Microtubule associated proteins and plasticity in the developing and diseased brain

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CHAPTER 1

General Introduction
**Microtubules and microtubule associated proteins**

A wide variety of cellular and organ functions is closely associated with, and often critically depends on, the characteristic shape and morphological make up of an individual cell. Neurons for example have a complex three dimensional architecture that allows them to synaptically connect to other cells and transmit electrical signals. For other cells, their morphological plasticity critically determines their function. Muscle cells e.g. can change their shape rapidly that enables movement and the transduction of force, while still remaining a very stable entity. Maintenance of cellular morphology in response to environmental challenges, is to a large extent attributed to the cellular cytoskeleton. Whether or not cells rely on morphological flexibility, they all require a constant maintenance and adaptation of their internal structural elements in order to meet external demands.

Particularly during development a highly adaptive structural morphology is required to enable cells to engage in processes as diverse as cell division, growth and neuronal migration. Next to these aspects of structural support, cytoskeletal elements allow a cell to orchestrate its extensive intracellular vesicle transport that is required for e.g. secretion or neurotransmission. In the next section we will further address cytoskeletal make up as well as some other important factors involved in the dynamic regulation of cytoskeletal stability.

**Microtubules**

In eukaryotic cells the cytoskeleton is composed of aggregated, filamentous structures that are categorized into different categories; microtubules (MTs), that are composed of tubulin proteins; intermediate filaments and microfilaments, that are composed of actin. In the nervous system each cell type has a unique composition of cytoskeletal proteins that is generally assumed to be important for the specific function of that cell type. These cytoskeletal components are by no means passive structural elements as their maintenance is continuously and dynamically regulated. The dynamic regulation of cytoskeletal plasticity is an essential prerequisite to enable and facilitate crucial cellular processes like cell division, migration and plasticity.

Of the different cytoskeletal components, the microtubuli are abundant and highly enriched in the nervous system, with tubulin subunits making up over 10% of the total amount of brain protein. These high expression levels are likely related to the complex architecture of the various specific classes of neurons, that may require special adaptations from their cytoskeleton. Electron microscopy studies have revealed MTs to consist of hollow tubular structures. The walls of these tubules are formed by linearly arranged globular subunits, that are composed of heterodimers of α and β tubulin subunits.

MTs are organized in a highly dynamic manner; growth is possible through the addition of tubulin dimers, but they can also depolymerize. Growth takes place primarily at the end of the MT and is determined by
polarity; the plus end is the fast growing side, whereas the minus end grows much slower. Most MTs are orientated with their minus end towards the center of the cell, but exceptions also exist. In dendrites for example, the orientation can be either way.

In the center of the cell lies the centrosome, also known as the MT-organizing center, which is important for microtubular attachment, cell orientation and polarity. Within the centrosome lies a pair of cylindrical structures called the centrioles. During cell division these centrioles duplicate and move towards opposite sides of the nucleus where they form the poles of the mitotic spindle. Besides a variety of posttranslational modifications including phosphorylation, the biochemical diversity of MTs is further increased by the binding of so-called MT associated proteins (MAPs) which can bind to the microtubular surface (Alberts et al., 1994; Brady et al., 1999).

**MAPs**

An important function of MAPs is that they promote microtubular stabilization. It has been proposed that the wide diversity of MAPs known to date might help to establish functionally different compartments of the cell. In neurons e.g., the MAP tau is enriched in axons, whereas MAP2 is selectively expressed in dendrites and the cell body. The MAPs kinesin and dynein are so-called MT-dependent motor proteins. These motor proteins can be used for organelle transport and migration. As such they are important in for example axonal transport, but they also play an important role during mitosis. Table 1 gives an overview of all brain specific MAPs excluding those that act as molecular motors.

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<tr>
<th>Spatial expression</th>
<th>Temporal expression</th>
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<tr>
<td>MAP1a</td>
<td>absent during development, increasing between p10-p20</td>
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<tr>
<td>MAP1b (MAP5)</td>
<td>early development, decreasing between p10-p20</td>
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<td>MAP2a (MAP2)</td>
<td>absent during early development, increasing after p20</td>
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<tr>
<td>MAP2b (MAP2)</td>
<td>abundant from late embryonic development</td>
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<tr>
<td>MAP2C tau</td>
<td>high in newborn rats, decreased after p20</td>
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<tr>
<td>MAP2C Lis1</td>
<td>dependent on isoform</td>
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<tr>
<td>MAP2C DCX</td>
<td>development and adult</td>
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<td>MAP2C DCLK</td>
<td>late prenatal development, adult GCL and SVZ</td>
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Table 1. Spatiotemporal expression of brain specific microtubule associated proteins (MAPs).

MAP1, MAP2 and tau are in this thesis referred to as classical MAPs. Comparisons of their expression patterns have been extensively described (Chauhan and Siegel, 1997; Dehmelt and Holpain, 2005) and references therein. The expression of different tau isoforms is described amongst others by Chauhan and Siegel (1997). Expression of LIS1 is described in several studies (Reiner et al., 1995; Shmueli and Reiner, 2000; Meyer, 2002). Expression of DCX is described in many studies (Des Portes et al., 1998; Matsuura et al., 1998; Francis et al., 1999; Gleeson et al., 1999; Qin et al., 2000; Nacher, 2001; Meyer, 2002; Brown et al., 2003; Capes-Davis et al., 2005, chapter 2; Couillard-Despres et al., 2005). Characterization of the DCLK gene is described in (Burgess et al., 2000; Lin et al., 2000; Vreugdenhil et al., 2001, Burgess et al., 2002; Engels et al., 2004; Deuel et al., 2006; Kozumi et al., 2006; Shu et al., 2006, chapter 2). DCD2 is described in (Meng et al., 2005). DCK2 is described in (Eakin et al., 2005). In this thesis we map the spatiotemporal expression of DCL.
Also MAPs without a specific motor function can be critically engaged in cell division. Most of these MAPs including MAP4 (non neuronal) XMAP230 and XMAP310 (both xenopus specific MAPs) increase MT stability and prevent MT collapse, a process in which MTs shrink rapidly. During mitosis these MAPs are localized in mitotic spindles, probably to arrange the spindle architecture. Mitosis is a process that requires tight regulation and coordination. One way by which this can be done is by phosphorylation by cell cycle dependent kinases (CDKs) of various plasticity related proteins. Generally speaking the affinity of MAPs for MT reduces upon their phosphorylation. There is now also one MAP known as XMAP215 that does not prevent MT collapse (Andersen, 2000), but increases MT dynamics and turn-over. Recent data show that lack of the XMAP215 Drosophila homologue "mini spindles" is essential to prevent MT pause in interphase (Brittle and Ohkura, 2005).

As expected, dysregulation in the expression or phosphorylation of these important proteins can have considerable implications for cellular functioning. Indeed, various MAPs have been implicated in both developmental as well as adult onset disorders like cancer, frontotemporal dementia and Alzheimer’s disease.

In this thesis, we will focus on the functional roles of two specific MAPs that are selectively expressed in the central nervous system. Chapter 2 of this thesis will focus on the recently discovered MAP called Doublecortin-like (DCL), a novel splice variant of the doublecortinlike kinase gene, that is abundantly expressed during early cortical development when structural plasticity, i.e. ongoing neurogenesis and migration, is extensive. The second part of this thesis will elaborate on the classic MAP tau, that is involved in cellular plasticity of developing cells but is best known for its involvement in the neurofibrillary tangle pathology in Alzheimer’s disease (AD) and frontal temporal lobe dementia with Parkinsonism (FTDP-17).

In the remainder of this introduction we will focus on the development of the early neocortex and the hippocampus, discuss specific stages of their pre- and postnatal maturation and further address the role of (hyperphosphorylated) tau in AD and FTDP-17.

I. The neocortex and DCL

The neocortex

The cerebral cortex is a prominent part of the mammalian brain. In the human cortex, only a relatively small proportion is involved in encoding sensory information and orchestrating movements. A much larger area is thought to be involved in attending to complex stimuli, the identification and selection of relevant stimuli, the recognition of related objects and the planning of appropriate responses. These functions are here collectively referred to as cognition, and the areas involved in these processes are named association cortices. A lot of these so called “higher” functions as well as language skills and abstraction are considered typically human. The
association cortices are typically affected in human disorders like aphasia, mental retardation, age related cognitive decline and dementia in which particularly these skills are disturbed.

Anatomically, the cerebral cortex consists of a relatively thin outer layer of multilayered neuronal tissue that spans the entire cerebrum, and covers extensive white matter tracts. The phylogenetically oldest parts of the cortex are the paleocortex and the hippocampal cortex, which consist of four and three layers respectively. However, the largest part of the cerebral cortex is from an evolutionary perspective, relatively novel and referred to as neocortex. This part of cortex typically consists of six different cell layers, which can be distinguished by differences in cell density, cell size and shape, and different inputs and outputs. These layers are interconnected on the vertical axes, through radial or columnar connections, but also in the horizontal plane, through lateral connections (Purves, 2000).

Neocortical development

The extensive morphological alterations that the neuroepithelium undertakes as it develops into a complex multilayered neocortex, have been studied for over a century now. Still, only very little is known about the molecular factors that control cell birth, neuronal differentiation, migration to the appropriate layers and the establishment of functional contacts. The developmental orchestration and establishment of this complex multilayered structure obviously requires equally complex regulatory mechanisms. Unraveling the determinants that control the orchestration of this laminated architecture may not only contribute to our understanding of the evolution of cortical functions during ontogeny but could also provide important insights in how cortical developmental disorders arise.

Cortical development originates from the thin neurepithelium out of which a complex 6-layered cortex is formed in a so-called inside-out manner. One of the first and inner layers, the ventricular zone (VZ) is characterized by rapid cell division and massive precursor population expansion starting at embryonic day (E) 8.5 in murine development (fig 1). The first neurons then leave the VZ and migrate in a radial direction towards the pial surface. They establish the preplate (PP) around E10-E11 which is separated from the VZ by a thin axonal layer called the intermediate zone (IZ). The preplate consists of both Cajal-Retzius cells and subplate cells. At E12-E13, a second wave of cells then splits the PP in a marginal zone (MZ) and the subplate (SP). The neuronal layer formed in between is called the cortical plate (CP). Within the MZ remain the Cajal-Retzius cells which are essential for the formation of the neocortex (Soriano and Del Rio, 2005). The remaining layers of the adult cortex are being formed by sequential waves of new neurons crossing the IZ and the CP between E14 and E18. Finally, during middle and late cortical development a second zone of mitotically active cells is formed between the VZ and the IZ, the subventricular zone (SVZ). With the exception of the CP, all these zones are specific for the embryonic brain, and have no direct
counterparts in the adult cortex anymore such that they are unrecognizable in the mature nervous system (Angevine and Sidman, 1961; Marin-Padilla, 1971; Rakic, 1974; Hattan and Heintz, 1999; Aboitz et al., 2001; Gupta et al., 2002).

Figure 1. Development of the mammalian neocortex

A. Schematic representation of the developing mammalian neocortex. There are 4 developmental stages recognizable: 1. During early corticogenesis (in the mouse starting around E8) the first cells born in the ventricular zone (VZ) start migrating towards the periphery. The VZ is characterized by its high mitotic activity throughout the prenatal period. 2. Around E10 the first wave of cells reaches its destination and forms the transient preplate (PP), the layer of migration is called the intermediate zone (IZ). 3. Around E13 a second wave of cells that has migrated away from the VZ splits the PP into the superficial marginal zone (MZ) and the deeper subplate (SP) to form the cortical plate (CP) in between. 4. Subsequent waves of mitosis and migration follow, these cells cross the previously formed layers of the CP and thus always ends up directly under the MZ. After (Aboitz et al., 2001).

B. Schematic representation of the 6 layered adult mammalian neocortex. Left drawing: Impression of a Golgi stained section showing the different neurons their neuritic extensions. Right drawing: Impression of a Cresyl violet stained section showing all cell bodies remaining in the cortex. I: lamina zonatis (embryonic marginal zone); II: lamina granularis externa; III: lamina pyramidalis; IV: lamina granularis interna; V: lamina ganglionaris; VI: lamina multiformis. Lamina II until VI originate from the embryonic CP. After K. Brodmann

Different modes of migration

In the later stages of cortical development, i.e. after E13 in mouse, the majority of the cortical neurons reach their destination using a pial-oriented mode of migration by which cells start from the ventricular side and migrate radially towards the outer pial surface. Also, while migrating, secondary divisions occur in higher zones as well. However, not all neocortical neurons are born in the VZ nor do they use radial migration selectively. At the same time, and perpendicular to the mainly excitatory pyramidal neurons, waves of GABAergic interneurons e.g., born in the ventral ganglionic eminence (the later striatum), migrate in a tangential manner (i.e. in a horizontal direction, following the ventricular wall) and enter the
neocortex in later stages of development where they interconnect and synapse onto the pyramidal cells of specific cortical layers (reviewed by Corbin et al., 2001; Kriegstein and Noctor, 2004).

Within the mammalian early neocortex, cortical patterning involves radially migrating cells that find their destination in a so-called inside-out manner. This refers to the principle that the first cells that are formed cross the IZ and stop migrating in the most inner layer. Subsequent cells produced later during development will have to cross and pass the IZ and other, earlier formed layers (Marin-Padilla, 1971). This developmental pattern contrasts e.g. with that of the reptilian cortex, which is formed in an outside-in manner where the first cells to be produced end up in the outermost layer, whereas younger cells are added to and remain in the inner layers. Also in the mammalian brain, other than the neocortex, cortical regions like the hippocampal dentate gyrus, are developed in an outside-in manner (reviewed by Aboitiz et al., 2001). In this section we will further focus on the prevailing inside-out mode of migration in the neocortex that is of relevance for chapter 2.

Radial migration in the mammalian neocortex is accomplished in two ways: nuclear translocation and locomotion. Traditionally, neuronal migration was thought to be exclusively radial glia-guided (Rakic, 1972). Radial glia cells attached to the border of the VZ send their fibers towards the pial surface and traverse the entire CP. As their terminal end feet are anchored near the outer surface of the pia, they span the entire width of the developing cortex and can function as a scaffold for subsequent waves of migratory neurons. The radial glia nucleus typically resides in the VZ or SVZ. During locomotion, newly formed neurons migrate away from their site of birth towards their destination in the cortical plate. They use the radial glia cell as scaffold to guide their migration, with a leading process in the direction of the pial surface and a VZ oriented trailing process.

Locomotion generally takes place after E15-E16 in mouse, whereas at earlier stages, when the cortical layers are still much smaller, the predominant mode of neuronal migration is nuclear translocation. This form of radial migration is glia-independent and is characterized by precursors which have their somata in the VZ and send their leading processes all the way to the pial surface, which is still possible around E12-E13 because the cortical plate is a very thin layer then and only relatively short distances need to be crossed. Shortening of the leading process results in movement of the nucleus towards the leading edge, until it reaches its destination in the preplate.

At later stages of development this type of migration continues to occur in the upper half of the cortical plate. At this time the cortical plate has expanded dramatically and neurons cannot extend their processes all the way. Instead, neurons first migrate by locomotion (radial glia dependent). Once their leading process has attached to the pial surface, the mode of migration switches towards radial-glia independent nuclear translocation (Rakic, 1972; Schmechel and Rakic, 1979; Rakic and 9, 1990;
Besides facilitating and guiding the process of radial migration it is now known that radial glia cells themselves can also function as neuronal precursors. Studies using viral mediated GFP transfection of mitotic cells in the embryonic VZ revealed that many of the mitotically active cells adopt a radial glial phenotype, while a population of tightly associated daughter cells adopts a neuronal phenotype. Subsequent labeling of GFP expressing cells with BrdU revealed that the only cells that remained mitotically active were the ones expressing the radial glia marker vimentin. Thus, radial glia takes on a new role as it can divide asymmetrically to produce glia as well as neurons. Although it was shown before that glia and neurons could be derived from the same progenitors in vitro, this was the first in vivo evidence showing the clonal relation between neurons and glia. Recent findings even show that these cells are the predominant neuronal precursors in the ventricular zone (Noctor et al., 2001; Anthony et al., 2004).

MAPs involved in cortical development

As stated previously, the field exploring the signaling pathways that regulate cortical development is relatively new. The first indications that specific molecular factors are crucial in this stage, were provided by studies on human neurodevelopmental disorders, that appeared often are the result of specific mutations. In various cortical development disorders, specific MAPs appeared to be involved. A few examples are discussed now.

LIS1

One of the best known cortical developmental disorders is lissencephaly (“smooth brain”). Pathologically, the disease is characterized by a thickened, disorganized cortex that lacks gyri and sulci. Patients with this disease suffer from severe mental retardation and epilepsy. In the early eighties, the disease was shown to be due to mutations on chromosome 17 (Dobyns et al., 1983; Reiner et al., 1993) and the gene was called after lissencephaly type 1; LIS1.

Subsequent studies have shown that LIS1 acts as a MT binding protein (MAP) (Sapir et al., 1997; Sapir et al., 1999). Remarkable insight into the function of LIS1 actually came from a homologue found in a filamentous fungus *Aspergillus nidulans*. During fungus development, reproduction is accomplished by nuclear division and subsequent nuclear migration. Analysis of many mutants has shown that the process of nuclear migration is mediated by MT stabilization requiring e.g. the aspergillus LIS1 homologue.

Doublecortin

A few years after the discovery of LIS1, a gene involved in another type of lissencephaly was characterized. As this gene is located on the X-chromosome, the syndrome in males is referred to as X-linked lissencephaly. It is characterized by a rudimentary four-layered cortex. In heterozygous females this mutation leads to a less severe disease called the double cortex
syndrome. In this syndrome the layering of the cortex is relatively normal, but within the white matter, an extra cortical layer is present. The protein it encoded was called doublecortin (DCX) after the most characteristic feature of the syndrome in females (des Portes et al., 1998).

Soon afterwards it was shown that doublecortin is a MAP that promotes microtubular stabilization and is selectively involved in neuronal migration (Francis et al., 1999; Gleeson et al., 1999). A more recent study used in utero application of RNAi to show that DCX expression is essential for all radially migrating neurons to cross the intermediate zone and enter the cortical plate (Bai et al., 2003). The function of doublecortin can be highly compromised by phosphorylation of two serines, one of which is known to be mutated in the doublecortin syndrome (Schaar et al., 2004; Tanaka et al., 2004). Doublecortin is also expressed in various adult brain areas where neurogenesis takes place, like the subventricular zone and subgranular zone of the dentate gyrus (Brown et al., 2003; Yang et al., 2004; Couillard-Despres et al., 2005), while it is also expressed in an isolated manner throughout the adult telencephalon (Nacher, 2001). It is now generally considered a reliable marker for newborn, migratory neurons (Brown et al., 2003; Yang et al., 2004; Couillard-Despres et al., 2005).

DCL

Soon after the discovery of DCX, a gene with high homology was detected in rat and human called doublecortin-like kinase (DCLK) because of the presence of a kinase domain at the C-terminal end of the gene (Matsumoto et al., 1999). Figure 2 shows the DCLK gene and its two MT binding splice variants. Only very recently has it been shown that both DCLK and DCX are essential for the formation of axonal projections across the midline, and for neuronal migration, indicating their roles might be partially redundant (Koizumi et al., 2006). Whereas single DCX or DCLK mutant mice displayed minor effects, DCLK/DCX double knockouts were perilethally and displayed a dysorganized cortex. Moreover, axonal defects were found in many brain areas. Also in culture axonal outgrowth and transport were disturbed (Deuel et al., 2006).

DCX-exons

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DCX-mRNA

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DCLK-exons

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Figure 2. DCLK genomic structure

For comparison also the DCX genomic structure is shown, black exons share high homology. For clarity only the DCLK splice products DCLK and DCL are shown, which both contain the N-terminal MT binding domain. There are also two mRNAs produced that do not contain this sequence, being CARP and CPG16. DCL does not contain the C-terminal calcium/calmoduline-dependent protein kinase domain of DCLK. DCLK proteins can either contain or lack exon 19. After (Burgess et al., 2002).
In contrast to DCX however, DCLK was found in the mitotically active VZ and was found to regulate the formation of bipolar mitotic spindles during mitosis (Shu et al., 2006). Another splice variant of this gene, called doublecortin like (DCL) has been described: this lacks the kinase C-terminus of the DCLK gene (Engels et al., 2004). Similar to DCLK, DCL is critically involved in migration providing radial process stability and plays a crucial role in mitosis. In the addendum of this thesis the functional role of DCL is characterized in more detail. Since to date a detailed distribution of any DCLK product particularly during early development in rodents was lacking we studied the spatio-temporal expression pattern of DCLK in Chapter 2. We compared DCL expression to that of DCX to provide insight in the differential and overlapping roles of the two genes.

Open question: What is the spatio-temporal expression of DCL and DCX protein during early cortical development? Does it indicate differential roles for DCL and DCX during this period? (Chapter 2)

Interestingly, both the function and expression of the second MAP studied in this thesis, namely protein tau, shows remarkable parallels to DCLK products. Not only is tau a MAP involved in axonal outgrowth, we also show in chapter 3 that tau plays a crucial role in neurogenesis. Like DCL and DCLK, tau is involved in both neurogenesis and growth of cellular extensions.

Moreover, DCLK plays a role in the earlier developmental stages of E9 to E13, whereas DCL is involved in later stages. Similarly, in the expression of tau isoforms a switch occurs from the smaller towards the larger isoforms (the latter having a higher affinity for MTs). This switch occurs much later in development, namely in the second postnatal week in mice. Tau might therefore be of particular relevance for brain areas that develop specifically in this period. Therefore, we set out to study the relevance of tau in hippocampal development (Chapter 3). In parallel to the previous section on DCL and cortical development we will now first describe the hippocampal formation, its development and functional relevance before linking it to tau changes.

II The developing hippocampus and protein tau

The Hippocampus

Hippocampal Anatomy
The hippocampus is phylogenetically one of the oldest parts of the cerebral cortex. In the rodent brain the hippocampus consists of 2 interlocking curved “C-like” structures that extend in a frontal-caudal orientation from dorsal-medial towards ventral-lateral. In a cross section, there are roughly four areas to be distinguished: the dentate gyrus (DG), the hippocampus proper or cornu ammonis (CA), the subicular complex and the parahippocampal cortex. In this thesis, the word hippocampus refers to the
hippocampus proper and the dentate gyrus combined (fig 3, see also section color figures).

Figure 3. Hippocampal layers and connectivity


B. The three synaptic circuitry of the hippocampus. Direction of signalling and synaptic transmission is indicated by arrows in axonal projections. LPP: lateral perforant path; MPP: medial perforant path; MF: mossy fibers; AC: anterior commisure; SC: schaffer collaterals

Development of the Dentate Gyrus

The majority of the brain's neurons are formed before birth. There are only few exceptions; for example the cerebellum is primarily formed postnatally (Goldowitz and Hamre, 1998; Wang and Zoghbi, 2001). Also, neurogenesis in the rostromigratory stream (RMS) (Doetsch et al., 1997; Garcia-Verdugo et al., 1998) and the dentate gyrus (DG) of the hippocampus continue to occur into adulthood. In both areas neurogenesis starts prenatally but a substantial part of the cells is formed after birth as well. In adulthood, the rate of neurogenesis declines after a few weeks and is then maintained at a low frequency up until old age (Schlessinger et al., 1975; Bayer and 1, 1980; Heine et al., 2004). Regarding its relevance for this thesis we will now further focus on the DG.

During late gestation, granule cell precursors originating from the wall of the lateral ventricle migrate into the immature hippocampus to reach the future dentate gyrus. While some cells differentiate into granule neurons, others remain a precursor phenotype and thus are capable of reproduction (Altman and Das, 1967). In rat, the peak of neurogenesis is in the first two weeks after birth and about 85% of the cells are generated after birth (Schlessinger et al., 1975; Bayer and 1, 1980). In mice this high rate of neurogenesis continues until at least the 3rd postnatal week (Altman and Bayer, 1975). From the end of the second postnatal week into adulthood, granule cell precursors, which are now located in the hilus/subgranular zone, divide and produce daughter cells, as will be discussed in the section "Adult neurogenesis".
The adult DG is composed of three main layers, the molecular layer, the granular layer and the polymorphous layer, also called hilus. The CA can be subdivided into CA3 and CA1. The transitional region between CA1 and CA3, called CA2, is small and, in conventionally stained tissue, only distinguishable to the experienced observer, and often neglected. All the subdivisions of the CA area are composed of the same layers when scanning from the outside towards the DG: the stratum oriens, stratum pyramidale, stratum radiatum and stratum lacunosum moleculare (fig 3a).

Both the DG and the CA have clearly defined borders where most neuronal cell bodies reside. Based on the characteristic shape of these cell bodies, this layer is called the granular cell layer in the DG and the pyramidal cell layer in the CA. The DG granular cells extend all their dendrites outwards into the molecular layer. The major source of input for these dendrites comes from the entorhinal cortex from where perforant path axons extend into the molecular layer. On the opposite side, axons extend from the DG granular cells traversing through the hilus (these axons are called the mossy fibers), making connections with the CA3 pyramidal neurons. CA3 pyramidal neurons send dendrites outwards (referred to as the basal dendrites) but also inwards (the apical dendrites) where they do not only synapse with the mossy fibers but also with the perforant path in the molecular layer. At the outer side the axons extend from the CA3 pyramidal cells into the stratum oriens. These axons cross the pyramidal layer and are connected to the apical dendrites of CA1 pyramidal neurons. These fibers are termed the Schaffer collaterals. The axons from these cells, also extending from the stratum oriens, project to the entorhinal cortex (fig 3b).

Most hippocampal pyramidal and granular cells communicate with each other via the excitatory neurotransmitter glutamate. There is also a small, diverse group of neurons, which are not restricted to the granular or pyramidal layer, that use the inhibitory neurotransmitter γ-aminobutyric acid (GABA). These neurons are located in e.g. the stratum radiatum and other areas and are referred to as interneurons (Lopes da Silva et al., 1990; Scharfman et al., 2000).

Hippocampal Function

Although the hippocampus, as a part of the limbic system, is critically involved in novelty and fear related responses, the best understood function of the hippocampus is in learning and memory. Earlier studies have provided several seminal observations, from which the case of H.M. is the most cited one. In this patient, damage of the medial temporal lobe, in particular the hippocampus, resulted in antrograde amnesia. These studies were the first to provide convincing evidence for the critical involvement of the hippocampus in the formation of new memories (Scoville and Milner, 1957).

Evidence from animal studies has shown that the hippocampus is also involved in spatial memory (Olton et al., 1978; Winson, 1978). One of the most widely used paradigms nowadays to test such abilities is the Morris Water maze, a paradigm in which rodents learn to find an escape platform in an opaque waterbath by navigation on spatial cues. After lesioning of the
hippocampus the animal cannot perform this task anymore (Morris et al., 1982). It has been shown that the hippocampus contains so-called place cells that fire only when the animal is at a particular location and it has also been shown that humans have these place cells (reviewed by (Leutgeb et al., 2005)). A famous study that made use of PET scans illustrated that in humans too, the hippocampus is involved in retrieving spatial information. It was shown that London taxi drivers show increased hippocampal activity when answering question concerning specific routes through the city (Maguire et al., 1997).

Although spatial memory is the most prominently studied function of the hippocampus, also other types of memory have been related to the hippocampus. For example object recognition in humans and primates is known to be related to the hippocampal formation and associated cortices (Zola-Morgan and Squire, 1993). Also in rodents, the object recognition test (ORT) can be used to test memory. In this test the animal's inborn curiosity is used to discriminate between a familiar object and a novel one. An animal remembering having explored a familiar object before, will have more interest in the novel one. These tests are attractive to apply in the study of memory in rodent since it involves low stress levels for the animal, the animal uses various cues including tactile, visual and odour stimuli, and the ORT requires little motor or sensory skills. Moreover, comparable tests for humans exist, facilitating the integration of rodent studies in the exploration of human memory.

**LTP a cellular basis for memory?**

Questions regarding the cellular basis for learning and memory, and how e.g. the hippocampus processes spatial types of information form an important focus of current research. It is generally thought that at least part of a memory trace is represented by changes in synaptic connectivity. Following a learning experience, the circuitry is thought to be remodelled in such a way that relevant information is transmitted more efficiently than previously (Cajal). Hebb suggested that repeated stimulation was the actual trigger for these changes to occur (Hebb, 1949). Bliss went on to show that such plastic changes can be brought about in the brain (Bliss and Gardner-Medwin, 1973). Brief high frequency stimulations in the hippocampal area can induce lasting increases in the excitatory postsynaptic potential. This form of potentiation can last for hours up to a year and is therefore referred to as long-term potentiation (LTP), and as such an attractive mechanism by which memory traces could be stored (Abraham et al., 2002; Abraham, 2003). Although a direct causal link between LTP and memory still has to be shown, many studies have shown that manipulations in LTP can affect spatial memory (Morris et al., 1986; Bach et al., 1995; Schmitt et al., 2005). Also recognition memory has been correlated to hippocampal LTP (Wang et al., 2004a).

It has to be noted though that there are also situations known in which LTP and spatial memory are not correlated (Vaillend et al., 2004; Niisato et al., 2005), indicating that memory might be a much more complex
phenomenon than we understand at present. For example next to LTP also long-term depression exists. Although the latter receives much less attention in literature it is likely to be as relevant for memory as LTP. Although slightly beyond the scope of this thesis, the intracellular mechanisms that can induce LTP will be briefly introduced. A central event, necessary for LTP to occur is a postsynaptic rise in intracellular calcium. This rise in calcium can induce morphological and functional changes pre- and post-synaptically which subsequently facilitate neurotransmission. This alteration in synaptic efficacy is regulated by many different signalling cascades which will not be elaborated upon here. In the glutamatergic synapse, which are the most common ones in the hippocampus, a rise in calcium can be accomplished following activation of the N-methyl-D-aspartate (NMDA) receptors. As explained, LTP is induced upon high-frequency stimulation. The NMDA receptor has specific features that makes it suitable to respond especially to these kinds of stimuli. Like other channels, it is a glutamate-driven cation channel, but it is unique in that it conducts calcium and only opens if a voltage dependent magnesium block is relieved. Thus its opening depends on a presynaptic signal (glutamate release) and a postsynaptic action (membrane-depolarization). Hence, situations in which this channel is opened can be accomplished by high frequency stimulation, while at the same time that the post-synaptic cell is still depolarized from a previous stimulus, glutamate is already released due to a novel pre-synaptic depolarization. However, NMDA is not the only mediator in LTP. For example in the CA3 region LTP can be induced in the presence of the NMDA-receptor blocker D-2-amino-5-5-phosphonovalerate (APV), showing that also other sources of calcium can induce LTP. Since it has been shown that both calcium entry and depolarization are essential features of this NMDA receptor dependent form of LTP, it is thought that it is mediated by voltage-dependent-calcium channels (VDCCs). LTP in the CA1 and medial perforant path as studied in this thesis is mediated by both NMDA-receptors and voltage gated calcium channels. For a seminal review about LTP and memory we refer to (Lynch, 2004).

One of the reasons why particularly calcium is such a potent mediator of LTP, is that it indirectly can exert genomic effects. Upon a calcium rise phosphorylation cascades are activated leading to the assembly of transcription factors on the DNA. Thus all kinds of proteins can be produced that can be involved in for example strengthening the synapse, facilitating the formation of new synapses or facilitating axonal transport. Also structural proteins can be produced leading to morphological changes that facilitate synaptic contact. In this regard dendritic spines form a very interesting field. Spines are small extensions of the dendritic tree and most synapses are located on spines. Rather than stable entities, spines are highly motile, and the spine density, their shape and size change continuously (Fischer et al., 1998). It has e.g. been shown that the spine size increases upon high frequency stimulation (Fukazawa et al., 2003; Lang et al., 2004; Matsuzaki et al., 2004; Okamoto et al., 2004). Also, the induction of LTP was shown to be associated with the formation of new spines (Engert and
Bonhoeffer, 1999; Goldin et al., 2001; Nagerl et al., 2004), reviewed by (Segal, 2005). LTP is a form of plasticity that mainly takes place at the level of the synapse, but this plasticity could also be mediated at the level of the individual cell or by the addition of new cells to the circuit. In this perspective, neurogenesis is currently receiving a lot of attention as a novel form of structural plasticity in the adult brain that could be involved in learning and memory.

In the next section we will discuss adult neurogenesis, which is now also seen as an attractive mechanism involved in memory storage. Unlike LTP which can to occur in many different areas of the brain, neurogenesis only takes place in the hippocampal DG and in the subventricular zone (SVZ), and therefore it can only be involved in types of memory that are directly or indirectly related to those locations. For the hippocampus it might be involved in spatial memory. Whether neurogenesis in the SVZ is also involved in learning, is less clear although there is some evidence that it might be involved in olfactory learning (Rochefort et al., 2002).

**Adult neurogenesis**

It has been a dogma for many years that the adult brain consists of a fixed population of neurons, which cannot be renewed anymore. Although already in the 1960s several studies using [H³] thymidine had demonstrated the occurrence of cell birth in selected regions of the adult brain (Altman, 1963; Altman and Das, 1965; Altman, 1969), this for a long time failed to change the traditional view that neurogenesis was an insignificant event in adult brain. This attitude is likely explained by its low frequency as determined by the methods available at the time, and by the lack of functional relevance, or parallels in primate or human brain. The field was significantly changed when adult neurogenesis in songbirds was identified and correlated to seasonal song learning (reviewed by (Nottebohm, 2004)). Another contribution was a methodological advance provided by the introduction of in vivo labeling of cytogenesis using bromodeoxyuridine incorporation (BrdU) (Gratzner and 4571, 1982), a synthetic thymidine analogue that is incorporated into the DNA and can later be detected by (double) immunohistochemistry. This technique has facilitated and increased the possibilities for phenotypic identification of newborn cells, the study of their maturation and survival, and their detailed quantification. This is why it remains an important method of choice nowadays, even though several other approaches have been developed.

**Methodological considerations**

Although BrdU allows to visualize adult cytogenesis in great detail and with relative ease, one of its disadvantages is that it is sensitive to peripheral metabolism and selective membrane passage e.g. by the blood brain barrier. BrdU is rapidly degraded by the liver and hence represents only a snapshot of 2 hours bio availability. Also, it will be diluted by subsequent divisions and becomes undetectable in later progeny of the newborn cells. As BrdU is based on in vivo labelling of cycling cells followed by specific
survival times, which requires injections in live animals it excludes this technique for e.g. use in post mortem human brain studies. As an alternative, various endogenous proliferation markers have been developed and characterized, many of which had already been extensively characterized in tumour biology and oncological research. These markers allow the study of cell birth, proliferation rate, early neuronal differentiation or specific stages of the cell cycle in adult brain tissue, without the need for prior in vivo injections.

One frequently used endogenous proliferation marker is Ki-67, a nuclear antigen that is expressed in all phases of the cell except G0 (Endl and Gerdes, 2000). To enable cellular differentiation into a neuronal phenotype, Ki-67 expression has to be downregulated. Ki-67 antisense treatment e.g. strongly reduces the thymidine uptake in cell lines, indicating an important role for Ki-67 in the cell cycle (Duchrow et al., 2001). The Ki-67 antibody MIB-1 is a well established marker for proliferation in tumor biology, and its expression pattern is highly comparable to BrdU labelling with short survival times (Kee et al., 2002). By the use of microwave antigen retrieval techniques it is now possible to study Ki-67 immunolabeling also in heavily fixed post-mortem human brain material.

Another relatively novel marker for neurogenesis is doublecortin (DCX), a MAP involved in the migration of young neurons. In the adult DG, it has proven very useful in identifying a young population of newly formed neurons. Using parallel BrdU doublelabeling, expression of DCX in the adult hippocampus was e.g. shown to start approximately 4 days after a cell is born. Its expression peaks at about 2 wks after the birth of an adult generated cell, and is absent again from 4 weeks of age onwards, more or less coinciding with the period when mature neuronal markers like NeuN start to become expressed (Brown et al., 2003).

Using combinations of these and other methods like viral-mediated gene transfer (van Praag et al., 2002), it has now been firmly established that functional neurogenesis does occur in the adult mammalian brain such as rodent and primate species, including humans, but is even conserved in invertebrates (Eriksson et al., 1998; Schmidt and Demuth, 1998; Zupanc, 2001; Scotto-Lomassese et al., 2003). Even though the exact role of neurogenesis in the mammalian brain is not known, its evolutionary conservation indicates an important role, possibly in learning.

The two most prominent areas where neurogenesis is found in the mammalian brain are 1) the subventricular zone, where neurons are born that migrate along the rostromigratory stream (RMS) into the olfactory bulb, and 2) the hippocampus. Especially the latter area receives a lot of attention because of its importance in learning and memory. Within the hippocampus the border of the dentate gyrus and hilus is the site where new neurons are generated during adulthood (fig 3). Cells are born in the sub granular zone (SGZ), a 3 cell layer thick border zone between the granular cell layer (GCL) and the hilus. Out of a population of precursor cells, adult generated cells proliferate before migrating into the GCL, where they, in a period of 2-3 weeks, mature and start to form dendrites and synapses by which time they
are fully and functionally integrated into the hippocampal circuit (fig 4, see also section color figures).

![Diagram showing adult neurogenesis in the hippocampal DG](image)

**Figure 4. Adult neurogenesis in the hippocampal DG**

A. Nissl stained section showing the dentate gyrus.

B. In a thin layer of cells on the border of the granular cell layer (GCL) and the hilus called the subgranular zone (SGZ) remain stem cells which continuously produce new neurons. After a daughter cell has left the cell cycle, it migrates into the GCL where it becomes a fully functional neuron in about 4 weeks time. Proliferating cells can be stained using Ki-67 antibodies. Immature neurons (at 3 days to 21 days old) can be stained using doublecortin antibodies. After [Christie and Cameron, 2006].

Interestingly, there are many factors that modulate hippocampal neurogenesis, either the proliferation or survival rate of the adult generated cells. Rodents housed e.g. in an enriched environment, or mice that were allowed access to a running wheel, all had increased numbers of new neurons after appropriate survival times, that was paralleled by an increased performance in hippocampus related learning paradigms ([van Praag et al., 1999; Kitamura et al., 2003; Kempermann et al., 2004]). On the other hand, stress is one of the most potent inhibitors of neurogenesis and aspects of learning ([Gould et al., 1997; Tanapat et al., 1998; Heine et al., 2004]). It is important to dissociate cell birth from cell maturation, since these different stages can be regulated by different mechanisms. Voluntary running e.g. is
known to stimulate proliferation of hippocampal progenitors (van Praag et al., 1999; Naylor et al., 2005).

Within the hippocampus resides a specific populations of neuronal progenitors (Type 2) recognizable by their morphological characteristics and nestin expression. These specific progenitors increase after running but not after enriched environmental housing (Kronenberg et al., 2003). Precursors that are past the nestin expressing stage, do not respond to running anymore. On the other hand enriched environment increased the number of new neurons without affecting proliferation of undifferentiated precursor cells. It is rather the fate of the newborn cells that was shifted by increasing the amount of neurons at the expense of gliogenesis (Kempermann et al., 1997, 1998; Nilsson et al., 1999; Kempermann et al., 2002).

Although these studies have revealed correlations between changes in neurogenesis and learning performance, the question remains whether this is a causal relation and how functionally relevant the phenomenon of adult neurogenesis is (Leuner et al., 2006). Similar to the above discussion on the correlation of learning and memory with LTP, neurogenesis is likely involved in certain aspects of memory, but the process of memory formation itself is probably too complex to allow a simple one-on-one correlation. In contradiction to synaptic types of plasticity which occur in many brain areas, neurogenesis is a very rare and selective process, and likely to only contribute to types of learning involving the hippocampus. One of the most constructive studies, stretching beyond a correlational line of arguing, is the observation that newborn cells are much more plastic than older ones, and, interestingly, show more LTP compared to older cells (Schmidt-Hieber et al., 2004). Thus if processes like LTP, dendritic growth and synapse formation are relevant for learning and memory, new neurons can play an important role since their individual capacity to facilitate such processes is larger.

Importantly, also damage to the hippocampus, as e.g. can occur selectively in epilepsy or ischemia, is known to stimulate neurogenesis (Parent et al., 1997; Covolan et al., 2000; Blumcke et al., 2001; Jiang et al., 2001; Jin et al., 2001; Sun et al., 2003). Although in epilepsy neurogenesis is known to be far from beneficial to the system, as aberrant rewiring occurs, this phenomenon has been regarded as an attempt to repair the brain. Also in Alzheimer’s disease (AD), a disorder in which the hippocampus is severely affected, increased expression of specific cell cycle proteins such as cyclins and PCNA has been observed (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). After a description of Alzheimer’s disease and how it might affect memory we will further discuss the possible relevance of neurogenesis related phenomena also in this disease.

Alzheimer’s disease.

Consistent with its important role in memory and cognition, selective damage to the hippocampal area is known to impair memory function. In
Alzheimer's disease (AD), progressive and severe memory impairment occurs, that is paralleled by reductions in hippocampal volume and specific neuropathological alterations in specific subregions of the hippocampus. Amongst the many other areas affected in AD, the hippocampus is one of the first and most severely damaged ones. While the diagnosis of AD partly relies on a clinical diagnosis by which the nature and severity of memory loss and cognition is established (Reisberg et al., 1982), a final diagnosis can only be made post mortem that consists of a quantitative rather than qualitative criteria that are a.o., the extent and numbers of two pathological hallmarks, namely senile plaques and neurofibrillary tangles in selected brain regions (Braak and Braak, 1991). Plaques are extracellular deposits of a protein called β amyloid (Aβ) that is derived from an amyloid precursor protein (APP), whereas tangles are intracellular inclusions consisting of heavily hyperphosphorylated tau proteins.

Another common feature of the AD brain is a marked reduction in acetylcholine levels, particularly of the cholinergic projections into the hippocampus. This is a.o. caused by alterations in neurons of the nucleus basalis of Meynert, which provide one of the major cholinergic inputs to the hippocampus. Remarkably, despite the widespread pathology and robust volume loss in the AD brain, actual cell loss have only been demonstrated in a few areas, like the locus coeruleus (Tomlinson et al., 1981; Hoogendijk et al., 1999; Zarow et al., 2003), the hippocampal CA1 area (Mizutani et al., 1990; West et al., 1994) and the nucleus basalis of Meynert (Geula and Mesulam, 1999; Wu et al., 2005) although contradictory evidence exists on the latter as well (Salehi et al., 1994).

Besides the specific neuropathological hallmarks, potent inflammatory responses take place in the AD brain that involve the activation of astrocytes and microglial cells. Presumably, gliosis takes place in response to the deposition of pathological proteins (Akiyama et al., 2000; Meda et al., 2001; Nagele et al., 2004). Glial activation is hence generally seen as a secondary maladaptation in AD that worsens the situation, rather than a primary causal event.

In almost all AD patients, additional and extensive amyloid pathology is present in the vasculature, which is referred to as cerebral amyloid angiopathy, and which correlates well with cognitive impairment (Mandybur, 1975; Jellinger, 2002). Recent literature even suggests that angiopathy might actually be an important factor contributing to dementia. Various causal mechanisms for cerebral amyloid angiopathy have been proposed in association with AD, including hypoperfusion, altered blood brain barrier function, vascular remodeling, aberrant angiogenesis and reduced Aβ clearance (Castellani et al., 2004; Nicoll et al., 2004; Zlokovic, 2005).

These neuropathological alterations are also observed in control groups and quantitative criteria determine the distinction between control and AD groups. As the best correlate of memory impairment is not made with the extent of the plaque or tangle load, but with the decrease in synaptic density (DeKosky and Scheff, 1990; Coleman and Yao, 2003), many
researchers believe that synaptic changes and synaptic loss is probably one of the underlying causes of memory impairment (Walsh and Selkoe, 2004). However, it is important to realize that immunocytochemical markers for synaptic density are often activity dependent, and could reflect an effect rather than the cause of memory impairment, particularly when studied in postmortem tissue.

**APP and AD**

For the larger part of the AD affected population the actual cause of the disease is unknown. However, in a small subset of patients, specific mutations are known to cause dementia. These familial cases of dementia have so far been linked to mutations in at least three genes: the Amyloid Precursor protein (APP), and the presenilin-1 and 2 genes. Presenilins are part of the larger γ-secretase complex which is involved in the cleavage of β-amyloid. Mutations in the above genes all favor the production of the larger, 42-43 residues long β-amyloid protein which accumulates in AD (reviewed by Walsh and Selkoe, 2004). The above has led researchers to hypothesize that amyloid deposition and plaques are a primary and central phenomenon in AD, that would subsequently be followed by tau alterations, eventually leading to functional deficits, and dementia. This is referred to as the “amyloid cascade hypothesis” (Hardy and Higgins, 1992). However, this is hard to combine with the observation that no linear correlation exists between plaque load and cognitive impairments in AD, nor with the many sporadic cases in which mutations do not play a major role, observations in transgenic mice in which plaques only develop after the occurrence of functional deficits (Moechars et al., 1999), an extensive but confusing in vitro literature in which both deleterious as well as growth promoting effects of amyloid (fragments) have been reported. Additional arguments in favor and against this hypothesis have been discussed elsewhere in detail (Van de Nes et al., 1994; Terry, 1996; Neve and Robakis, 1998; Salehi et al., 1998; Robinson and Bishop, 2002).

It was shown that not the plaques per se, but rather the smaller soluble accumulations of β-amyloid, called oligomers, are highly toxic (Walsh et al., 2002). Since APP is a large transmembrane protein that can undergo alternative splicing, different parts of the protein have been studied in response to injury and have been proposed to e.g. act as adhesion molecule, regulator of neuronal processes (outgrowth, arborization, synaptogenesis etc), signalling molecule and regulator of cell survival/death. Currently, it remains unknown how exactly either β-amyloid or APP interfere with neuronal transmission, or what their normal, physiological role is (reviewed by Reinhard et al., 2005).

**Neurofibrillary Tangles and tau**

In addition to the APP- and PS-based familial dementias, mutations in tau protein are known to cause a specific and rare type of dementia, called frontotemporal dementia with parkinsonism (FTDP-17). Clinically, the first alterations noticable are behavioral inhibition leading to profound
personality alterations, paralleled by "Parkinson-like" motor disturbances. As the disease further develops, also memory becomes severely impaired (reviewed by (Foster et al., 1997)). Neuropathologically, these patients are characterized by a profound volume loss of the frontal cortical regions, that is accompanied by an extensive neurofibrillary tangle pathology. As plaques are largely absent, FTDP-17 provides a clear example of a "tangle only" disorder, and suggests that tau dysfunctioning is involved in memory impairment. Yet, similar to APP, the exact functions of tau, and the mechanisms by which it causes dementia are not fully understood.

Tau is a MAP that plays an important role in the stabilization of microtubuli. One of the characteristics of the gene is a C-terminus that contains 4 MT binding repeat sequences. Alternative splicing can lead to tau proteins with either 3 or 4 repeat sequences (tau-3R or -4R), the latter having a higher affinity for MT. Besides these C-terminal repeat sequences, tau also contains 2 N-terminal repeat sequences, the function of which is so far poorly understood. Hence, upon splicing, 6 tau isoforms with either 0, 1 or 2 N-terminal sequences (tau-0N, 1N or 2N), combined with 3R or 4R can be produced (Buee et al., 2000; Lee and 5534, 2001; Goedert and Jakes, 2005) (fig 5). These different isoforms have different MT binding affinities and can thereby differentially modulate MT stability. In addition, MT affinity of tau can be altered by post-translational processes, the most important of which is phosphorylation. In general, tau phosphorylation reduces its affinity for MT thereby allowing a more flexible cytoskeleton.

![Figure 5. Tau genomic organization and adult brain protein products.](image)

A. Tau gene. Of 14 exons, exons 2, 3 and 10 (white box) are alternatively spliced. Constitutive exons are shown in black.
B. The six tau isoforms produced in adult mouse brain. Exons 9–12 each encode a MT-binding repeat sequence (grey box). By alternative splicing E10 can be deleted giving rise to tau with 3 repeat sequences (tau-3R). Otherwise tau containing 4 repeat sequences is produced (tau-4R). Furthermore, tau mRNA can also be spliced at the N-terminus, thus splice variants can be obtained containing neither exon 2 or 3 (0N), only exon 2 (1N) or both exon 2 and 3 (2N). Amino acid numbers are to the right. After (D'Souza and Schellenberg, 2005).
Of interest, extensive phosphorylation and alternative splicing of tau occur also during early development when a plastic cytoskeletal make up is a prerequisite to allow division and e.g. migration of cells. During early postnatal development, a characteristic shift in the main tau isoforms occurs. Whereas mainly tau-3R isoforms are being expressed in the early stages, at approximately 12 days after birth, tau 3R expression diminishes and is replaced by mainly tau-4R isoforms in mouse brain. In the adult murine hippocampus the switch towards tau-4R isoforms is complete, whereas in human adults tau-3R and 4R isoforms are expressed in equal amounts (Goedert et al., 1989; Kosik et al., 1989; Larcher et al., 1992; Takuma et al., 2003).

During development, phosphorylation is furthermore extensive, whereas in healthy adults, phosphorylation is generally low, except when neuropathology occurs and tau becomes hyperphosphorylated. Indeed tau phosphorylation is high also in proliferating cells (Pope et al., 1994; Preuss et al., 1995; Illenberger et al., 1998; Tatebayashi et al., 2006). The lower MT affinity of tau during development due to the expression of 3R isoforms and phosphorylation, likely reflects the need for a plastic cytoskeleton that is required for motility, migration and e.g. division of young neuronal precursors.

Phosphorylation of tau is difficult to control. However, isoform expression can be relatively easily and specifically altered by transgenic approaches. In Chapter 3 we tested the longstanding hypothesis that the tau-3R to 4R isoform switch in the second postnatal week is indeed involved in maturation of the brain in general and neuronal differentiation in particular. Our focus was especially on the hippocampus because the DG is known to develop largely in the first two postnatal weeks and because of the occurrence of adult neurogenesis.

Open questions: What are the structural consequences and functional relevance of the tau 3R to 4R isoform switch during early postnatal development? (Chapter 3)

In contrast to the healthy adult situation, tau is heavily phosphorylated at many specific residues in the AD and FTDP-17 affected brain. Certain epitopes that are only phosphorylated in the diseased brain are considered disease specific. This phenomenon is referred to as tau hyperphosphorylation. It has been suggested that especially the hyperphosphorylation of tau is crucial for the development of memory impairments (Avila, 2006). Various studies indicate a strong positive correlation between the extent of tangle pathology in e.g. the hippocampus and cortex and the severity of the cognitive decline. Not only have alterations in various specific protein phosphatases been found in AD brain (Liu et al., 2005), experimentally modulating kinase or phosphatase activity was reported to cause an increased phosphorylation of tau, neuropathology and memory impairments (Arendt et al., 1998). However, these kinases are not very selective, and these results have proven difficult to reproduce (Van Dam et al., 1998). In addition to phosphorylation, alternative splicing of tau is
important in dementia since several of the FTDP-17 mutations are known to favour tau-4R production. Also in AD, most tangles contain tau-4R, that is overrepresented relative to 3R (Hutton et al., 1998; Spillantini et al., 1998; D’Souza et al., 1999; Hasegawa et al., 1999). However, there is also a population of FTDP-17 mutations which does not affect splicing. Since AD and all forms of FTDP-17 and several other taupathies all involve tau hyperphosphorylation this is thought to be an essential event leading to memory impairments. In Chapter 4 the effect of such a tau mutation is tested in the absence of tau hyperphosphorylation.

AD and FTDP-17 transgenic mouse models

Following the discovery of the involvement of APP, PS and tau mutations in dementia, many transgenic mice models have been created in order to mimic aspects of the pathology of dementia, to better understand its etiology and to test putative pharmacological compounds. With the wide range of possibilities these transgenic mice offer, animal modeling has become a prominent field within dementia research. This section is not aimed to give a complete overview but will highlight only some important aspects of the current animal models within the scope of this thesis, and put the currently used tau mice models in perspective. Recent reviews cover this in more detail (Gotz et al., 2004; Spires and Hyman, 2005).

Tau transgenic mouse models can roughly be divided in two groups: Models developed to recapitulate FTDP-17 or AD symptomatology, and those aimed to understand the biological role of tau protein. Starting with the latter group, tau-KO models are worth mentioning. As stated above, the MAP tau, and especially tau-4R is a potent stabilizer of MT. Tau has also been shown to promote neuritic outgrowth, morphogenesis and synaptic connectivity in vitro (Caceres and Kosik, 1990; Ebneth et al., 1998; Gonzalez-Billault et al., 2002; Mandelkow et al., 2003). Therefore, it was quite surprising that tau KO mice failed to show a severe phenotype (Harada et al., 1994; Dawson et al., 2001), and only some minor defects in fear conditioning and axonal outgrowth have been reported (Ikegami et al., 2000). Thus it was concluded that tau is functionally redundant as the role of tau is likely replaced by other MAPs like MAP1B (Takei et al., 2000; Dawson et al., 2001).

The lack of a major phenotype in tau-KO mice suggests that a gain of function rather than a loss of function is involved in FTDP-17. To test this hypothesis many models have been generated, over-expressing one or more isoforms of either normal or mutated tau, in the absence or presence of endogenous tau (Lee et al., 2005). FTDP-17 mouse models recapitulate many of the symptoms of the human disorder and display severe tauopathy, often with premature death, memory impairment (Tachibana et al., 2000; Arendash et al., 2004; Pennanen et al., 2004) and severe hyperphosphorylation of tau at later ages (Chen, 2005; Terwel et al., 2005).

Generally, these tau transgenic models were developed to induce tangle formation. However, recent research has shown that tangles per se are not essential to induce memory impairments (Santacruz et al., 2005;
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Taniguchi et al., 2005). Therefore, in this thesis the traditional question of how tangle-formation takes place and how it can be stopped, is rephrased. In chapter 4 we questioned what aspects of FTDP-17 pathology are actually causing memory impairments in the first place. To this aim we studied a model that displays tau-hyperphosphorylation and pathology, but only at later ages. Rather than reconfirming that tau-phosphorylation is associated with memory deficits, we choose to study these animals well before the appearance of any such pathological deficits to address the question whether it is the tau phosphorylation that is essential for memory deficits to occur, or whether mutated tau itself can cause these effects.

**Open questions:** What are the structural and functional consequences of the FTDP-17 related tau P301L mutation itself, as opposed to the ensuing tau hyperphosphorylation as it develops over time? (Chapter 4)

Alzheimer's disease and neurogenesis

In the AD hippocampus various cell cycle markers are expressed. As to its occurrence, most authors reasoned that the expression of e.g. cyclins or PCNA does not reflect the birth of new cells but would rather reflect an aberrant response of damaged cells that attempt to (apparently unsuccessfully) re-engage in cell cycle. The so-called ectopic expression of these markers in AD is regarded as a pre-stage of apoptosis (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). One paper describes an increase of cell-cycle markers but also of DCX (Jin et al., 2004a) in a cohort of senile AD patients, suggesting that neurogenesis is increased in the hippocampal CA1. In chapter 5 this issue is addressed in more quantitative detail in a younger, presenile AD cohort.

**Open questions:** Is neurogenesis altered in the hippocampus of presenile AD cases? (Chapter 5)

Also in the animal literature changes in neurogenesis have been related to both APP and PS mutations (Jin et al., 2004b; Chevallier et al., 2005). This literature is far from conclusive as both reductions and increases in neurogenesis have been found in mice models with altered expression of APP / Abeta or PS variants (Dong et al., 2004; Wang et al., 2004b; Wen et al., 2004). Especially in PS mutants this is surprising as preseniilins do not only affect APP cleavage but also Wnt signalling, which is involved in neurogenesis (Chevallier et al., 2005). Interestingly, to date there are no reports in which changes in tau expression are correlated with changes in neurogenesis. Given the prominent role many other MAPs play in mitosis (Andersen, 2000), including DCL and DCLK which directly affect neurogenesis (Shu et al., 2006) (Vreugdenhil et al., submitted), and the putative role of tau in neurodevelopment, alterations in (mutant) tau expression probably could
provide an interesting direct link to neurogenesis. Therefore, we paid particular attention to the role of neurogenesis in tau transgenic models (Chapter 3 and 4).

**Aim and outline of this thesis.**

In this thesis, we focus on two proteins, i.e. the novel MAP DCL and the more well known protein tau, and their roles in structural plasticity during early cortical development and in relation to structural and functional effects on hippocampal development, respectively. Extensive further characterization of DCL is presented in the addendum.

Following the general introduction, Chapter 2 constitutes a detailed spatio-temporal mapping of DCL protein expression during early cortical development. This protein shares common functions and expression patterns with both DCX and DCLK. In this study we also found some striking differences in the expression of DCX and DCL pointing to a unique role for DCL in precursor mitosis and radial migration that is different from that of DCX, particularly during the earliest stage of cortical development prior to E 10.

In chapter 3 we focus on the structural consequences and functional relevance of the tau 3R to 4R isoform switch during early postnatal development. It has been argued for a long time that this switch must be involved in the postnatal maturation of the brain. To test this hypothesis we studied the consequences of interference with this isoform switch. This was done by making use of a mouse that represents a humanized tau 4R transgene in a mouse tau knock-out background. These mice lack all six mouse isoforms of tau and only express the longest human tau 2N/4R isoform from approximately PND12 onwards.

Following this focus on MAPs during brain development, we subsequently addressed the role of tau and neurogenesis in (hippocampal) pathology in chapters 4 and 5. The P301L mouse model reflects many of the symptoms observed in human FTDP-17. In addition to its endogenous tau levels, these mice over-express a mutated form of tau (P301L) that causes FTDP-17 in humans. To separate the structural and functional consequences of the tau mutation from the ensuing tau hyperphosphorylation that develops over time in these mice, we address in chapter 4 on plasticity related parameters like LTP, memory, dendritic arborization and neurogenesis assessed in young P301L mice at ages when the tauopathy and age-related accumulation of tau hyperphosphorylation is still absent.

Having shown a role for tau in neurogenesis in the KOKI model, and given the frequent occurrence of cell cycle changes in AD, we subsequently questioned whether neurogenesis is altered in the hippocampus of presenile AD cases as reported in chapter 5.

In chapter 6, our results are discussed in relation to the current literature and future possibilities.