Understanding doublecortin-like kinase gene function through transgenesis

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Chapter 1

General introduction outline

General introduction

The mammalian brain consists of billions of nerve cells, which have even more connections between them. Neurons have a complex morphological structure that is determined by the presence of cytoskeletal proteins, which are the building bricks of cells. The morphology of neurons largely determines their ability to function and to establish synaptic contacts with other cells. Neurons, and the networks they form, are not static, but are subject to changes in local protein function and availability and to alterations in the expression of genes that encode proteins that are able to affect e.g. cytoskeletal architecture and neurotransmission. This plasticity of the brain is critically important for brain function during basal conditions, but also for the ability to make adaptive changes when neurons are faced with a challenge. Therefore, neuronal plasticity is crucial to neuronal viability. Thus, alterations in gene expression that cause shifts in protein levels can affect both the functionality and viability of nerve cells and the networks they support, ultimately leading to changes in central nervous system output, such as memory formation and behaviour. In this thesis, research will be described, in which the consequences of neuronal over-expression of a plasticity related gene, called Doublecortin-Like Kinase (DCLK), are studied in the mouse hippocampus on the genetic, network and behavioural level.

1. The Doublecortin (DCX) Superfamily

The DCX-repeat gene family is composed of eleven paralogs in human and in mouse. Its expression is found across vertebrates, invertebrates, and is also traced to unicellular organisms (Reiner et al., 2006). Members of this gene family are microtubule (MT) associated proteins (MAPs), and they contain at least one evolutionary conserved tubulin binding domain that stimulates tubulin polymerization. Importantly, mutations in several members of this protein superfamily are linked to genetic diseases of the brain. Mutations in the X-linked gene doublecortin (DCX) were first discovered (Gleeson et al., 1998; des Portes et al., 1998) and they result in subcortical band heterotopia (SBH) or lissencephaly. SBH is a disorder in which bilateral bands of gray matter situated in the white matter between the cortex and the lateral ventricles are found. This disorder is also

referred to as ‘double cortex syndrome’; hence the gene and protein are named ‘doublecortin’. Lissencephaly is a related disorder characterized by severe brain malformation and absent or decreased convolutions (‘smooth brain’) accompanied by thickening of the cortex. This disease is characterized by mental retardation and frequent epileptic attacks. In general, lissencephaly and SBH are neuronal migration disorders (des Portes et al., 1998; Gleeson et al., 1998; Sapir et al., 2000; Taylor et al., 2000). SBH is very common among females with mutations in DCX (des Portes et al., 1998; Gleeson et al., 1998). The DCX gene is expressed primarily in post mitotic neurons during cortical development, both during periods of neuronal migration as well as during neurite formation (Gleeson et al., 1999; Francis et al., 1999). Two other genes of the DCX superfamily that are related to human disease are a product of the retinitis pigmentosa-1 gene (RP1) and DCDC2. Mutations in these genes are implied in progressive blindness and dyslexia respectively (Reiner et al., 2006).

A gene product closely related to DCX is doublecortin-like kinase (DCLK) (Burgess and Reiner, 2000; Lin et al., 2000). Previously, Vreugdenhil et al. have cloned the DCLK gene from a differential display study screening for challenge-induced hippocampal transcripts (Vreugdenhil et al., 1999). This gene contains two putative promotors corresponding to two transcription initiation sites and is subject of massive alternative splicing (Sorray-Alaoui and Srivastava 1999; Burgess and Reiner, 2002; Engels et al., 2004). The DCLK gene spans 20 exons and several major DCLK gene transcript types can be distinguished (Figure 1).

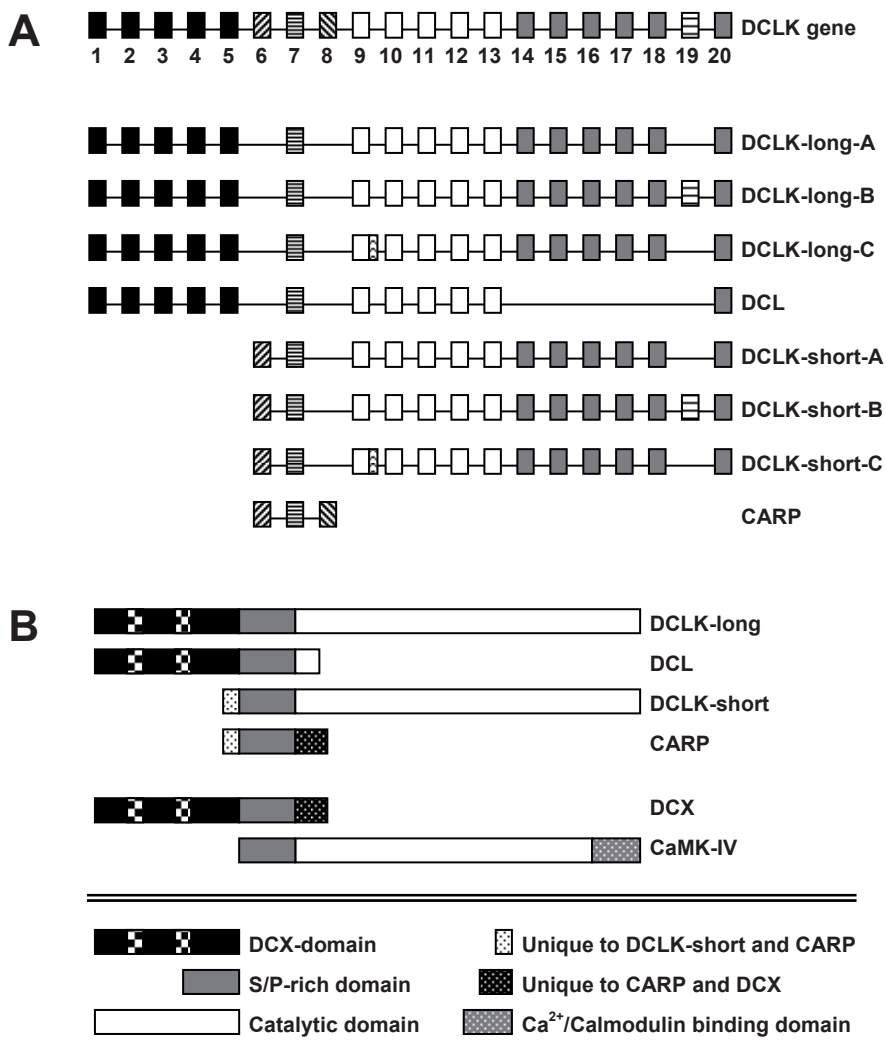

Figure 1. (A) Genomic organization of the DCLK gene. The addition to exon 9 in the 'C' variants represents a nuclear localization signal. (B) Functional domains of proteins generated by the DCLK gene. DCLK-long and DCL are expressed during embryonic development (Deuel, Liu et al. 2006; Koizumi, Tanaka et al. 2006; Shu, Tseng et al. 2006), while DCLK-short is mainly expressed in the adult brain (Engels, Lucassen et al. 1999; Engels, Schouten et al. 2004). CARP is expressed at very low levels but induced in the hippocampus by kainate-induced seizures (Vreugdenhil, Datson et al. 1999), in striatal neurons by D1-agonists and cocaine (Berke, Paletzki et al. 1998) and after BDNF-LTP (Wibrand et al., 2006).

Most of the major DCLK splice variants are expressed in the developing brain, including the hippocampus (Vreugdenhil et al., 2001; Koizumi et al, 2006; Deuel et al., 2006; Tanaka et al., 2006; Vreugdenhil et al., 2007; Tuy et al., 2008), but the exact role for DCLK in hippocampal functioning is unknown. Both DCLK and DCX are microtubule (MT) associated proteins (MAPs), and they contain two evolutionary conserved tubulin binding domains that stimulate tubulin polymerization (Gleeson et al., 1999; Francis et al., 1999, Kim et al., 2003a; 2003b) as well a serine-proline (SP)-rich domain (Vreugdenhil et al., 1999; Burgess and Reiner, 2000; 2002). Recently, an additional member of this protein family, doublecortin kinase 2 (DCLK2), has been described and was found to posses MT binding activities (Edelman et al., 2005). In addition, DCLK3 has also been described, however, DCLK3 proteins contain only a single DCX domain, which is highly similar to the C-terminal DCX domain from mammals and invertebrates (Reiner et al., 2006). A significant portion of the DCX-domain containing proteins has a kinase domain attached at the C-terminal end. This group includes DCLK, and DCLK2 and DCLK3. Orthologs of these proteins are also found in invertebrates. The *C. elegans* DCLK ortholog ZYG-8 has been studied extensively. In nematodes, ZYG-8 was found to be important for assembly of astral microtubules. Mutant ZYG-8 phenotypes were observed with several different mutations in the DCX domain, but also the kinase domain, therefore suggesting a role for kinase activity in regulating MT assembly (Gönczy et al., 2001).

2. Products of the DCLK gene

2.1 DCLK-long

Full-length products of the DCLK gene, the DCLK-long transcripts, encode proteins of 700 amino acids (AAs) long of which 300 AAs at the N-terminus exhibit 80% identity with DCX. The N-terminal DCX domains of DCLK bind to microtubules and stimulate microtubule polymerization (Lin et al., 2000; Silverman et al., 1999). The C-terminal region of DCLK-long proteins shows substantial amino acid homology with members of the Ca^{2+}/calmodulin dependent protein kinase (CaMK) and/or serine/threonine protein kinase family. Through alternative splicing three slightly different forms of this kinase domain exist. The corresponding gene products are

named DCLK-long-A, B and C, and they have different kinase activities and can undergo auto-phosphorylation (Vreugdenhil et al., 2001; Burgess and Reiner, 2002; Engels et al., 2004; Ohmae et al., 2006). DCLK-long proteins exhibit similar biochemical and biophysical characteristics as DCX (Lin et al., 2000) and both DCX and DCLK-long are coexpressed in the developing brain starting from embrionic day (ED) 11 (Sossey-Alaoui and Srivastava, 1999; Burgess and Reiner 2000; Capes-Davis et al., 2005). Recently, it was reported that DCLK-long controls mitotic division by regulating spindle formation and also determines the fate of neural progenitors during cortical neurogenesis (Shu et al., 2006). In cultured neurons, DCLK-long localization to microtubules overlaps with DCX, with the strongest expression in neurite tips (Burgess and Reiner, 2000) and in the vicinity of the cell soma around the nucleus (Lin et al., 2000). Furthermore, in cultured cortical neural progenitors, DCLK-long RNAi mediated knockdown also disrupts the structure of mitotic spindles, causing increased levels of progenitors that differentiate into neurons (Shu et al., 2006). Taken together, the homology and expression pattern of DCLK supports potential functional overlap with DCX during development (See section 4 of the introduction for more details).

2.2 Doublecortin-Like (DCL)

A second type of transcript that is, like the DCLK-long variants, derived from the first transcription start site of the DCLK gene, contains the highly conserved DCX domains, but does not have a kinase domain (Burgess and Reiner, 2002; Engels et al., 2004; Vreugdenhil et al., 2007; Boekhoorn et al., 2008). Similar to DCX, this protein is 363 AAs in length, compared to 366 AAs for DCX, and because of its 73% amino acid sequence identity with DCX this transcript is known as doublecortin-like (DCL). Like DCX, DCL is a phosphoprotein which is associated with microtubules thereby stabilising the cytoskeleton and is able to induce microtubule polymerization. DCL and DCX are coexpressed in populations of migrating neurons during brain development, but in contrast to DCX, DCL is already expressed during development at embryonic day 8, a time point at which massive proliferation occurs but no neuronal migration (Boekhoorn et al., 2008). In neuronal cell lines DCL is colocalized with mitotic spindles and centrosomes, which

control correct spindle formation during cell division (Vreugdenhil et al., 2007). Interestingly, DCL is not found in the adult brain with the exception of the subventricular zone and the hippocampus (Boekhoorn et al., 2008), two areas which are capable of continuous neurogenesis throughout life (Taupin, 2005), underscoring the potential role for this DCLK gene splice variant in brain development.

2.3 DCLK-short

Another transcript type, consisting of DCLK-short A, B and C, is a product generated from the second transcription initiation site, which is located upstream of exon 6. Consequently, these proteins lack the DCX domain and encode proteins that consist of the CaMK-like domains that are identical to the kinase domains found at the C-terminal part of the DCLK-long variants. Like the DCLK-long proteins, DCLK-short A, B and C encode different proteins with different kinase activities. Interestingly, C-terminal truncation of the kinase domain increases its activity 10 fold, through deletion of the auto-inhibitory domain (Burgess and Reiner, 2002; Engels et al., 2004; Ohmae et al., 2006). DCLK-short-A was first identified in a hippocampal screen similar to that performed by Vreugdenhil et al. and initially termed candidate plasticity gene 16 (CPG16) (Hevroni et al., 1998; Vreugdenhil et al., 1999). Since the DCX domains are absent in DCLK-short, it does not colocalize with MTs and has a diffuse cytoplasmic localization. In contrast to DCLK-long, the DCLK-short variants are not expressed during development but are abundantly found in limbic structures of the adult brain (Vreugdenhil, Engels et al. 2001; Burgess and Reiner 2002; Engels, Schouten et al. 2004). DCLK-short shares in several features with CaMks, in particular CaMK-IV. DCLK-short and CaMK-IV are both able to phosphorylate myelin basic protein (MBP) (Lin et al., 2000; Silverman et al., 1999), syntide and autocamtide, two highly specific CaMK substrates (Engels et al., 2004). In addition, they have a similar distribution pattern in the brain (Engels et al., 1999). Interestingly, both DCLK-short and CaMK-IV contain an extended S/P-rich N-terminal domain of approximately 60 amino acids. This S/P-rich domain is also present at the C-terminal part of DCX and DCL and in the middle of the DCLK-long proteins. Recently, DCLK-short phosphorylation of the

S/P-rich domain has been shown to take place following nerve growth factor (NGF) stimulation *in vitro* (Dijkmans et al., 2009), placing DCLK-short downstream of neurotrophic factor signalling.

2.4 CaMK Related Peptide (CARP)

Alternative splicing of the DCLK gene also generates a transcript encoding a 55-amino-acid peptide, called CaMK-related peptide (CARP) (Vreugdenhil et al., 1999); also called Ania-4 (Berke et al., 1998). CARP largely overlaps with the SP-rich N-terminal domain of DCLK-short, but lacks the DCX and catalytic kinase domains and therefore does not exert any MT binding properties or kinase activity. Under normal circumstances, CARP is expressed at extremely low levels or is not present at all. In contrast, CARP mRNA is highly up-regulated by kainate-induced seizures in the hippocampus while DCLK-short is not (Vreugdenhil et al., 1999). CARP is also induced in striatal neurons by D1-receptor agonists and by the psychostimulant cocaine (Berke et al., 1998; Glavan et al., 2002). In addition, CARP levels are highly elevated following brain derived neurothropic factor induced long term potentiation (BDNF-LTP; Wibrand et al., 2006). This raises the possibility that CARP has a specific function during times of neuronal activity and challenge, since its expression is only increased when neurons are faced with strong stimuli, i.e. kanic acid, cocaine and high frequency stimulation. The structural overlap of CARP and the SP-rich N-terminus of DCLK-short suggests involvement of CARP in neuronal apoptosis. In fact, the caspase-cleaved SP-rich N-terminal fragment of DCLK-short exacerbates serum-deprived induced apoptosis in neuroblastoma cells while the catalytic domain-containing C-terminal domain does not (Kruidering et al., 2001). Apart from these observations, very little is known about the function of CARP *in vivo*.

3. The hippocampus

DCLK splice variants are expressed in the adult hippocampus (Vreugdenhil, Engels et al. 2001; Burgess and Reiner 2002; Engels, Schouten et al. 2004) and are important for hippocampal development (Koizumi et al, 2006; Deuel et al., 2006;

Tanaka et al., 2006; Vreugdenhil et al., 2007; Tuy et al., 2008). The hippocampus is part of the limbic system and is involved in learning and memory processing, probably contributing to the transition from short-term to long-term memories. This is demonstrated by the fact that particularly simultaneous lesions of several cortical areas together with the hippocampus produce impairments in spatial navigation and the ability to learn new facts. It is proposed that information processing and memory formation is shared by several brain areas, such as the amygdala and cortical areas, that act as a functional system, and that the hippocampus is an important part of this system. It plays a supportive role in associating complex information and generating new memory traces (Hölscher, 2003).

The hippocampal formation consists of the subfields of the cornu ammonis (CA) and the dentate gyrus (DG), which are folded into each other and form a trisynaptic circuit (Figure 2). Both fields contain densely packed cell layers; the CA contains pyramidal neurons, whereas the DG is composed of smaller granule neurons. In addition, both layers contain inhibitory interneurons. Information flow through this trisynaptic circuit enters the DG from different brain areas, mostly from the enthorinal cortex, which projects onto the granule cells of the DG via the perforant path. Mossy fibers project from DG neurons onto the pyramidal cells of the CA3 region, which in turn project via Schaffer collaterals to the pyramidal neurons of the CA1. Another important input to the CA1 cell layer comes directly from the perforant path, without the involvement of the DG and CA3 regions. Hippocampal output is mainly directed via the subiculum to the cortex and the prefrontal cortex. Hippocampal neurons mostly rely on the excitatory neurotransmitter glutamate for neurotransmission, but they are also innervated by monoaminergic projections, e.g. serotonin (5-HT), dopamine (DA) and noradrenalin (NE). Monoamines strongly suppress the perforant path input to the CA1 hippocampal region with minimal effects on Schaffer collateral input. For inhibitory interneurons, γ-amino-butyric-acid (GABA) is the most common neurotransmitter. These excitatory and inhibitory inputs are capable of modulating information processing of the hippocampal network and strongly determine the excitability and also affect the plasticity and viability of neurons (Otmakhova et al., 2005).

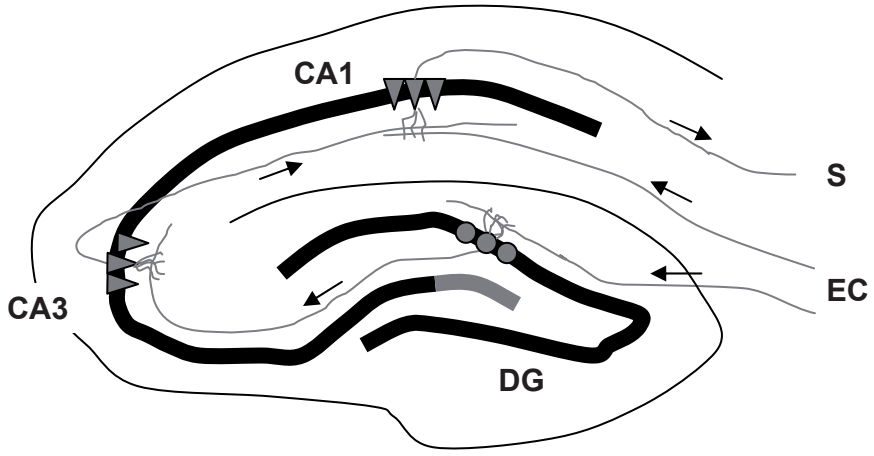

Figure 2. Schematic representation of the hippocampal formation. EC=Entorhinal Cortex; DG=Dentate Gyrus; CA=Cornu Ammonis; S=Subiculum. Cicles represent DG granule cells, triangles represent CA pyramidal cells. Arrows indicate the direction of neuronal transmission/information flow. For details see text.

3.1 Corticosteroids and neuronal viability in the hippocampus

Crucial players in controlling hippocampal plasticity and functioning are glucocorticoid hormones, which are secreted by the adrenals in a circadian rhythm and during periods of stress. The main glucocorticoids in humans and rodents are cortisol and corticosterone, respectively. Receptors for these hormones, the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), are abundantly found in the hippocampus, making this structure a major target for corticosteroid action (de Kloet et al., 1998; Nichols et al., 2001). Following binding of ligand to these receptors, they form dimers that can bind to Glucorticoid-Responsive-Elements (GREs) in the DNA, thereby affecting expression of target genes. This mechanism is called transactivation. Alternatively, monomers of ligand bound receptors can interact with other transcription factors, such as AP-1, CREB and NFκ-B, independently of DNA-binding, to regulate gene transcription (Heck et al., 1994; Ray and Prefontaine, 1994). This mode of action is called transrepression. Corticosteroids have an important impact on hippocampal physiology and on

hippocampus dependent memory formation (Reviewed in de Kloet et al., 1998). Corticosteroids are also able to affect the morphology and viability of hippocampal neurons. Chronic stress exposure or chronic administration of corticosterone results in remodelling of dendrites of the CA3 pyramidal neurons and in reduced numbers of synapses on these neurons (Magariños et al., 1996; Fuchs et al., 2006). In contrast, removal of freely circulating corticosteroids by adrenalectomy (ADX), leads to apoptosis of granule cells in the DG (Sloviter et al., 1993; Hu et al., 1997). In addition, stress inhibits the process of neurogenesis in the DG, while corticosteroid depletion, leading to apoptosis, coincides with increased neurogenesis and neuronal migration. Interestingly, it has been demonstrated that these newborn, migrating neurons in the subgranular zone of the DG express DCX. Hippocampal changes like CA3 atrophy, apoptotic cell death and neurogenesis that occur in the DG of the adult hippocampus are modulated by glutamatergic transmission and subsequent N-Metyl D-apartate (NMDA) receptor activation and fluctuations in Ca^{2+} levels, in concert with corticosteroid hormones (Gould, 1994; Gould et al., 1994; Cameron and Gould, 1994; Cameron et al., 1998; Karst and Joels, 2001). Molecular studies show that corticosteroids modulate expression of genes involved in neuronal differentiation and structural remodelling, probably to enable the hippocampal network to rearrange its connections in order to cope with changing requirements from both the internal and external milieu.

4. Redundancy in DCX and DCLK function during development

Several recent studies using knockout mice and RNAi-mediated knockdown of target genes, indicate that DCX and DCLK have overlapping functions during cortical and hippocampal development in mouse (Koizumi et al, 2006; Deuel et al., 2006; Tanaka et al., 2006). Germline DCX targeting is associated with a hippocampal lamination defect, but cortical lamination is unaffected (Corbo et al., 2002), whereas acute RNAi-mediated inactivation of DCX by electroporation of a target plasmid results in a doublecortex phenotype with defects in neuronal targeting to the cortical plate (Bai et al., 2003). As a result, neurons are aberrantly located in the intermediate zone of the brain and display alterations in neuronal morphology. The lack of a cortical defect in the DCX germline knockout suggests

that members of the DCX domain family (i.e. DCLK) may function in a redundant way during cortical development. In fact, the DCLK gene functions in a partially redundant pathway with DCX in the formation of axonal projections and migration of cortical neurons (Koizumi et al., 2006; Deuel et al., 2006). Like DCX knockout mice, DCLK null mice also exhibit a rather mild cortical phenotype, but DCX/DCLK double mutant mice display severe cortical defects, with disorganized neocortical layering, profound hippocampal disorganization and neurons with abnormal axon outgrowth and dendritic structure (Koizumi et al., 2006; Deuel et al., 2006; Tanaka et al., 2006). Surprisingly, RNAi-mediated knockdown of either DCX or DCLK results in similar severe cortical migration defects. In addition, targeted deletion of DCLK shows no appreciable developmental defect in the hippocampus, but removal of both genes shows severe hippocampal lamination defects involving the entire CA and DG fields that resemble the phenotype observed in human (Tanaka et al., 2006). More specifically, Deuel et al. demonstrate that DCX/DCLK-deficient neurons show defects in axonal transport of synaptic vesicle proteins. Thus, DCX and DCLK may directly or indirectly regulate MT-based vesicle transport, a process critical to both neuronal migration and axonal outgrowth (Deuel et al., 2006). These observations clearly indicate overlapping roles for DCX and DCLK during development.

5. DCLK beyond neuronal development: scope and objectives

A wealth of information is available on the function of DCX and DCX-domain containing DCLK splice variants during development. However, since the DCLK gene encodes multiple, different transcripts, some of which are expressed in the adult brain or in response to neuronal activity, the DCLK gene may have additional functions beyond neuronal development (Burgess et al., 1999; Burgess and Reiner, 2002; Hevroni et al., 1998; Silverman et al., 1999; Vreugdenhil et al., 1999; Wibrand et al., 2006). Surprisingly little is known about those splice variants that do not contain the DCX-domains, DCLK-short and CARP. Therefore, we have generated transgenic mice with over-expression of either CARP or a constitutively active form of DCLK-short, called δC-DCLK-short, in the hippocampus of C57BL/6j mice. This has opened up the possibility to study the effect of over-expression of

these DCLK transcripts in the adult hippocampus. The work described in this thesis aims to determine the phenotypes of these transgenic mice at different functional levels, such as the genetic, network and behavioural level.

5.1 Experimental outline

Firstly, since the caspase-cleaved SP-rich N-terminal fragment of DCLK-short exacerbates serum-deprived induced apoptosis in neuroblastoma cells and CARP and the SP-rich N-terminus of DCLK-short are highly homologous, CARP itself may play a role in neuronal apoptosis (Kruidering et al., 2001). In **Chapter 2** we set out to determine the involvement of CARP in the apoptotic process in the DG following corticosteroid depletion by adrenalectomy. **Chapter 3** describes the first of three transgenic lines that were examined, namely a transgenic mouse line with high expression levels of CARP throughout the brain, designated high-CARP. In **Chapter 4** high-CARP mice and a second transgenic line with a more restricted neuronal expression profile, called low-CARP, are characterized at the behavioural level by fear conditioning. **Chapter 5** is dedicated to characterization of the third transgenic strain; δC-DCLK-short. Mice from this background have brain specific expression of a truncated form of DCLK-short, making this kinase constitutively active (Engels et al., 2004). Using the elevated plus maze test we investigated if δC-DCLK-short mice display altered anxiety-related behaviours. Finally, **Chapter 6** consists of a general discussion where we aim to establish the functions and involvements of the DCLK gene splice variants CARP and DCLK-short, based on literature and our current findings using transgenesis.

6. References

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