C1 (only red channel shown)), indicating effective DCL protein knockdown. Control plasmids did not alter DCL immunoreactivity (C2 and also J).

D+E: After control plasmid delivery, the SVZ/VZ showed numerous dividing (arrows) and migrating precursors (inset), with straight radial fibers (arrowheads). DCL knockdown reduced progenitor number, with the remaining ones displaying an aberrant, disorganized and often multipolar cell shape (arrows, inset). Processes of these cells, if any, were aberrant and generally very short (arrowheads in E, inset).

F: Overview of the aberrant organization of cells in the PZ and the shortened and aberrantly oriented processes in IZ and CP after DCL knockdown.

G: Detail of a straight, long radial fiber in the IZ after control vector delivery.

H: A similar fiber after delivery of the pSuper-DCL183 mismatch control. A straight and long process with tight endfeet at the pial surface is seen (arrowhead).

I: Detail of a cell transfected with control vector, with a normally appearing process without any detectable effect on adjacent DCL immunoreactivity (red).

J: Details of the radial processes in the CP and their endfeet (arrowhead) at the pial surface after control plasmid delivery. Knockdown of DCL caused very few, if any endfeet to reach the pia (no detail shown, but see B and F).

K + L: Detail of the IZ after DCL knockdown (left is SVZ/VZ, to the right is CP), showing many aberrantly organized, shortened, curved, twisted, and occasionally obliquely oriented (lower right arrow in L) individual processes (arrows) and small cellular elements in the inner zones (arrowhead in K).

M: Example of a clearly disorganized cell shape in the SVZ/VZ after RNAi for DCL.

N: Example of a curved and turning (arrow) process in the IZ after RNAi for DCL.

O: Numbers of electroporated cells in the proliferative zone (PZ) and IZ are significantly reduced in the PZ. In the CP, no differences were found (not shown).

Bars represent 20 μm (A), 15 μm (C+F), 10 μm (D+K), 3 mm (G,J,M,N,L) and 7 μm (H).

Furthermore, a profound reduction in the extent and number of individual radial processes was apparent, particularly in the IZ and CP, where many shortened and twisted radial processes (Figure 11B, C) were observed. Also, very few endfeet had reached the pial surface as compared to empty vector or mismatch controls (Figure 11A and B). Individual processes in the IZ were often distorted, twisted and sometimes obliquely oriented (Figure 11E, F, G, H). The straight, long processes observed in both empty vector and mismatch controls, were never seen in embryos electroporated with pSuper-DCL183 indicating a crucial role for DCL in the stabilization of microtubules as part of the cytoskeletal organization inherent of normal radial glia fiber formation. Together, these data demonstrate a critical role for DCL not only in mitotic spindle formation but also in the stability of the radial fiber network.

**Discussion.**

We have shown that the DCLK splice variant DCL is a predominant MAP during early corticogenesis. DCL regulates mitotic spindle stability, precursor proliferation and integrity of the radial fiber network, which will form the early radial scaffold, enabling neuronal migration during later developmental stages.
Our results provide an important addition to the current knowledge on DCLK, as they specifically address the role of DCL during the early stages of cortical development. Microtubule stabilization of the early pial oriented processes allows somal or nuclear translocation, a process by which neuroblasts translocate their somata along radially oriented processes. The processes are typically restricted to E10-13, the period when DCL is abundantly expressed. During that period, DCX is either absent, or expressed in a pattern that does not overlap with DCL, suggesting an important and exclusive role of the DCLK gene during early corticogenesis.

Aside from the expression in radial fibers, DCL is selectively found in RGC/neural progenitors. Recent data has shown that RGCs are the source of most, if not all, neurons generated during cortical development, with neuronal progeny being derived from clonally related RGCs (Malatesta et al., 2000; Alvarez-Buylla et al., 2001; Noctor et al., 2001; Gotz et al., 2002; Malatesta et al., 2003; Rakic, 2003; Anthony et al., 2004). The selective expression of DCL in vimentin-positive neurogenic RGCs in mitotic cells and in neuroblastoma cell lines is consistent with the concept suggesting DCL is a RGC/precursor-specific MAP. Indeed, microtubules but not microfilaments, were already shown to be critically involved in the polarized morphology of RGCs (Li et al., 2003). This centralization of neurogenesis and radial glia raises the interesting possibility that one cell type early in development is critical for division, initial neuronal production, radial process extension and hence for cortical architecture.

Recent findings by us and others (Shu et al., 2006) on the role of DCLK in mitotic spindle formation substantiate a crucial role for spindle stabilization in neuronal proliferation and differentiation. The stability and length of the mitotic spindle had already been shown to affect the fate of daughter cells in D. melanogaster: the generation of daughter cells with different sizes and phenotypes depends on the asymmetric formation of the spindles during anaphase (Kaitschmidt, 2000). Similarly, in C. elegans, the orthologue of the mammalian DCLK gene zyg-8 is involved in asymmetric division of the one-cell stage in embryos. Also, zyg-8 is associated with mitotic spindles and promotes microtubule assembly during anaphase (Gonczy, 2001). Mutations in zyg-8 disrupt division of the one-cell stage of C. elegans embryos, suggesting evolutionary conserved functions for the mammalian DCLK and zyg-8 with regards to spindle formation.

Neurogenic radial glia can generate neurons directly through asymmetric cell division, or indirectly through generation of intermediate progenitors or transit amplifying cells that migrate to the subventricular/intermediate zone (SVZ/IZ), where it divides symmetrically to produce two daughter neurons. The vimentin+, DCX+ and DCL+ triple stained cells in the SVZ/IZ (see Figure 6) represent the secondary, neurogenic divisions of migratory intermediate progenitors (Haydar et al., 2003a; Haubensak et al., 2004; Kriegstein and Noctor, 2004; Noctor et al., 2004). This illustrates interactions between DCL and DCX in these layers (Deuel et al., 2006; Koizumi et al., 2006). In the inner VZ, DCL, but no DCX expression occurs.
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in mitotic cells, with horizontal or vertical cleavage planes (Figure 6), indicating no preference for a specific mode of division.

The nearly complete depletion of the radial fiber network after in utero knockdown of DCL demonstrates a critical role in the regulation of radial fiber stability. Interestingly, electroporation of DCL RNAi, but not of empty vector controls, at ED13.5 resulted in 0% survival rate. A similar rate was obtained at survival times longer than 32 h after DCL knockdown at E14.5. Together, this indicates an essential role of the initial radial fiber network in normal development. In recent in utero knockdown studies considerably longer survival times were obtained after knockdown of DCLK. RNAi was applied at E13 and could be studied 3 to 6 days later. In addition, no changes in radial fiber integrity were reported in this study. A possible explanation may lie in their RNAi construct that targets the full length DCLK long transcript but is not capable of targeting the DCL transcript (Shu et al., 2006). Thus, these different phenotypes may pinpoint DCL as a selective protein necessary for radial fiber formation (Koizumi et al., 2006; Shu et al., 2006). Other possible explanations include the precise time-point of analysis (ED17 versus ED14 in our study) as well as the methodology used (in utero lentiviral delivery versus in utero electroporation of a large cortical swath in our study). In theory, DCL knockdown could also have contributed to the poor survival rates by affecting precursor proliferation. However, a recent study showed that despite changes in precursor number after DCLK manipulation in utero, survival of these embryos was not affected (Shu et al., 2006). As radial fibers were not altered, it is tempting to speculate that it is the radial fiber loss after DCL knockdown that influences embryo survival. The exact mechanism of how disturbances in the radial fiber network may induce premature death requires further study.

Given the early, prominent role for DCLK, one would expect a severe or even lethal phenotype in DCLK mutants. Yet, loss of the full length DCLK, the DCLK DCX-like isoform, or the kinase domain containing isoforms of DCLK, results in a preserved neocortical lamination (Deuel et al., 2006; Koizumi et al., 2006). Similar discrepancies between acute RNAi mediated knockdown and germ line knockout have been described for the DCX gene. These result in a severe or very mild phenotype, respectively (Corbo et al., 2002; Bai et al., 2003). This suggests that other genes could have compensated for the loss of DCX function in DCX knockout mice. Indeed, only double DCLK/DCX null mice display disrupted cortical lamination (Koizumi et al., 2006; Shu et al., 2006). Recent evidence (Ramos et al., 2005) further indicates that the RNAi effect for DCX is rat-specific, as knock down of DCX in mice failed to induce subcortical band heterotopia. This bears considerable relevance to the relatively mild phenotypes in DCLK mouse mutants (Deuel et al., 2006; Koizumi et al., 2006), which could relate to genetic redundancy with another recently described DCK2 gene (Edelman et al., 2005). The levels of DCK2 were indeed not altered in DCLK homozygous null mouse brains (Koizumi et al., 2006).

Various genes associated with the centrosome and spindle formation are key elements in mitosis regulation. Mutations in the genes for
DCX and DC L are expressed that both promote microtubule stability. But, in contrast to recent studies on the DCLK gene, DCX and DCL differ in their early spatio-temporal expression pattern and function. Although their functions at later stages of corticogenesis appear synergistic and largely overlapping, DCX is mainly involved in the stabilization of microtubules during neuronal migration after E12. DCL expression, in contrast, starts earlier, i.e. around E8, and is involved in stabilization of radial processes and in mitosis of neurogenic precursors through spindle stabilization. This early developmental expression, in contrast to other DCLK splice variants, is of particular general relevance as progenitor pool expansion and neuronal production are extensive during this period. Interference with these processes may ultimately determine cortical number and brain size. We demonstrate that DCL is crucial for precursor proliferation and stability of the early radial glial scaffold and propose it may prove to be of great interest for our understanding of early neurogenesis and of disorders associated with alterations in initial neuronal production, like microcephaly.

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