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Voor Rob en Anneke

Table of Contents

Abbreviations

ACSF	Artificial Cerebrospinal Fluid
ADX	Adrenalectomy
BDNF	Brain Derived Neurotrophic Factor
CA	Cornu Ammonis
Calb2	Calbindin2; Calretinin
CaMK	Calcium/Calmodulin dependent protein kinase
CARP	Calcium/Calmodulin dependent protein kinase Related Peptide
CS:	Conditioned Stimulus
D1	Dopamine 1
DCL	Doublecortin-Like
DCLK	Doublecortin-Like Kinase
DCX	Doublecortin
DEPC	Diethylpyrocarbonate
DG	Dentate Gyrus
Epac2	Exchange protein activated by cyclic AMP 2
EPM:	Elevated Plus Maze
ERK	Extracellular signal-Regulated Kinase
fEPSP	field Excitatory Post Synaptic Potential
GABA	g-Amino-Butyric Acid
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GDP	Guanosine Diphosphate
Gef	Guanine exchange factor
Grb2	Growthfactor receptor-bound protein 2
GTP	Guanine Tri Phosphate
HPA	Hypotalamic Pituitary Adrenal
LT-82	Clostridium *Sordelli* Lethal Toxin-82
LTD	Long Term Depression
LTP	Long Term Potentiation
MAPK	Mitogen-Activated Protein Kinase
NMDA	N-Methyl-D-Aspartic acid
NT-3	Neurotrophin-3
PP	Paired Pulse
PSA	Population Spike Amplitude
ROD	Relative Optical Density
SH	Src Homology domain
Sos	Son of Sevenless
SP	Serine/Proline
TG	Transgenic
Trk	Tropomyosin Receptor Kinase
UCS	Unconditioned Stimulus
WT	Wild-Type

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).

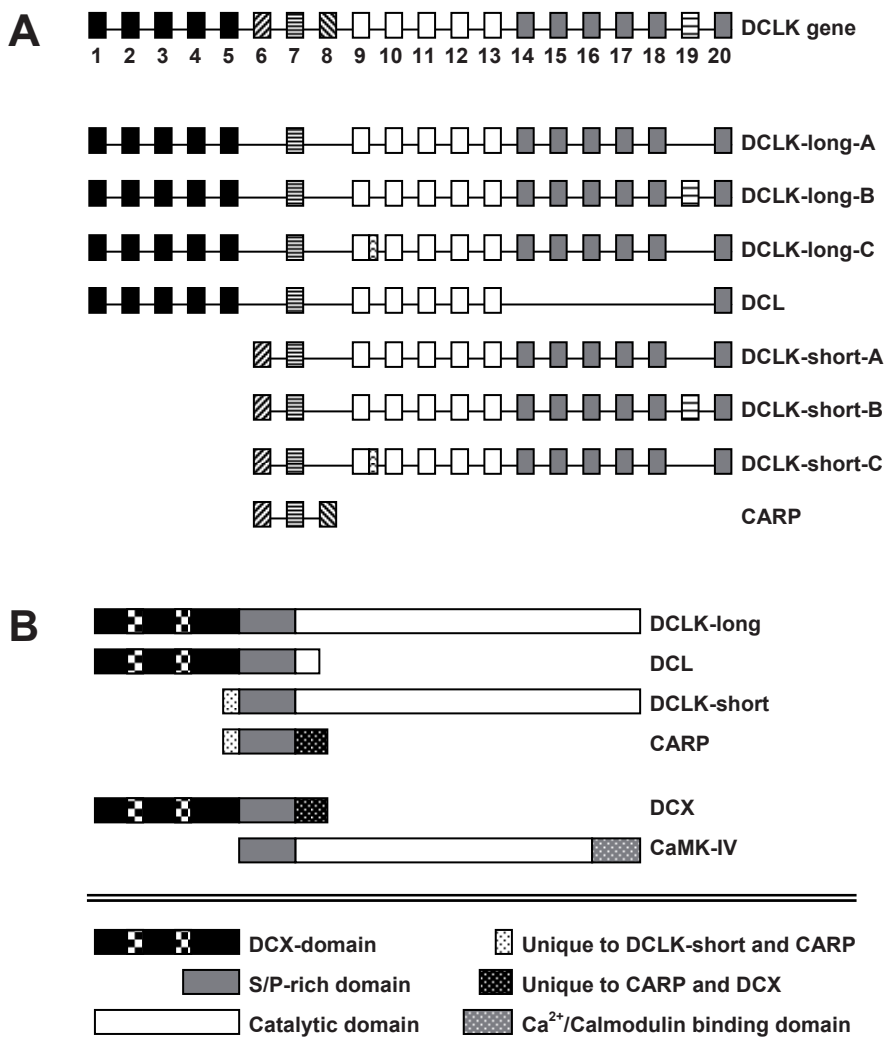

Figure 1. (A) Genomic organization of the DCLK gene. The addition to exon 9 in the 'C' variants represents a nuclear localization signal. (B) Functional domains of proteins generated by the DCLK gene. DCLK-long and DCL are expressed during embryonic development (Deuel, Liu et al. 2006; Koizumi, Tanaka 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).

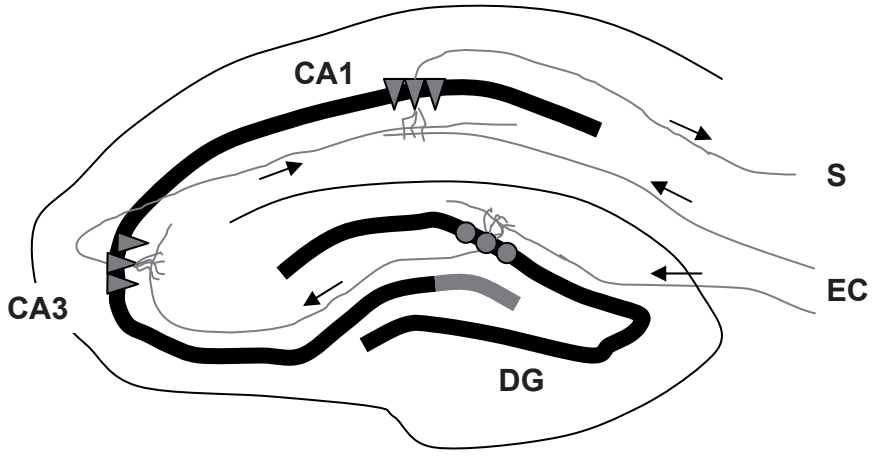

Figure 2. Schematic representation of the hippocampal formation. EC=Entorhinal Cortex; DG=Dentate Gyrus; CA=Cornu Ammonis; S=Subiculum. Cicles represent DG granule cells, triangles represent CA pyramidal cells. Arrows indicate the direction of neuronal transmission/information flow. For details see text.

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.

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Chapter 2

A potential role for calcium/calmodulin-dependent protein kinase related peptide in neuronal apoptosis: *in vivo* and *in vitro* evidence

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Abstract

Previously, we have established that a product of the Doublecortin-like kinase (DCLK) gene, DCLK-short, is cleaved by caspases during serum deprivation. Subsequently, the N-terminal cleavage product of DCLK-short facilitates apoptosis in the neuroblastoma cell line NG108. As this N-terminal cleavage product is highly homologous to CaMK-Related Peptide (CARP), another DCLK gene splice-variant, we aimed to determine possible apoptotic properties of CARP *in vivo* and *in vitro*.

We report highly specific CARP expression in apoptotic granule cells in the rat dentate gyrus after adrenalectomy relative to healthy granule cells. CARP is significantly up-regulated in the supra pyramidal blade of the dentate gyrus, with varying levels of up-regulation, depending on the extent of adrenalectomy-induced apoptosis. Similar to the caspase-cleaved N-terminus of DCLK-short, CARP over-expression itself facilitated apoptosis in serum-deprived NG108 cells. Furthermore, CARP facilitated polymerization of tubulin *in vitro* and was capable of interacting with Grb2, an intracellular protein involved in vesicle trafficking. Together, our data demonstrate a facilitating role for CARP in the apoptotic process in granule cell populations sensitive to adrenalectomy and suggest that this pro-apoptotic effect is mediated by increasing the stability of the microtubule cytoskeleton.

Introduction

Granule cells that are destined to die through apoptosis are known to have, when compared to healthy cells, altered electrophysiological, morphological and cytoskeletal characteristics, which are accompanied by triggering of specific gene expression profiles (Stienstra and Joels 2000; Nair, Karst et al. 2004). A well-established model for the induction of apoptosis in the rat dentate gyrus (DG) is adrenalectomy (ADX). ADX-induced apoptosis typically affects only a small subset of dentate granule neurons, whereas most surrounding cells remain viable (Sloviter, Sollas et al. 1993; Hu, Yuri et al. 1997).

Previously, we have identified the expression of the Doublecortin-Like Kinase (DCLK) gene in the hippocampus of ADX rats (Vreugdenhil, de Jong et al. 1996; Vreugdenhil, de Jong et al. 1996). This gene contains a doublecortin (DCX) domain as well as a Calcium/calmodulin dependent protein kinase (CaMK)-like domain, and is subject of alternative splicing (Vreugdenhil, Engels et al. 2001; Burgess and Reiner 2002). DCLK-long and Doublecortin-Like (DCL), exhibit high homology with DCX and are both expressed during development where they control neuronal migration and neurogenesis (Figure 1) (Deuel, Liu et al. 2006; Koizumi, Tanaka et al. 2006; Shu, Tseng et al. 2006). DCLK-long and DCX have similar biochemical and biophysical characteristics (Lin, Gleeson et al. 2000). These proteins function as microtubule associated proteins thereby affecting cytoskeleton stability (Gleeson, Lin et al. 1999; Burgess and Reiner 2000).

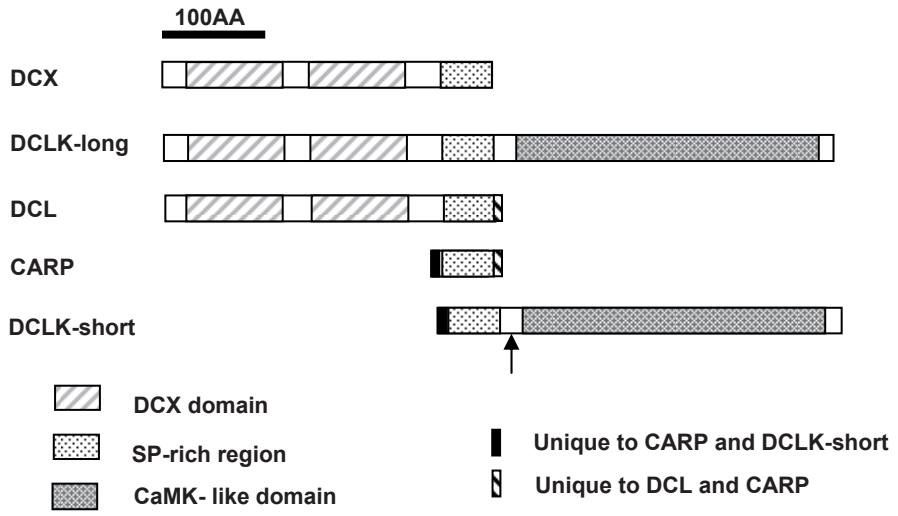


Figure 1. The main proteins generated by the DCLK gene. DCLK-long and DCL are mainly expressed during embryonic development (Deuel, Liu et al. 2006; Koizumi, Tanaka et al. 2006; Shu, Tseng et al. 2006), while DCLK-short is mainly expressed in the adult brain (Engels, Lucassen et al. 1999; Engels, Schouten et al. 2004). CARP is expressed at very low levels but induced in the hippocampus by kainate-induced seizures (Vreugdenhil, Datson et al. 1999) and in striatal neurons by D1-agonists and cocaine (Berke, Paletzki et al. 1998). The arrow indicates the location of the caspase cleavage site (Kruidering, Schouten et al. 2001).

DCLK-short contains a CaMK-like catalytic domain and is abundantly expressed in limbic structures of the adult brain (Vreugdenhil, Engels et al. 2001; Burgess and Reiner 2002; Engels, Schouten et al. 2004). The DCLK gene also encodes a transcript that lacks both the DCX and CaMK-like domains. This 55-amino-acid peptide, called CaMK-related peptide (CARP) (Vreugdenhil, Datson et al. 1999); also called Ania-4 (Berke, Paletzki et al. 1998), largely overlaps with the serine/proline (SP)-rich domains of DCLK and DCL (Figure 1). CARP expression is below detection levels under normal conditions. In contrast, CARP mRNA is highly up-regulated by kainate-induced seizures in the hippocampus (Vreugdenhil, Datson et al. 1999). CARP is also induced in striatal neurons by D1-receptor agonists (Berke, Paletzki et al. 1998; Glavan, Sket et al. 2002). Interestingly, it has been shown that the caspase-cleaved SP-rich N-terminal fragment of DCLK-short exacerbates apoptosis in NG108 neuroblastoma cells (Kruidering, Schouten et al.

2001). Therefore the structural overlap between CARP and the SP-rich N-terminus of DCLK-short may predict involvement of CARP in apoptosis in neuronal cells. Moreover, the homology between DCLK-long/DCL and CARP and the recent observations that the DCX-domain containing products of the DCLK gene (DCLK-long, DCL) are crucial for DCLK gene function (Shu et al., 2006) has lead us to investigate the effect of CARP on DCL-induced microtubule polymerization. Additionally, we have screened for candidates for CARP protein-protein interactions. Since CARP was originally identified in the hippocampus of ADX rats (Vreugdenhil et al, 1999), we used this model to investigate the possible role of CARP in neuronal apoptosis *in vivo*. We have also studied the effect of CARP over-expression on the fate of NG108 cells *in vitro*. Together, our results indicate that CARP has pro-apoptotic properties in neuroblastoma cells *in vitro* and in granule cells in the DG of ADX rats *in vivo*.

Materials and Methods

Aimals and Surgery

26 Male Wistar rats, weighing 150-170 g, were housed two per cage (12h/12h light/dark cycle, lights on 9 AM). Animals had access to food and water *ad libitum* and were handled and weighed daily (9h-10h AM). After 10 days all animals (then weighing 200-250g) were adrenalectomized between 9 and 12 AM to ensure low circulating corticosterone levels. ADX was performed under isoflurane anaesthesia as described (Meijer and de Kloet 1995). After ADX, all animals had free access to 0.9% saline. Two days after ADX (day 12), a tailcut blood sample was obtained in EDTA-coated capillaries and kept on ice. Samples were centrifuged at 10.000 rpm for 10 min. and plasma was stored at -20 °C until use. Three days after ADX (day 13) animals were decapitated and trunk blood was collected in EDTA-coated tubes and kept on ice. Blood plasma was obtained by centrifuging at 3000 rpm for 15 min. at 4 °C and stored at -20 °C for determination of plasma corticosterone levels by radio immuno assay as described (Karssen, Meijer et al. 2005). Animals were considered properly adrenalectomized if corticosterone values were below 1.00 μg/dl on day 12. Of the 26 rats that were adrenalectomized, 6 had a plasma corticosterone level of 1,00 μg/dl or higher. These animals were excluded from the

experiment (Supplementary Table 1). Brains were quickly taken from the skull and snap-frozen in isopentane on a mixture of ethanol absolute and dry ice. Coronal sections (20 μm) were cut using a cryostat and thaw-mounted on poly-L-lysine coated slides. Sections were stored at -80 °C until use. All animal treatments were approved by the Leiden University Animal Care and Use Committee (UDEC# 01022).

Constructs

The DCLK-short construct has been described previously (Kruidering, Schouten et al. 2001). The CARP expression plasmid was contructed using CCAGGATCC ACCATGGGCCCTGGGGAAGAAGAGTC as a sense oligonucleotide and GCAGAATTCTTACACTGAGTCTCCTGAGTCCAAATC as antisense oligonucleotide and *Pfu*I as a proofreading polymerase. After purification on QuiaQuick columns, the fragment was digested with *Bam*HI and *Eco*RI (underlined in oligonucleotides) and subcloned into the corresponding sites of pcDNA3.1 (InVitrogen, Groningen, The Netherlands).

In situ hybridization was performed using oligonucleotides as described (Meijer, Steenbergen et al. 2000). DCLK-short was detected using 45mers recognizing the 3'untranslated region of the DCLK-short transcript and CARP was detected using a 45mer recognizing the 3'-untranslated region of the CARP transcript. Mismatch oligonucleotides with 4-5 substitutions were used as control. The DNA sequences are:

1. TGGTAGTAGTCCAAAGACCTTGATCTCTGGATGGTAAACCCGTGG
2. TGGTAGAAGTCCATAGACCGTGATCTCTGCATGGTATACCCGTGG
3. GATGCTTGCTTAGGAAATGGGAAACCTTGATCCCATCACAAACCA
4. GATGCTTGATTAGGAAACGGGAAACCTCGATCCCATTAAAACCA

No. 1 is the perfect match recognizing DCLK-short, No. 2 its mismatch control, No. 3 is the perfect match recognizing CARP and No. 4 its mismatch control (substitutions are underlined). Following labelling of the oligonucleotides, **slices** were exposed to an X-OMAT AR film (Kodak) for approximately 5-7 days. Films were scanned and relative optical densities (RODs) of hippocampal subfields CA1, CA3 and DG and background (area between the cell layers of CA1 and DG) were

measured using NIH Image 1.62. The background was subtracted from the RODs of the corresponding areas.

Dipping slices in photographic emulsion

Photographic emulsion in a glass container was liquefied at 42 °C for 30 min. in a water bath and kept at 42 °C during the entire dipping procedure. Hybridized slices were dipped into the emulsion and placed in an upright position in order to dry overnight in the dark and exposed for approximately 3 weeks. The emulsion was developed as follows: developer for 10 min., distilled water for 1 min., 5% acetic acid for 1 min., distilled water for 1 min., fixer for 5 min. and finally distilled water for 1 min. After developing the slices were kept under running water for 1h and air-dried. Sections were counterstained by Nissl staining: cresyl violet solution (0.5 %) for 10 min. and dehydrated in a graded series of ethanol 50, 70, 80, 100, 100% for 30 sec. and 4 times 1 min. respectively. Sections were air-dried, covered with permount and a microscopic coverslip (24x50 mm) and analysed by bright field microscopy using polarising light. Counting both healthy and picnotic nuclei, as observed with Nissl staining, the percentage of apoptotic cells in the supra pyramidal blade of the DG was investigated. Picnosis is a well-known hallmark of neurons that are dying through programmed cell death (Sloviter, Sollas et al. 1993). Picnotic DG granule cells are characterized by small, round, densely stained nuclei that are fragmented (Insert in Figure 2C). Both healthy and apoptotic nuclei of the supra pyramidal blade of the DG were counted in one microscopic field, at a magnification of 400x. The percentage of apoptosis was estimated (% apoptosis = (number of apoptotic cells/total number of cells) x 100) and plotted against the measured RODs of the corresponding *in situ* hybridization. The 20 properly adrenalectomized animals were included in this experiment (see above). Of these adrenalectomized animals, 16 displayed apoptosis, while 4 did not.

Cell Culture and Microinjection Experiments

NG108-15 cells were grown as described previously (Kruidering, Schouten et al. 2001). All cell culture chemicals were obtained from Life Technologies, Inc.

Transient transfection experiments were performed with Superfect (Qiagen, Valencia, CA) according to the protocol of the manufacturer. Cells were exposed to staurosporine 2 h after transfection, and the viability was assessed by microscopy based on cell morphology.

Cells were seeded on glass-bottomed coverslip dishes (Matteck Corp., Ashland, OR) 24–48 h prior to injection. Nuclear microinjection was performed using an automated microinjection system (Eppendorf Transjector 5246, micromanipulator 5171). Identical standardized conditions of pressure (150 hectopascals) and time (0.1 s) were used for microinjection in all experiments. DCLK-short and CARP DNA plasmids were mixed 1:5 with EGFP-N1 reporter plasmid (CLONTECH, Palo Alto, CA) in ultrapure water to a final concentration of 100 ng/μl of plasmid. Cells were injected, and the next morning the number of green, EGFP-N1 expressing, viable cells was counted (*t*=0). Cells were washed three times with serum-free medium to remove all serum and kept in serum-free medium. Cells were counted again 48 h after serum withdrawal. Viability was expressed as EGFP positive cells at a given time after serum withdrawal as percentage of green cells at *t*=0. For each construct, at least 400 green cells were counted from an average of six independent injection experiments. Injection of pcDNA 3.1 plasmid served as control.

Tubulin polymerization assay

To quantitatively analyze microtubule polymerization, 100 µl of pure tubulin at 1 mg/ml in G-PEM buffer (80mM Pipes pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 10 mM GTP) plus 5% (v/v) glycerol was added to 10 µl of recombinant wild type DCL protein and/or synthetic CARP peptide at different concentrations (30 mg/μl DCL, 20 μg/ml or 100 μg/ml CARP). Taxol was used as a positive control. According to manufacturer's instruction, tubulin polymerization was detected by measuring the absorbance of the solution at 340 nm at 37 ^{o}C kinetically for 60 minutes (HTS7000 spectrophotometer, BioRad).

Immunoprecipitation and Western blotting

Because CARP was predicted to interact with Grb2 (see results section), we investigated a possible protein-protein interaction between CARP and Grb2 by incubating increasing concentrations of CARP peptide (0; 0.5; 1; 5; 10 and 15 μg) with COS cell protein lysates. Total protein was extracted by lysing a million cells in lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Triton. Subsequently immuno precipitation was performed using a Grb2-agarose conjugate (Santa Cruz): Grb2 protein conjugated to agarose beads in PBS containing 0.1% azide, 0.1% BSA and 10% glycerol. For detection of CARP and Grb2, equal amounts of protein were separated by electrophoresis on an SDS-polyacrylamide gel (12%) and semidry electroblotted to a polyvinylidene difluoride membrane, Immobilon-P (Millipore Corp., Bedford, MA). Blots were blocked with blocking buffer (Tris-buffered saline, 0.2% Tween (TBS-T), 10% milk) and incubated with primary antibodies (1:1000) for 1h at room teperature in blocking buffer. The anti-CARP antibody was produced in rabbits by injection of a 55-amino acid-long synthetic peptide corresponding to the N-terminal domain of DCLK, designated CARP. The anti-CARP antibody is capable of recognizing DCLK-long and -short and DCL in addition to the CARP peptide (Vreugdenhil et al., 1999). The anti-Grb2 antibody and horseradish peroxidase (HPA)-conjugated secondary antibodies (used at 1:5000) were from Santa Cruz. Blots were washed three times with blocking buffer, incubated with secondary HPA-conjugated antibodies for 1 h at room temperature, and washed five times. Binding was detected by enhanced chemiluminescence.

Analysis and Statistics

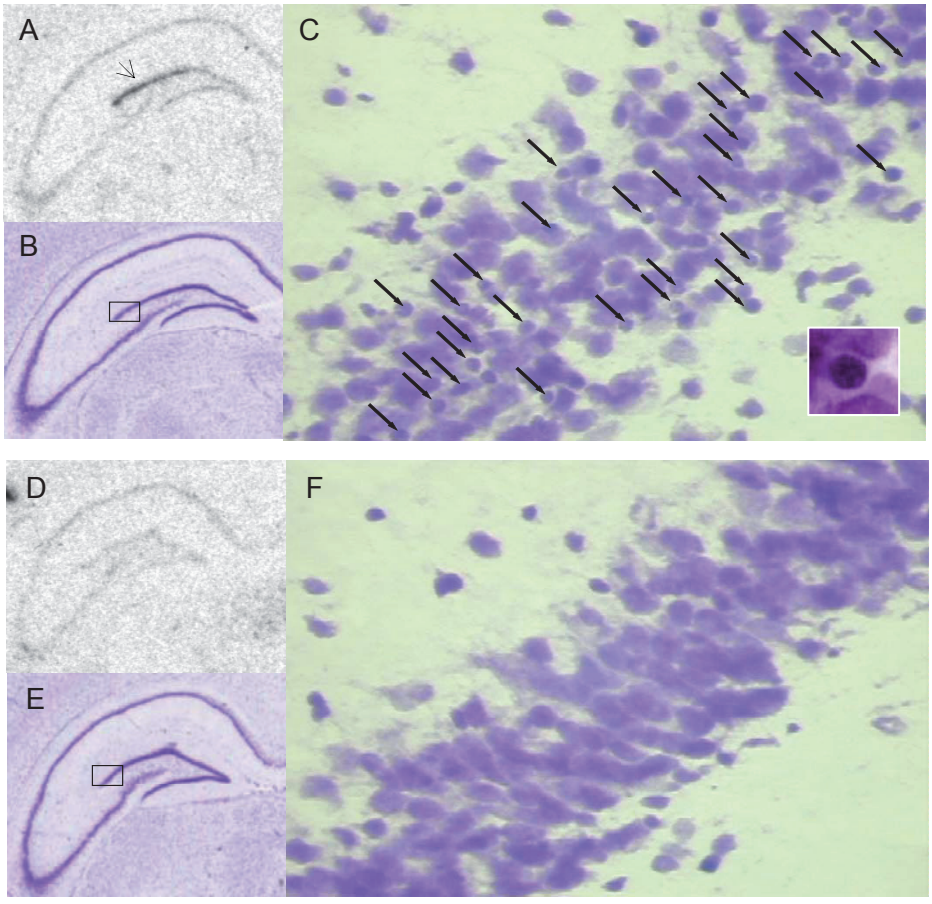
A motif scan of the full length DCLK protein sequence was performed using http://scansite.mit.edu/motifscan_seq.phtml. Corticosterone radioimmunoassay data were analysed using the SECURIA II program. *In situ* hybridization relative optical densities (RODs) of hippocampal subfields CA1, CA3 and DG and background were measured using NIH Image 1.62. The background signal was subtracted from corresponding measurements for each of the areas studied. Western blot RODs were analyzed in a similar manner. Significant differences were determined with one-way anova, with posthoc Tukey HSD (honest significant

difference) test. Pearson's Correlation test was used to calculate correlation coefficients and to determine significant correlations. For the output of the tubulin polymerization assay the maximal level of tubulin polymerization for the indicated samples after 60 minutes of incubation was statistically analyzed using one-way anova with Tukey-Kramer multiple comparisoms test. For all tests probability level of 5% was used as the minimal criterion of significance.

Results

CARP expression in the DG of ADX animals

Adrenalectomy has been widely used to induce apoptosis in adult dentate gyrus granule cells (Sloviter et al, 1989; 1993). To study possible involvement of CARP in neuronal apoptosis, we have studied CARP expression in ADX animals with different levels of apoptosis in the DG. We observed an increase in CARP mRNA expression level specifically in the supra pyramidal blade of the DG in ADX rats (Figure 2A). No changes in CARP expression were observed in the other subfields of the hippocampus (CA1 and CA3). Using the same tissue sections, both the extent of apoptosis (Figures 2B/C and 2E/F) and CARP mRNA expression were investigated (Figures 2A and 2D). The extent of apoptosis ranged from 0% to 30% while ROD measurements for CARP varied from 5 to 110 (arbitrary units). A significant and positive correlation (correlation coefficient 0.66; $p<0.01$) was found between CARP mRNA expression and the relative number of picnotic nuclei representing apoptotic cells. Specifically, high levels of apoptosis were correlated with high CARP expression and vice versa (Figure 2G). No significant change in DCLK-short expression was found in the DG of ADX animals, regardless of the presence or absence of corticosterone along with no correlation between the level of DCLK-short mRNA expression and the percentage of apoptosis (data not shown). Brightfield microscopy using polarising light revealed that silver grains, representing CARP transcripts, were exclusively colocalized with apoptotic cells while the number of silver grains found in healthy granule cells was equal to background (Figures 2H and 2I), suggesting a role for CARP in the process leading to ADX-induced apoptosis in DG granule cells.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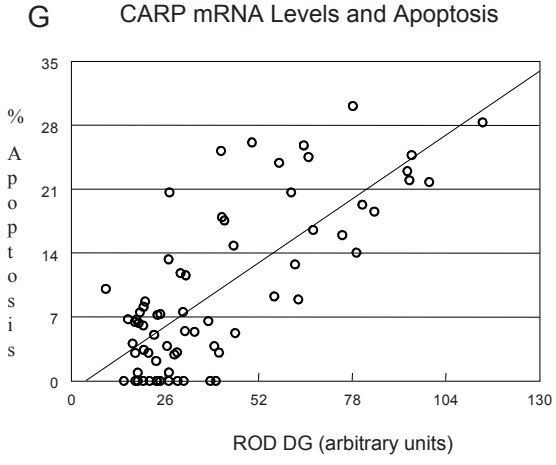
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CARP mRNA Levels and Apoptosis
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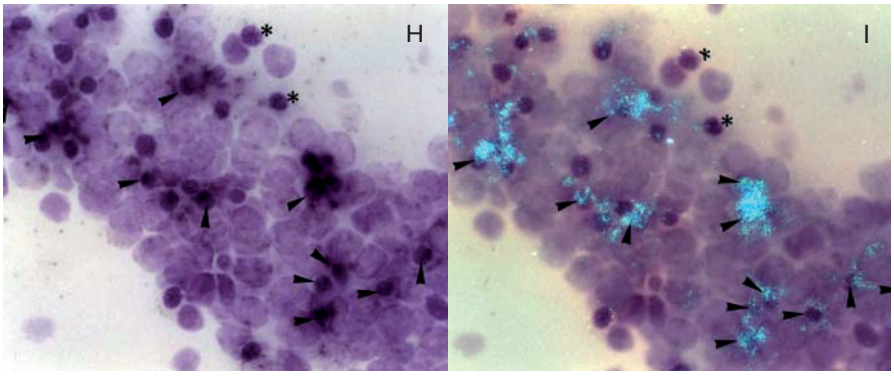

Figure 2. CARP is specifically expressed in apoptotic granule cells in the hippocampus of ADX rats. A-F: in situ hybridization analysis of CARP in ADX animals with high (A-C) and low (D-F) numbers of apoptotic cells. A and D are CARP in situ hybridization autoradiograms. Note the up-regulation of CARP mRNA in the supra pyramidal blade of the dentate gyrus (indicated by arrowhead) in ADX rats with apoptotic cells (A) compared to ADX animals without apoptotic cells in the DG (D). B, C and E, F are the corresponding Nissl-stained sections to visualize apoptotic cells (arrows and insert in F). G: Correlation between the percentage of apoptosis in the suprapyrimidal blade and the expression level of CARP (correlation coefficient 0.66; p<0.01). Apoptotic cells were counted in 6 rats with at least 8 sections per animal and the corresponding RODs of the hybridization signal is indicated. See text for further details. H: Microscopical view visualizing nissl-stained nuclei. Apoptotic cells can clearly be seen by their picnotic appearance (arrows). I: Same section as H but exposed to polarising light to reveal silver grains representing CARP transcripts (arrows). Note that CARP expression is located in, or very near to apoptotic cells although some at low levels (indicated by asterisks) and that the hybridization signal in healthy granule cells is below detection levels.

CARP micro-injection in NG108 cells

Previously, we have shown that DCLK-short is cleaved by activated caspases and that the N-terminal cleavage product facilitates staurosporine-induced apoptosis in NG108 cells. As 38 out of the 55 amino acids of the CARP peptide are identical (63%) within the 60 amino acids-long N-terminal cleavage product of DCLK-short (Figure 1), we decided to study the effect of CARP over-expression on staurosporine-induced apoptosis in NG108 cells. With that aim we have micro-injected CARP, DCLK-short and control constructs in NG108 neuroblastoma cells and monitored their fate. Cells micro-injected with control vector showed 74.5% viability after 24 hours of serum deprivation, whereas only 55.4% of all NG108 cells were viable after micro-injection of the CARP-expressing construct when exposed

to 24 hours of serum deprivation. In contrast, injection of the DCLK-short construct did not alter the number of apoptotic cells after serum deprivation (Figure 3). When injected in healthy, non-serum deprived NG108 cells the CARP construct did not decrease viability (data not shown). These observations suggest that CARP acts as a facilitator of apoptosis in neuronal cells, but has no apoptosis-inducing properties of its own.

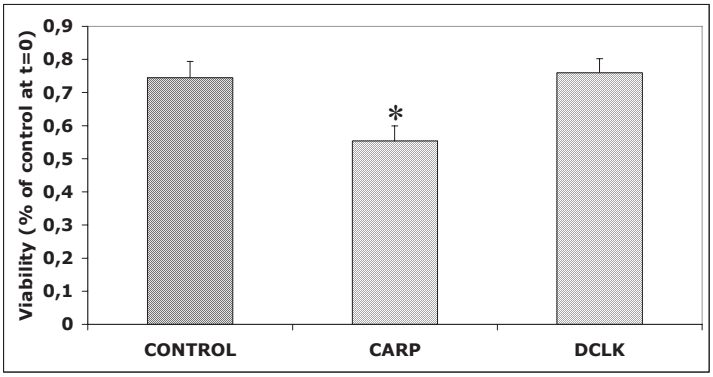

*Figure 3. Effect of microinjected CARP and DCLK-short constructs on neuronal cell death induced by serum withdrawal. Cells were injected with different constructs mixed with eGFP as described. At t=0, green viable cells were counted and serum was withdrawn. Cells were re-counted 24 hrs after serum withdrawal. Viability is expressed as % of green cells at t=24 relative to t=0. For each construct at least 400 cells have been counted in at least 4 injection series. Injection of empty vector served as control. Cells were injected with CARP and DCLK-short. * Statistically significant relative to control (T-test, p<0.05).*

CARP and DCL-induced tubulin polymerization

The DCLK gene, which generates CARP by alternative splicing, has been shown to be associated with the stability of microtubules (Kim et al, 2003, Shu et al, 2006, Vreugdenhil et al, 2007), a process that is severely affected by apoptosis. DCL is a microtubule-associated protein with high homology to CARP (Vreugdenhil et al., 2007). Therefore, we have studied *in vitro* the effect of CARP on DCL-induced polymerization of microtubules. Recombinant DCL was incubated with purified tubulin in the presence or absence of different concentrations of synthetic CARP peptide (20 or 100 μg/ml) and the degree of polymerization was measured (Figure 4). As a positive control for this assay Taxol was used. Taxol was able to induce

polymerization, with a similar level of polymerization as observed with the addition of DCL alone after 60 minutes. Recombinant DCL directly affected tubulin polymerization and an increase in the total amount of polymerized tubulin was observed in all samples containing DCL (Figure 4A). We found that addition of the highest concentration of CARP (100 μg/ml) facilitated DCL-induced polymerization of tubulin, whereas the lowest concentration (20 μg/ml) did not, indicating a dose dependent effect of CARP on DCL-induced tubulin polymerization. In contrast, CARP in the absence of DCL did not positively affect polymerization. This is more clearly illustrated by Figure 4B, which shows the maximal level of tubulin polymerization for the indicated samples after 60 minutes of incubation.

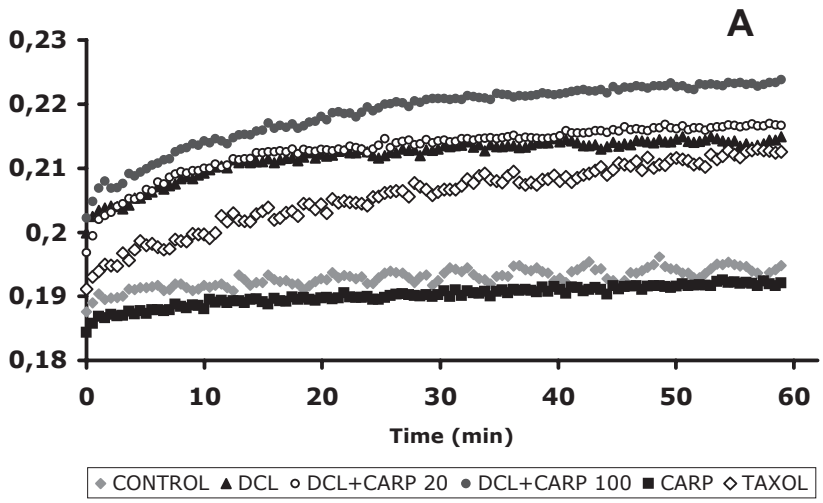

Figure 4. Effect of CARP on DCL-induced microtubule polymerization. Two concentrations (20 μg/ml or 100 μg/ml) of synthetic CARP were incubated with recombinant DCL protein (30 mg/μl) and purified tubulin (1 mg/ml). The turbidity of the DCL/tubulin mixture was monitored at 340 nm for 60 min. Taxol was used as a positive control (different from control ($p<0.01$)). Addition of synthetic CARP to the DCL/tubulin mixture increased DCL-mediated tubulin polymerization, while addition of CARP in the absence of DCL did not facilitate polymerization (A).

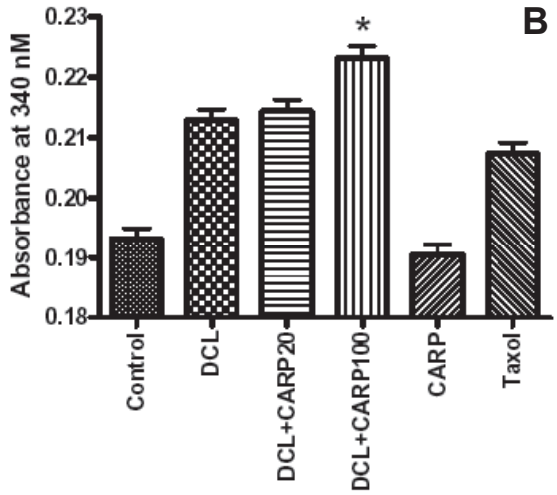

*Figure 4. (Continued) The maximal level of tubulin polymerization for the indicated samples after 60 minutes of incubation is shown in B. *, significantly different from DCL ($p<0.05$). All DCL containing samples were significantly different from control ($p<0.001$). The graphs are representative of two independent experiments with similar results.*

CARP and Grb2 interaction in vitro

The primary amino acid sequence of CARP does not contain obvious protein motifs linking it to specific biological functions e.g. transcription or enzymatic activity. As the CARP peptide is also small in size (55 amino acids) and the S/P-rich C-terminal parts of DCX and DCLK, to which CARP is highly homologous, are implicated in protein interactions (Friocourt et al., 2001; Moores et al., 2004), we speculated that CARP also exerts its effect by interacting with other proteins. To identify potential interacting proteins we conducted an in silico search using a motif scan (http://scansite.mit.edu/motifscan_seq.phtml). This motif scan of the full length DCLK protein sequence revealed a high concentration of protein-phosphorylation motifs within the CARP domain (for details see supplementary figure S1). In addition, the CARP domain is predicted to interact with SH3 domain containing proteins, in particular with Grb2. To study a possible protein-protein interaction

between CARP and Grb2 we incubated increasing concentrations of CARP peptide (0, 0.5, 1, 5, 10 and 15 μg) with COS cell lysates to more closely mimic a cellular context. Immuno precipitation using Grb2 protein coupled to agarose beads was performed to specifically pull-down Grb2-interacting proteins. Western blot analysis using a DCLK/CARP specific antibody of Grb2-captured lysates showed a 10 kD immunoreactive band that co-migrated with the synthetic CARP peptide and a 50 kD band corresponding to the Grb2 protein (Figure 5A). In addition, quantification of the RODs revealed that CARP-Grb2 interaction was dependent on the absolute concentration of added synthetic CARP peptide (Figure 5B), whereas the amount Grb2 protein was equal in all samples (Figure 5C). Thus, *in vitro*, CARP is able to interact with Grb2 in a dose-dependent manner.

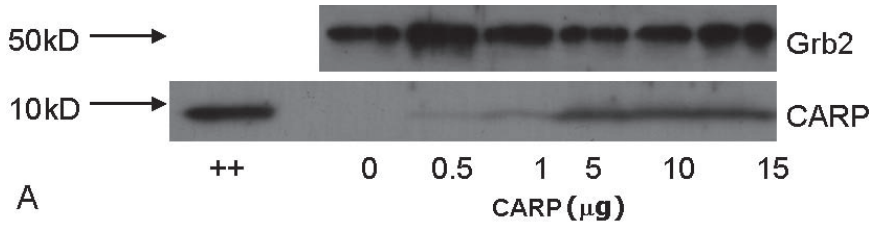

Figure 5. CARP protein-protein interaction with Grb2. A: Western blot showing CARP co-precipitation with Grb2 protein. Increasing concentrations of synthetic CARP peptide (0; 0.5; 1; 5; 10 and 15 μg) and synthetic CARP peptide as a positive control (++) are indicated. The 10 kD band corresponds to CARP and the 50 kD band corresponds to Grb2.

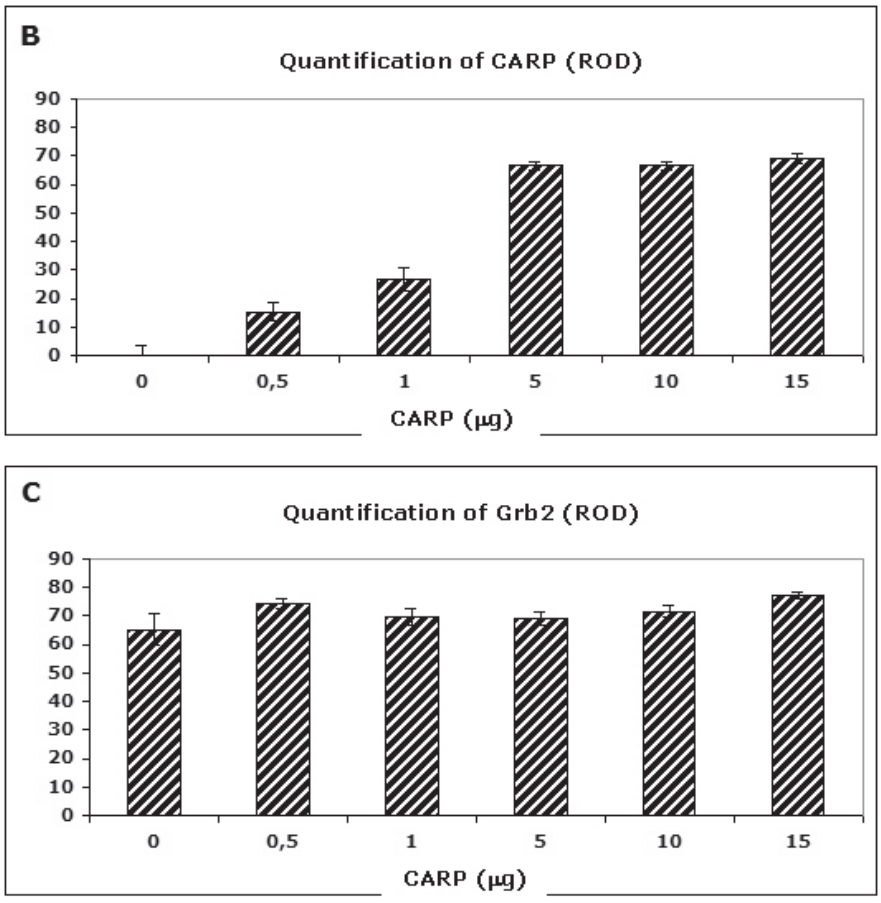

Figure 5. (Continued) B: Relative quantification (ROD) of CARP peptide levels shows dose dependency. Note that 5 µg of CARP peptide is sufficient to saturate the Grb2-agarose conjugate. 0 µg CARP is used as base line. C: Relative quantification (ROD) of Grb2 protein levels shows equal levels of Grb2 in all samples.

Discussion

We have investigated the role of the DCLK gene in neuronal apoptosis by studying CARP expression in the hippocampus of rats with varying degrees of apoptosis, 3 days after ADX. Under physiological conditions, CARP expression is low or even below detection levels in the adult brain. Previously, induction of CARP mRNA has been associated with kainate-induced seizures in hippocampal neurons (Vreugdenhil et al., 1999) and with administration of D1-agonists in striatal neurons (Berke, Paletzki et al. 1998; Glavan, Sket et al. 2002). However, during these processes, CARP induction has not been associated with neuronal apoptosis. Here, we show a novel association, i.e. a correlation between CARP mRNA expression and ADX-induced apoptosis in DG granule cells and specific expression of CARP in these apoptotic neurons. Moreover, CARP over-expression in neuronal cells facilitated apoptosis neuronal cells. CARP was also able to simulate DCL-induced tubulin polymerization *in vitro*. Thus, our data for the first time demonstrate a pro-apoptotic role for this non-DCX domain-containing splice product of the DCLK gene. These findings may be of importance in understanding the functions of members of the DCLK gene family and the molecular basis of apoptosis in specific neuronal populations.

CARP is specifically expressed in apoptotic DG cells following ADX

The extent of changes in CARP expression varied considerably across animals. This may be a consequence of the variability among animals in the number of degenerating cells after ADX (Sloviter, Sollas et al. 1993). This variability allowed us to examine the relation between CARP expression and the presence of apoptotic cells in the DG. We demonstrated a significant and positive correlation between CARP mRNA expression and ADX-induced apoptosis in DG granule cells. This suggests that either cells that have become apoptotic produce large amounts of CARP transcripts or that CARP represents a pro-apoptotic signal, consequently enhancing the rate of apoptosis. To investigate these possibilities we have exposed brain sections to photographic emulsion. Importantly, high levels of CARP transcripts were found in or close to apoptotic cells, but were absent in healthy granule cells. This strongly indicates that the observed increase of CARP in the DG

of ADX rats is a consequence of expression of CARP in apoptotic granule cells specifically. This observation raises the question whether CARP is involved in the initiation of the apoptotic process or whether the apoptotic process induces the expression of CARP. Since most, but not all, picnotic granule cells contained CARP transcripts and non-picnotic granule cell (including pre-apoptotic cells) were completely lacking CARP transcripts, it seems more likely that the apoptotic process induces the expression of CARP. This suggests that CARP exerts its function, albeit presently unknown, in an apoptotic context. This hypothesis is strengthened by our findings in NG108 neuroblastoma cells.

CARP exacerbates apoptosis in NG108 cells

Previously, we have shown that the N-terminus of DCLK-short, when cleaved from the full length protein, facilitates serum-deprived apoptosis in neuronal cell lines (Kruidering, Schouten et al. 2001). Since the 38 N-terminal amino acids of CARP are identical to this N-terminus, CARP itself may have similar pro-apoptotic effects. In line with the hypothesis that CARP has a function mostly in an apoptotic context, is the finding that CARP over-expression in non-serum deprived NG108 cells did not alter the number of apoptotic cells. In contrast, CARP over-expression in NG108 cells endangered by serum deprivation exacerbated apoptosis. CARP may therefore have pro-apoptotic properties exclusively in neuronal cells that are programmed to die via an apoptotic process. Given the high expression of DCLK gene products in the hippocampus (Engels et al., 1999; Vreugdenhil et al., 1996b; 1999) CARP may enhance apoptosis in a similar manner in granule cells destined to die through apoptosis after ADX.

CARP interacts with Grb2 *in vitro*

CARP is highly homologous to the S/P rich C-terminal parts of both DCX and DCLK, which are implicated in protein interactions (Friocourt et al., 2001; Moores et al., 2004). In addition, the DCLK gene has recently been implicated in intracellular vesicle trafficking (Duel et al., 2006). In this respect it is important to note that CARP was found to interact with Grb2, most likely through SH-3 domain binding.

Inspection of the primary amino-acid structure of CARP did not reveal any known homology domains that could link a possible biological role of CARP to enzymatic, katalytic, transcriptional or any other activity. The sequence GKSPSPSPTSPGSLR of CARP was predicted to interact with Grb2, an intracellular adapter protein containing a SH3-SH2-SH3 configuration that has been implicated in tyrosine-kinase receptor signalling (for review see (Tari and Lopez-Berestein 2001) and regulation of the actin cytoskeleton (for review see (Buday, Wunderlich et al. 2002). Grb2 is part of the Trk-receptor complex, where it tranduces neurotrophin-binding to activation of the Ras GTP-exchange factor Sos (Lowenstein, Daly et al. 1992; Egan, Giddings et al. 1993) and becomes internalized in and transported by signalling vesicles (Howe and Mobley, 2005), ultimately leading to activation of the Ras-ERK kinase cascade (for review see (Katz and McCormick 1997). From this perspective, CARP may influence the viability of granule cells in the DG in concert with a change in the availability of neurotrophic factors and their receptors after ADX (Schaaf, De Kloet et al. 2000; Nichols, Agolley et al. 2005). Interestingly, Neurotrophin-3 is reported to be down-regulated after ADX in the DG (Chao, Sakai et al. 1998; Hansson, Cintra et al. 2000) but not in other hippocampal subfields (Hansson, Cintra et al. 2000). Moreover, injection of Neurotrophin-4/5 grants protection against ADX-induced apoptosis of DG cells (Qiao, Hughes et al. 1996), which suggests a negative correlation between CARP expression and neurotrophin levels. CARP is also highly induced in the rat DG by kainate-induced seizures (Vreugdenhil, Datson et al. 1999), a phenomenon that is accompanied by induction of several neurotrophins (Gall and Lauterborn 1992; Lindvall, Kokaia et al. 1994). The fact that CARP expression is not associated with apoptosis in this seizure model leaves open the possibility that CARP induction is under control of distinct signalling cascades and that its induction, as well as its function, are highly context dependent. In accordance with this is the observed pro-apoptotic effect of CARP during serum deprivation in NG108 cells. Thus, CARP might facilitate apoptosis only in a small subset of the granule cell population in the DG that is deprived of growth factors. Together, our data indicate *in vitro* interaction of CARP and Grb2 in a dose-dependent fashion. As such, binding of CARP to Grb2 might lead to a downstream shift of neurotrophin signalling cascades and/or availability *in vivo*.

CARP enhances DCL-induced tubulin polymerization

The mechanism by which CARP affects neuronal viability is presently unknown. The doublecortin family consisting of DCX, DCLK-1 and DCLK-2 are known to induce microtubule polymerization and stabilisation (Francis, Koulakoff et al. 1999; Lin, Gleeson et al. 2000; Edelman, Kim et al. 2005). This, in combination with the structural overlap between DCX, DCLK-1 and DCLK-2 on the one hand and CARP on the other hand, raises the possibility that CARP affects DCLK gene-induced microtubule polymerization and thus cytoskeleton stability, thereby affecting neuronal viability. In addition, Shu et al. propose that the DCX domain containing isoforms of the DCLK gene are responsible for its functions (Shu et al., 2006), suggesting regulation of these isoforms (i.e. DCL) may be of importance. Using a tubulin polymerization assay we found that DCL was capable of tubulin polymerization, an observation that is well in line with previous results (Vreugdenhil et al., 2007). In this assay, CARP increased DCL-induced polymerization of tubulin in a dose dependent fashion. Thus, CARP influences the stability of microtubules *in vitro,* suggesting that in addition to DCX-domain containing isoforms, DCLK gene products without DCX-domains also have biologically relevant functions. Taxol, a well-known microtubule-polymerizing compound with anti-tumorigenic properties, was used as a positive control. Arrest of the cytoskeleton underlies the tumor-suppressing properties of this agent. Taxol is also able to selectively kill granule cells in the DG *in vivo* (Kim, Mitsukawa et al. 2002). Similarly, CARP may exert its pro-apoptotic properties through stabilization of the microtubule skeleton. CARP mRNA is induced in hippocampal neurons during kainate-induced seizures (Vreugdenhil, Datson et al. 1999) in striatal neurons by D1-receptor agonists (Berke, Paletzki et al. 1998; Glavan, Sket et al. 2002) and in apoptotic cells in the DG of ADX rats (this study). A common feature of these three challenges is the requirement of cytoskeleton rearrangements underlying the plasticity of specific neuronal circuits (Morimoto, Fahnestock et al. 2004; Everitt and Robbins 2005; Luo and O'Leary 2005). In this respect it is of interest to note that the DCLK gene is also found in a screen for candidate plasticity genes in the hippocampus by Hevroni et al., where it is designated Candidate Plasticity Gene 16 (CPG16) (Hevroni et al., 1998). CARP may function as a modulator of these rearrangements, perhaps by affecting neurotrophin signalling and/or cytoskeleton

stability. Interestingly, CARP shares 70% amino acid identity with the C-terminus of DCX. Mutations in the human DCX gene are associated with the doublecortex syndrome which is a result of malfunction of the DCX protein in rearrangement of the cytoskeleton during the development of the central nervous system (reviewed in (Feng and Walsh 2001). Some of the patients carry naturally occurring DCX mutations that lead to a truncated DCX protein with a deletion of the S/P-rich CARP-like C-terminus while the two microtubule binding domains are not affected. This C-terminus of DCX may promote protein-protein interactions or regulate the microtubule-interacting effects of DCX (Taylor et al., 2000; Friocourt et al., 2003). In line with the observed effect of CARP on DCL-induced microtubule polymerization *in vitro*, these types of DCX mutations may indicate an important role for the 'CARP-like' domain in the function of DCX, i.e. condensation and polymerization of microtubules.

CARP and DCLK-short in apoptosis

Previously, we reported that DCLK-short is a substrate for caspases *in vitro* and *in vivo* and that DCLK-short cleavage by caspases is necessary for apoptosis to proceed. The finding that calpain, a protease with a wider variety of substrates than caspases, is capable of breaking down DCLK proteins also suggests a role for the DCLK gene in apoptotic processes or the prevention thereof (Burgess and Reiner 2001). Moreover, a recent study in zebrafish demonstrates that gene knockdown of DCLK (zDCLK) induces a significant increase of apoptotic cells in the central nervous system (Shimomura et al., 2007). Interestingly, DCLK-short cleavage by caspases generates a N-terminal fragment that overlaps largely with CARP in its primary structure and has similar pro-apoptotic properties as CARP when studied during serum-deprived apoptosis (Kruidering, Schouten et al. 2001). This suggests that CARP and the N-terminus of DCLK-short share a common motif that is responsible for the observed pro-apoptotic properties. Apparently, cleavage of this N-terminal motif from DCLK-short is a crucial step in revealing its pro-apoptotic characteristics. CARP mRNA is specifically up-regulated by kainate-induced seizures in the hippocampus, in striatal neurons by D1-receptor agonists and in apoptotic DG cells after ADX. These processes are not accompanied by up-

regulation of DCLK-short, raising the possibility that the extreme induction of CARP (also called ania-4, Berke et al., 1998)) is a consequence of a shift in mRNA splicing. Interestingly, alternative splicing of ania-6 in the striatum is under control of distinct signalling cascades (Berke, Paletzki et al. 1998). A similar mechanism may be responsible for our findings regarding the DCLK gene.

In conclusion, we here demonstrate that CARP mRNA is up-regulated in the supra pyramidal blade of the DG in rats after ADX and that this induction of CARP is specific for apoptotic granule cells. Furthermore, CARP over-expression in serum deprived NG108 cells exacerbated apoptosis. CARP is also able to simulate DCL-induced polymerization of tubulin *in vitro*. CARP may play a role in these *in vivo* and *in vitro* models, perhaps by affecting neurotrophin signalling and/or cytoskeleton stability (Figure 6). Thus, our data for the first time demonstrate a pro-apoptotic role for this non-DCX domain-containing splice product of the DCLK gene. As such, and given its highly specific neuronal expression, CARP may be an important modulator of processes underlying the molecular basis of apoptosis in neuronal populations.

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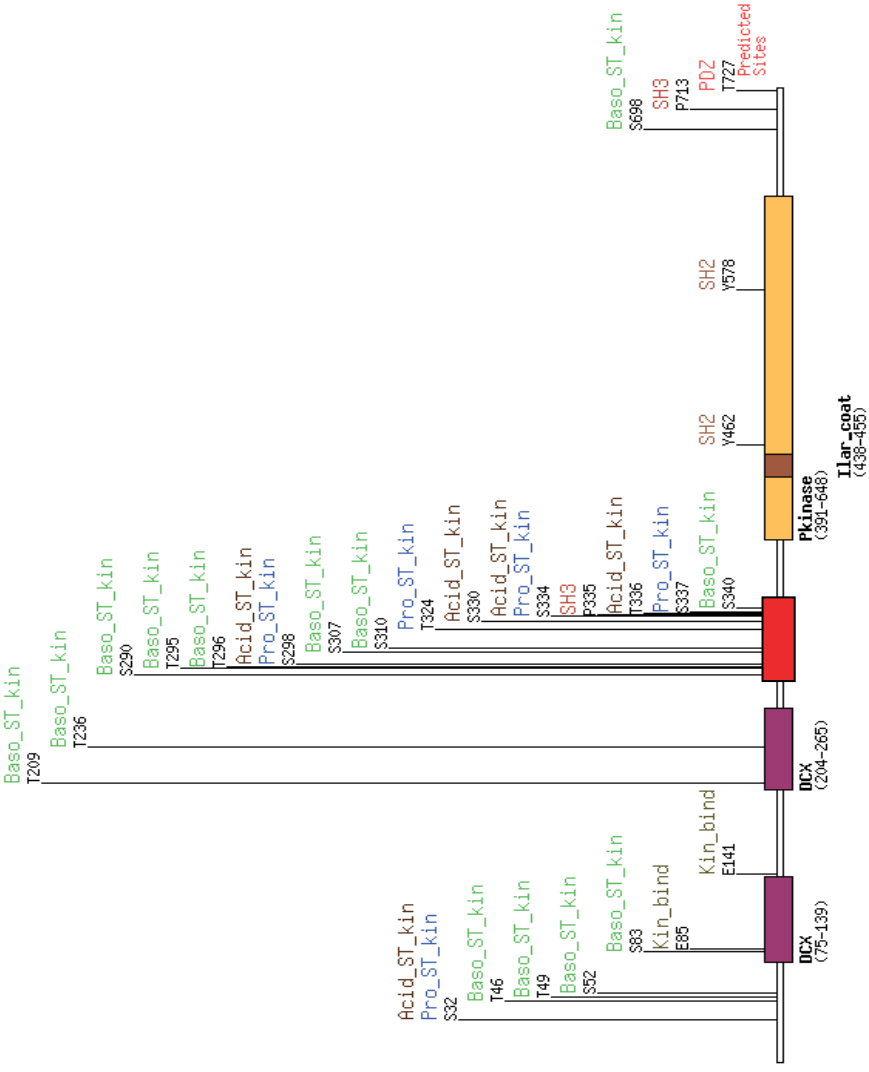
Supplementary Material

rat	[cort.] μg/dl	rat	[cort.] μg/dl
1	0.0946383	14	0.656573
2	0.6425383	15	0.467452
3	0.540385	16	0.169645
4	0.044715	17	0.081877
5	0.0537533	18	0.294958
6	0.2518267	19	**10.17755**
7	0.2400767	20	**3.962373**
8	0.282525	21	0.732467
9	0.1361233	22	0.91378
10	**1.2043333**	23	0.60218
11	**1.41662**	24	0.704987
12	**1.39972**	25	0.235597
13	**1.13592**	26	0.336365

Suplementary table 1. Corticosterone levels of the 26 adrenalectomized rats. 6 animals were excluded from the experiment, as their corticosterone levels were above 1,00 μg/dl (bold text).



Supplementary Figure S1. Predicted interaction sites within the full-length DCLK gene. The two DCX domains are indicated (purple), as well as the C-terminal kinase domain (yellow). Note the extremely high number of potential interaction sites located within the S/P-rich CARP domain (red).

Chapter 3

Over-expression of the DCLK gene transcript CARP decreases CA3/CA1 network excitability

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Abstract

Products of the Doublecortin-Like Kinase (DCLK) gene are implicated in cortical migration and hippocampal maturation during embryogenesis. However, one of its splice variants, called CaMK Related Peptide (CARP), is expressed during adulthood in response to neurological stimuli, such as kainic acid-induced seizures and BDNF-LTP. The function of this transcript of the DCLK gene is poorly understood. To elucidate its function during adulthood we have created transgenic mice with over-expression of CARP in the brain. To study potential functions of CARP in the hippocampus we performed an electrophysiological characterization of the CA3/CA1 network of transgenic and wild-type mice and show that field excitatory post synaptic potentials (fEPSPs) are highly increased in transgenic mice, while population spike amplitudes (PSAs) remained equal between genotypes. Consequently, hippocampal CA3/CA1 network excitability was decreased in transgenic mice. In addition we show a 2-fold up-regulation of the Ca^{2+}-binding protein calretinin and a down-regulation of Rapgef4, a guanine exchange factor for Rap1, in the hippocampus. Given previously established conditions during which CARP is induced and our current data, we propose that this DCLK gene product affects glutamtergic neuronal transmission in response to neurological stimuli.

Introduction

The Doublecortin-Like Kinase (DCLK) gene is expressed during neuronal development and has high homology to doublecortin (DCX); it encodes two conserved microtubule-binding DCX domains as well as a catalytic domain, and is subject of massive alternative splicing. Major splice variants include the full length transcripts (DCLK-long), the DCX domains containing transcript doublecortin-like (DCL) and the kinase-only variants (DCLK-short; (Vreugdenhil, Engels et al. 2001; Burgess and Reiner 2002). Interestingly, several studies using knockout mice and RNAi-mediated knockdown of target genes, suggest 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; Vreugdenhil et al., 2007).

Since the DCLK gene also encodes transcripts that are expressed in the adult brain, but not during embryogenesis, 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). Alternative splicing of the DCLK gene also produces a transcript encoding a 55-amino-acid peptide, called Ca^{2+}/Calmodulin dependent protein kinase (CaMK)-related peptide (CARP) (Vreugdenhil et al., 1999); also called Ania-4 (Berke et al., 1998). CARP expression is typically associated with the hippocampus and is below detection limits under basal conditions. In contrast, CARP mRNA is highly up-regulated by kainate-induced seizures in the hippocampus while DCLK-short is not (Vreugdenhil et al., 1999). Additionally, CARP is up-regulated in dentate gyrus (DG) granule neurons that are destined to die through apoptosis (Schenk et al., 2007). Interestingly, CARP is also highly expressed following brain derived neurotrophic factor (BDNF)-induced long-term potentiation (LTP; Wibrand et al., 2006). Despite these previously described associations of CARP induction, the function of CARP during adulthood is poorly understood. To better study the function of this DCLK gene product *in vivo*, we have generated a transgenic mouse line, called high-CARP, which has brain specific over-expression of the CARP transcript. These mice have a behavioural phenotype that is characterized by increased freezing in the contextual fear conditioning paradigm (Chapter 4). Since CARP has previously

been related to seizures and LTP (Vreugdenhil et al., 1999; Wibrand et al., 2006), processes closely related to glutamatergic neurotransmission, we aimed to characterize these mice at the electrophysiological level by recording field potentials from the CA3/CA1 network. We observed consistently increased field excitatory postsynaptic potentials (fEPSPs) in high-CARP mice, albeit without an increase in population spike amplitudes (PSAs). Subsequently, we performed an initial examination of hippocampal gene expression in high-CARP mice. We validate that expression levels of the Ca^{2+}-binding protein calretinin (calbindin 2; Calb2) are up-regulated, while RapGef4, a guanine exchange factor for the GTPase Rap1, is down-regulated. Taken together, our observations suggest that CARP expression indeed strongly affects glutamatergic neurotransmission in the CA3/CA1 network.

Materials and methods

Generation of transgenic high-CARP mice

A cDNA construct containing the DCLK gene kozac sequence and the sequence encoding the rat CARP transcript was generated. A pTSC expression construct was used. This vector contained an 8.1 kb EcoR1 fragment comprising the mouse Thy-1.2 gene. A 1.5 kb Ban1/Xho1 fragment (located in exon 2 and exon 4, respectively) was replaced by the CARP cDNA (Vidal et al., 1990; Moechars et al., 1996). The Thy-1.2 promotor specifically drives expression in neurons that starts at postnatal day 6, leaving embryonic development unaffected (Vidal et al., 1990; Hirrlinger et al., 2005). The CARP expression construct was then microinjected into a C57BL/6j background and backcrossed to C57BL/6j for at least 10 generations to produce transgenic offspring. Several criteria were used to select a suitable transgenic line, including fertility of the offspring, size of litters, relation of transgene expression to gender and the expression levels and distribution of CARP mRNA in the brain. The presence of the transgenic CARP transcript was initially confirmed by PCR analysis of DNA isolated from tail biopsies. The sense (5'-GTC CAA ATC ATC CGA CGA GAG A-3') and the anti-sense (5'-GCA GTC AGC TCT CCA CTC CG-3') primers were used to amplify a 150-bp fragment of CARP DNA. The number of integrated copies was determined by Southern blot analysis. All

transgenic (TG) mice used in the present study were heterozygous. Non-transgenic littermates were used as wild-type (WT) controls for all experiments. The transgenic line (designated high-CARP) is fertile with normal frequency and size of litters and stably transmits the transgene to both male and female offspring. All animal treatments were approved by the Leiden University Animal Care and Use Committee (Udec# 01022).

Animals

For electrophysiological experiments heterozygous animals were used. WT (n=11) and heterozygous TG high-CARP (n=11) male animals of 6 months old were used. Mice were individually housed one week prior to the experiment. Animals had access to food and water *ad libitum* and were kept under standardized housing conditions with a 12h/12h dark/light cycle (lights on 8am). For validation of a selected set of genes found to be differentially expressed in high-CARP mice by the initial large scale screen, a combined *in situ* hybridization and qPCR approach was used. For *in situ* hybridization experiments mice that were housed under similar conditions were used (WT n=5, TG high-CARP n=5). Additionally, using a qPCR approach we investigated the hippocampal expression of the CARP transcript in WT and high-CARP mice of different ages. Mice of 10 days old (D10), 2, 6 and 12 months old (M2, M6 and M12 respectively) were examined for transgenic CARP mRNA expression. These mice (n=5 for each group) were similarly housed one week prior to the experiment, with exception of the D10 groups, which were group housed.

Electrophysiology

Animals were decapitated and brains were dissected and chilled in ice-cold (4 °C) artificial cerebrospinal fluid (ACSF, containing (in mM) NaCl (120), KCl (3.5), NaH_2PO_4 (1.25) $NaHCO_3$ (25), $CaCl_2 \cdot 2H_2O$ (2.5), $MgSO_4 \cdot 7H_2O$ (1.3), glucose (10)) gassed with 95% O_2 and 5% CO_2. Hippocampi were carefully dissected out and transverse hippocampal slices (300 μm) were prepared using a tissue chopper (Krugers et al., 1997). After keeping slices at room temperature (21 ºC) for at least

1 h, one slice at a time was transferred to the recording chamber and submerged in ACSF (32ºC; perfusion rate was 2-3 ml/min) and gassed (95% O_2 and 5% CO_2). Field potential recordings were performed in at least four hippocampal slices per animal and one animal from each group was measured daily, to a total number of 11 animals per group. The CA1 network of the hippocampus was activated using bipolar stainless steel extracellular stimulation electrodes (tip diameter 60 µm) at the Schaffer collaterals. Synaptic input to the CA1 area was monitored with ACSF-filled glass electrodes placed in the stratum pyramidale and stratum radiatum. Field excitatory postsynaptic potentials (fEPSPs) were observed in the stratum radiatum as downward deflections of the recorded potential (Figure 3B). With the stronger stimulation intensities the evoked fEPSP can elicitate in the stratum pyramidale a population spike (PS) which is observed as a downward deflection of the recorded potential (Figure 3A). The PS is superimposed on the fEPSP, which is in the stratum pyramidale observed as an upward deflection of the recorded potential. The field potentials amplitudes (PSAs) and fEPSP amplitudes were analyzed using in-house developed software (Figure 3A and B).

Electrophysiology stimulation protocols

Four different stimulation protocols were used during the electrophysiological recordings. The four protocols (called 20-Input/Output (IO), 80-IO, IO-20 and IO-80) consisted of 10 paired pulses (PP) with varying intensity ranges. Stimulation intensities ranged from threshold (set to 0%; absolute stimulation intensities 22.1±1.3 µA and 18.4±0.8 µA; WT and high-CARP, respectively) to maximal (set to 100%; absolute stimulation intensities 235.1±10.4 µA and 218.6±9.8 µA; WT and high-CARP, respectively). The 20-IO and 80-IO protocols consisted of a fixed conditioning pulse (20% or 80% of the maximal stimulation intensity, respectively), followed 20 ms later by a second pulse which was increased with each of the 10 steps from threshold to maximal (Figure 3C). In protocols IO-20 and IO-80 the conditioning pre-pulse was increased each time (from threshold to maximal), while the second pulse (applied 20 ms later) remained constant at 20% and 80% of the maximal stimulation intensity, respectively. A period of 10 (s) separated each of the 4 different protocols as well as the PPs. For all protocols, the biphasic stimulus

pulse duration was 250 µs, while the time between the pre-pulse and the second pulse was 20 (ms) to monitor (γ-amino-butyric acid) $GABA_A$ mediated inhibition of the network (Figure 3C).

Microarray experimental design

To initially characterize the molecular alterations in the hippocampus of TG mice, a large scale screen for differential gene expression was performed. The hippocampi from both hemispheres of WT and TG mice were dissected at 4 °C and transferred to ice-cold Trizol (Invitrogen). Hippocampi were homogenized for each biological sample seperately using a tissue homogenizer (Salm&Kipp, Breukelen, The Netherlands) and total RNA was isolated according to the manufacturer's protocol. After precipitation, RNA was purified with Qiagen's RNeasy kit with on column DNase digestion. The quality of the RNA was assessed with the RNA 6000 Labchip kit in combination with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), using the Eukaryote Total RNA Nano assay according to the manufacturer's instructions. Total RNA was amplified using Ambion's MessageAmp kit, with incorporation of DIG-UTP. Labeled cRNAs of 2 individual WT and 2 TG high-CARP mice of 6 months old were hybridized on the Applied Biosystems (ABI) expression array system. Each individual RNA sample was hybridized to one microarray according to the manufacturer's instructions (ABI chemiluminiscent detection and RT-IVT labeling kit V.2.0). Microarray data analyses were performed with the software packages Abarray, BRB Array Tools, developed by the Biometric Research Branch of the US National Cancer Institute (http://linus.nci.nih.gov/BRBArrayTools.html) and with Spotfire Decisionsite (Spotfire) as described previously (Dijkmans et al., 2008). A probability level of 5% was used as the minimal criterion of significance for differential gene expression ($p<0.05$).

Quantitative PCR

Validation of differential hippocampal gene expression in WT and TG high-CARP mice was investigated by qPCR using a LightCycler 2.0 (Roche). Tissue samples

were homogenized in Trizol (Invitrogen Life Technologies) and RNA was isolated and dissolved in RNAse-free, diethylpyrocarbonate (DEPC)-treated water. Samples were subjected to DNAse treatment and cDNA was synthesized as described (Morsink et al., 2006). RNA quality and concentrations were assessed using the LabChip RNA 6000 Nano Assay on the 2100 Bioanalyzer (Agilent Technologies) and Nanodrop technology (Agilent Technologies) according to manufacturer's instructions. Preparations of PCR reactions were performed as described (Dijkmans et al., 2008). Briefly, the LightCycler FastStart DNA Master PLUS SYBR Green I (Roche) reagentia, were used according to manufacturer's instructions. Final primer concentration was 5 µM per primer, with thermal cycling settings: annealing at 60 degrees for 10 seconds, amplification at 72 degrees for 10 seconds and dissociation at 95 degrees for 10 seconds. The PCR program was followed by a melting curve with a temperature rise from 65 degrees to 95 degrees, with continuous SYBR Green emission monitoring. Specificity of amplification of primer pairs was controlled by BLAST search of primer pair sequences versus the mouse genome, determination of actual PCR product size by gel electrophoresis and comparison with predicted product size. To calculate relative mRNA concentrations of differentially expressed genes, a standard curve was prepared for each gene. Normalization of the measurements was achieved by dividing the relative mRNA concentrations of each experimental sample by the average of the relative mRNA concentrations of two housekeeping genes, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Cyclophillin. The following primer pairs were used for the detection of selected genes. For each primer pair the sequences are indicated as anti-sense / sense:

1) 5'-GCA GTC AGC TCT CCA CTC CG-3' / 5'-GTC CAA ATC ATC CGA CGA GAG A-3'
2) 5'-AGG CAA GAG TCT AGA CAT CTC-3' / 5'-ACA GGC CCT ATG ATG AAC C-3'
3) 5'-AGG ATC CAT CAA GCT CTC AAA C-3' / 5'-GCA CTA ACG TGG GAG AAA CTG-3'
4) 5'-CCA GTG AGC TTC CCG TTC A-3' / 5'-GAA CAT CAT CCC TGC ATC CA-3'
5) 5'-TTA TGG CGT GTA AAG TCA CCA CCC-3' / 5'-GCA GAC AAA GTT CCA AAG ACA GCA-3'

1) CARP, 2) CR, 3) Rap1gef2, 4) GAPDH, 5) Cyclophillin.

In situ hybridization

Brain tissue samples were collected between 9am and 12am to ensure low circadian corticosterone levels. Brains were quickly taken from the skull and one hemisphere was snap-frozen in isopentane *au bain marie* on ethanol absolute and dry ice for *in situ* hybridization experiments. Brains were stored at -80 °C until use. The hippocampus from the other hemisphere was dissected quickly at 4 °C and transferred directly to a tube containing ice cold Trizol (Invitrogen Life Technologies) for qPCR experiments (see below). Coronal sections (20 μm) were cut using a cryostat (CM1900 Leica Microsystems, Wetzlar, Germany) and thaw-mounted on poly-L-lysine coated slides. Slides were stored at -80 °C until use. CARP and Calretinin (CR) were detected using 40-45mers. Mismatch oligonucleotides with 5-6 substitutions were used as control. The oligonucleotide sequences are:

1) 5'-GCC GCC ACT GTG CTG GAT ATC TGC AGA ATT CTT ACA CTG A-3'
2) 5'-GCC GCG ACT GTC CTG GAA ATC TGG AGA ATA CTT ACT CTG A-3'
3) 5'-AGG CAA GAG TCT AGA CAT CTC TGA GAG ACC CAA TTT GCC ATC TCC-3'
4) 5'-AGG CAA CAG TCT ACA CAT CTG TGA GAG TCC CAA TTA GCC ATC TCC-3'

1) is the perfect match recognizing CARP with 2) as its mismatch control, 3) is the perfect match recognizing CR and 4) its mismatch control (substitutions are underlined). *In situ* hybridisation was performed as described (Meijer, Steenbergen et al. 2000). Subsequently, slides were exposed to an X-OMAT AR film (Kodak) for approximately 14 days. Films were scanned (at 1200 dpi) using Umax MagicScan and relative optical densities (RODs) of hippocampal subfields CA1, CA3 and dentate gyrus (DG) and background (the area between the cell layers of CA1 and DG) were measured using Scion Image. The background was subtracted from the RODs of the corresponding areas.

Analysis and Statistics

For the electrophysiology experiments an unpaired t-test was used to determine significant differences between experimental groups. For the qPCR experiments

significant differences were determined with the Mann-Whitney test. *In situ* hybridization relative optical densities (RODs) of hippocampal subfields CA1, CA3 and DG and background were measured using Scion Image. To determine the background threshold, the background signal was measured in 10 different slides by selecting a background area in the tissue section (between the CA1 and DG) to calculate the threshold factor. The measuring threshold was then set to minimize interference of the background signal. One-way ANOVA was used to determine significant differences in RODs between WT and TG hippocampal mRNA expression. For all tests a probability level of 5% was used as the minimal criterion of significance.

Results

Characterization of CARP expression in transgenic mice

A transgenic line with over-expression of the DCLK gene transcript CARP was generated by injection of a Thy-1.2 promoter driven expression construct into fertilized eggs and subsequent *in utero* implantation. This transgenic line was designated high-CARP. Mapping of CARP expression was performed in heterozygous male mice from this line and their wild-type (WT) littermates served as a control. A schematic representation of the CARP expression vector is shown (Figure 1A). High-CARP brains were characterized by robust expression of the CARP transcript throughout the different layers of the hippocampus (Figure 1E-G). Expression of CARP was not limited to the hippocampal formation as its expression was also found in other limbic areas, such as amygdaloid and thalamic nuclei (for example see Figure 1F), and also in several layers of the cerebral cortex (Figure 1C-G). Typically, no expression of CARP was observed in the striatum (Figure 1C-D). Also see Table 1 for a more detailed overview of area's that have or lack expression of CARP mRNA. In WT control subjects, CARP expression levels did not exceed the background signal.

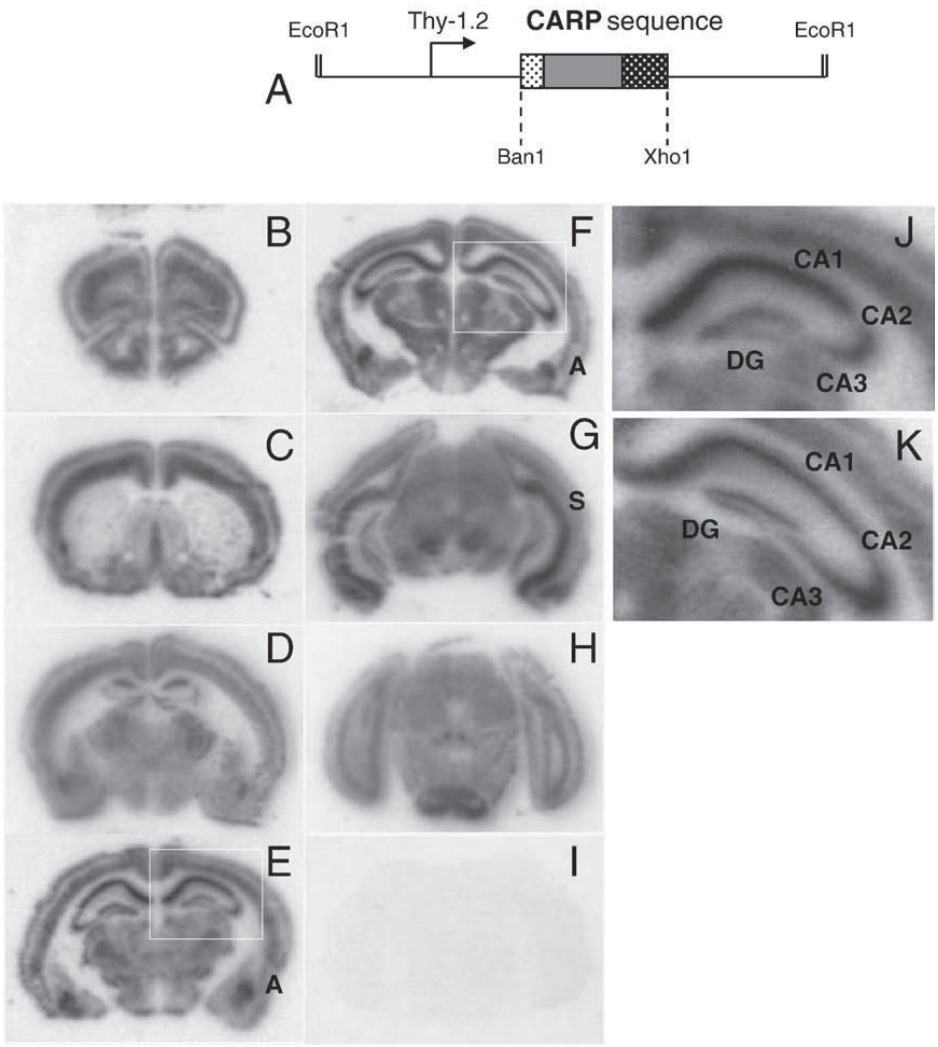

Figure 1. CARP expression in transgenic high-CARP mice. The CARP pTSC expression construct is indicated in (A). The following domains are indicated from left to right: domain that is unique to CARP and DCLK-short (white dotted box), serine/proline-rich domain (gray box) and the domain that is unique to CARP and DCL (black dotted box). This vector contained an 8.1 kb EcoR1 fragment comprising the mouse Thy-1.2 gene. A 1.5 kb Ban1/Xho1 fragment (located in exon 2 and exon 4, respectively) was replaced by the CARP cDNA (Vidal et al., 1990; Moechars et al., 1996). A coronal overview from rostral (B) to caudal (H) of CARP expression is shown. Note the high expression in several layers of the cortex (C-G), hippocampus (D-G), amygdaloid and thalamic nuclei (E-F), which is typical for the Thy-1.2 promotor (Vidal et al., 1990; Hirrlinger et al., 2005). (I) shows the autoradiogram of a section treated with the mismatch control. The white rectangles in E and F show enlarged pictures of the hippocampus in J and K, respectively. See Table 1 for an overview of CARP mRNA expression in high-CARP brain. CA= Cornu Ammonis; DG= Dentate Gyrus; A= central Amygdala; S= Subiculum.

Brain area	CARP Expression
Anterior commissure	-
Orbital cortex	-
Anterior olfactory nucleus	+
Periform cortex	+
Motor cortex	+
Dorsal endoperiform nucleus	+
Corpus callosum	-
Medial septal nucleus	+
Caudate putamen	-
Amygdaloid nucleus	+
Basolateral amygdaloid nucleus, anterior	+
Basolateral amygdaloid nucleus, posterior	+
Basomedial amygdaloid nucleus, posterior	+
Hippocampus	+
Dentate gyrus	+
Cornu ammonis 1	+
Cornu ammonis 2	+
Cornu ammonis 3	+
Cerebral cortex	+
Ventrolateral thalamic nucleus	+
Ventromedial thalamic nucleus	+
Anteromedial thalamic nucleus	+
Ventral posterolateral thalamic nucleus	+
Ventral posteromedial thalamic nucleus	+
Posterior thalamic nuclear group	+
Subparafascicular thalamic nucleus	+
Parafascicular thalamic nucleus	+
Lateral hypothalamic area	+
Lateral globus pallidus	+
External capsule	-
Internal capsule	-
Fimbria hippocampus	-
Stria terminalis	-
Medial lemniscus	-
External medullary lamina	-
Fasciculus retroflexus	-
Zona incerta	+
Ventrolateral geniculate nucleus	+
Dorsal lateral geniculate nucleus	+
Red nucleus, parvocellular	+
Red nucleus, magnocellular	+
Subiculum	+
Amydalopiriform transition area	+
Substantia nigra	+
Dorsal hippocampal commissure	-
Ventrolateral tegmental area	+
Reticulotegmental nucleus pons	+
Medial longitudinal fasciculus	+

Table 1. Overview of CARP mRNA expression in high-CARP mouse brain. Expression of CARP mRNA (+) or lack thereof (-) is indicated for several brain structures. Localization is based on the mouse brain atlas by Franklin and Paxinos (Franklin and Paxinos, 1997). Semi-quantification is based on the in situ hybridization images shown in Figure 1.

In addition, using a qPCR approach we investigated the hippocampal expression of the CARP transcript in high-CARP and littermate control mice of various ages. Mice of 10 days old (D10), 2, 6 and 12 months old (M2, M6 and M12 respectively) were examined for CARP mRNA expression. In littermate controls, CARP expression levels were below the detection threshold. In contrast, expression of CARP in the TG groups was found from D10 onwards and reached maximal expression levels in the hippocampus of 6 months old animals (Figure 2).

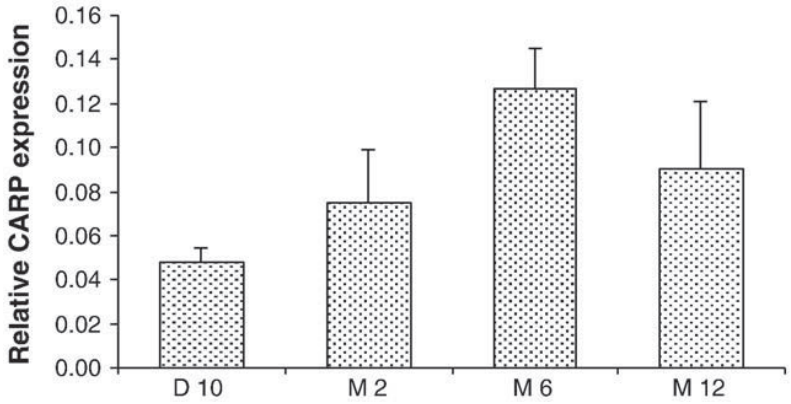

Figure 2. Relative CARP expression in high-CARP mice of different ages. Expression of CARP was measured in the hippocampus by qPCR. CARP is detected from day 10 (D10) onwards and reaches maximal expression levels after 6 months (M6). CARP expression was normalized to expression levels of GAPDH and Cyclophillin. Expression of CARP in WT littermates was below detection levels.

Glutamatergic transmission

High levels of CARP mRNA are found in the hippocampus after kainic acid administration (Vreugdenhil et al., 1999). Kainic acid treatment is a well-known model for the induction of epileptic seizures (Hellier et al., 1998). In addition, CARP mRNA is robustly up-regulated following BDNF-induced LTP (Wibrand et al., 2006). Both processes are associated with increased glutamate mediated neuronal excitability. Since the high-CARP transgenic line has over-expression of the CARP transcript in the hippocampus, neuronal transmission may be affected in these mice. To elucidate this, we subjected hippocampal slices of 6 months old high-

CARP and WT animals to 4 different stimulation protocols. We stimulated hippocampal slices at the Shaffer collaterals, and recorded field potential responses at the strata radiatum and pyramidale. fEPSP and PS amplitudes were measured in these areas, respectively (Figure 3). For each of the 4 stimulation protocols (Figure 3C) responses were compared between high-CARP and their WT littermates.

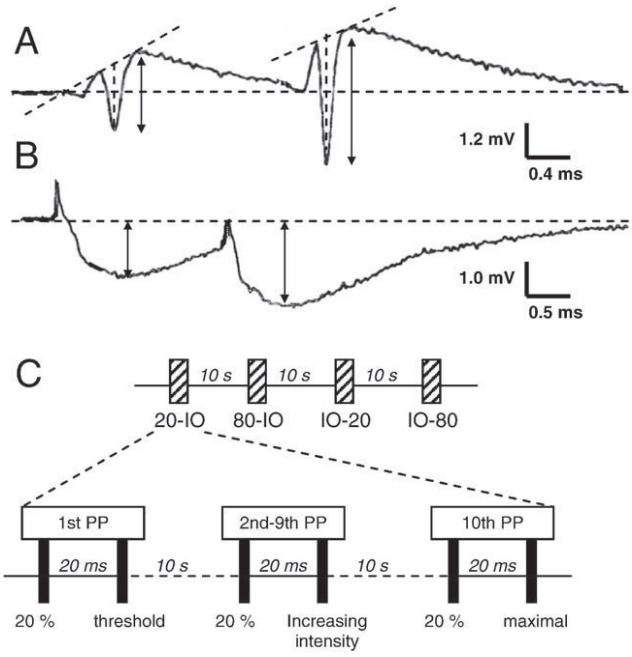

Figure 3. Overview of electrophysiology data analysis and stimulation protocols. The CA1 network of the hippocampus is activated using bipolar extracellular stimulation electrodes at the Schaffer collaterals. Evoked fEPSPs were observed by measuring in the stratum radiatum. Synaptic input to CA1 neurons is monitored by recording population spikes (PS) in the stratum pyramidale. Subsequently, PS amplitudes (indicated by vertical double-headed arrows in A) and fEPSP amplitudes (indicated by vertical double-headed arrows in B) were analyzed. Stimulation artifacts are removed for illustrative purposes. Four different paired pulse (PP) stimulation protocols are used to investigate CA3/CA1 neuronal transmission. For details on the stimulation protocols, see the Experimental Procedure section. A period of 10 (s) separated each of the 4 different protocols as well as the PPs. For all protocols, the biphasic stimulus pulse duration was 250 μs, while the time between the pre-pulse and the second pulse was 20 (ms). The time between the pre-pulse and the second pulse was 20 (ms) in order to detect potential (γ-amino-butyric acid) $GABA_A$ mediated inhibition of the network (C). Stimulation intensities ranged from threshold (absolute stimulation intensities 22.1±1.3 μA and 18.4±0.8 μA; WT and high-CARP, respectively) to maximal (235.1±10.4 μA and 218.6±9.8 μA; WT and high-CARP, respectively).

Figure 4 shows the basal, non-conditioned responses evoked by stimulation intensities ranging from threshold to maximal (dotted lines; black symbols). These basal responses were compared to conditioned pulses where a fixed 20% (Figure 4A and C) or 80% (Figure 4B and D) of maximal stimulation intensity pre-pulse preceded the stepwise increasing second pulse. The solid lines (white symbols) show the recorded outputs of the conditioned pulses at the indicated relative stimulation intensity shown on the x-axis. The stimulation intensities of the second of each of the 10 PPs ranged from threshold (absolute stimulation intensities 22.1±1.3 μA and 18.4±0.8 μA; WT and high-CARP, respectively) to maximal (235.1±10.4 μA and 218.6±9.8 μA; WT and high-CARP, respectively). Absolute stimulation intensities were not significantly different between genotypes. Comparing basal and conditioned pulses by applying a 20% of maximal pre-pulse resulted in a mild trend towards facilitation (Figure 4A; relative stimulation intensities 10% and 15%, respectively), whereas using an 80% pre-pulse induced a reduction in amplitude of the conditioned PSA, which was statistically significant for the highest stimulation intensities (Figure 4B; relative stimulation intensities 80% (WT $p<0.05$ and TG $p<0.05$) and 100% (TG $p<0.05$), respectively). However, no significant differences for the PSA were found between genotypes, regardless of the intensity of the applied pre-pulse (Figure 4A and B). With conditioning pre-pulses (solid lines; white symbols) the fEPSPs evoked with the second pulses were increased in amplitude, both for the wild-type (triangles) and high-CARP (squares) mice, indicative of a facilitating process (indicated by the gray rectangular inserts in Figure 4C and D; relative stimulation intensities 10-20% and 0-50%, respectively). In addition, significant differences were observed between genotypes for fEPSPs, where we observed that fEPSP amplitudes were highly increased in high-CARP mice when compared to WTs ($p<0.05$). Basal responses differed between genotypes at higher stimulation intensities (Figure 4C and D). Conditioned IO curves with the 20% pre-pulse also differed between genotypes at higher stimulation intensities (Figure 4C), whereas the conditioned fEPSPs with the 80% pre-pulse of the high-CARP mice were increased in amplitude over the full stimulation intensity range as compared with the wild type mice (Figure 4D).

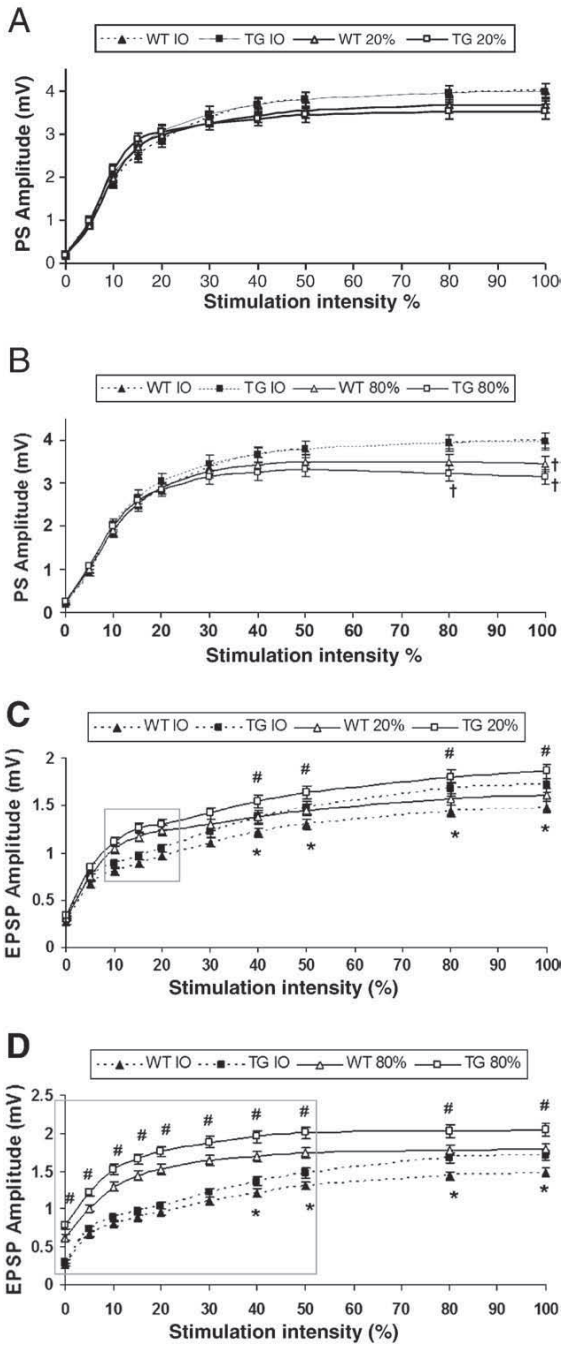

*Figure 4. Neuronal responses of high-CARP and WT hippocampal slices (4-6 slices per animal (n=11) were used) observed with stimulation paradigms 20_IO (A and C) and 80_IO (B and D). Basal responses are compared to conditioned pulses where a fixed 20% or 80% of maximal stimulation pre-pulse preceded the stepwise increasing second pulse. Field potential amplitudes are represented by the points connected with lines in the graph, with the corresponding relative stimulation intensities (of the second pulse) shown on the x-axis. The dotted curves represent the non-conditioned responses, while the solid curves show the conditioned responses. The PSA is shown in A and B. Note that conditioned responses (solid lines; white symbols) where a fixed 80% of maximal stimulation pre-pulse precedes the stepwise increasing second pulse are significantly different from the basal responses (dotted lines; black symbols) at the highest stimulation intensities (B; † $p<0.05$). However, no significant differences between genotypes are found for PSAs. The fEPSP amplitude is shown in C and D. With conditioning pre-pulses (solid lines; white symbols) the fEPSPs evoked with the second pulses were significantly increased in amplitude, both for the wild-type (triangles) and high-CARP (squares) mice, indicative of a facilitating process (points within the gray rectangular inserts in C and D; $p<0.05$). In addition, evoked fEPSPs measured in slices of high-CARP animals are significantly larger in amplitude as compared to WTs (C and D; non-conditioned responses (dotted lines; black symbols), * $p<0.05$; conditioned responses (solid lines; open symbols) # $p<0.05$).*

By subjecting hippocampal slices to protocols that consisted of an increasing first pulse (again ranging from threshold to maximal) followed by a fixed second pulse at 20% or 80% of maximal stimulation, we aimed to gain better insight into potential differences in the extent of paired pulse facilitation and inhibition between genotypes. Occurrence of both processes was observed, although no significant differences were found between genotypes. For the second PS evoked with 20% stimulation intensity facilitation was observed with the lower intensity conditioning pulses (Figure 5A, dotted lines; relative stimulation intensities 0-30%). With increasing stimulation intensities of the conditioning pulses a small decrease in PSA was observed, indicative of an inhibition process (Figure 5A, dotted lines; relative stimulation intensities 30-100%). For the second PS evoked with an 80% stimulation intensity (which evokes a PS of almost maximal amplitude) only a slight reduction in amplitude was observed (Figure 5A, solid lines). No differences for PSAs were observed between TG and WT animals using these two stimulation protocols. The fEPSPs evoked with these protocols only showed a slight increase in amplitude with increasing conditioning pre-pulse intensities. However, while the PSAs were not significantly different between the high-CARP and WT groups, again the fEPSP amplitudes recorded in the slices of high-CARP (squares) animals were largely increased compared to those recorded in slices from WT (triangles) animals (Figure 5B, $p<0.05$). For the IO-20 protocol these differences were observed with stimulation intensities (of the conditioning pulses) ≥30%, whereas for the IO-80 protocol the amplitude of the conditioned fEPSP of CARP mice was increased over the full stimulation intensity range as compared with the fEPSPs of wild type mice.

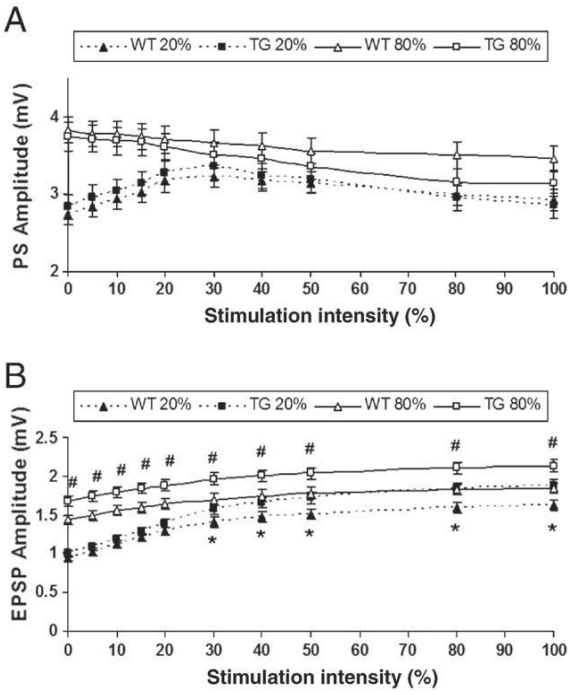

*Figure 5. Neuronal responses of high-CARP and WT hippocampal slices (4-6 slices per animal (n=11) were used) observed with stimulation paradigms IO_20 and IO_80. These protocols consist of an increasing first conditioning pulse (from threshold to maximal) followed by a fixed second pulse at 20% or 80% maximal stimulation. (A) With increasing stimulation intensities of the conditioning pulses a small increase of the PSA evoked with the 20% second pulse was observed (until ~30% stimulation intensity); with higher stimulation intensities the PSA was reduced (dotted lines). The PSA evoked with the 80% stimulation intensity pulse was slightly reduced over the whole stimulation intensity range of the conditioning pulse (solid lines). No significant differences were found between genotypes. (B) fEPSP amplitudes evoked with the 20% and 80% stimulation intensities (dotted and solid lines, respectively) were slightly increased over the whole stimulation intensity range of the conditioning pulse. Significantly larger fEPSP amplitudes for high-CARP animals were measured as compared with WT animals. Evoked fEPSPs that are significantly different from WTs are indicated; 20% fixed second pulse (dotted curves; black symbols) * $p<0.05$; 80% fixed second pulse (solid curves; open symbols), # $p<0.05$.*

Although with all protocols used an increased fEPSP amplitude was recorded in slices of high-CARP mice (as compared to WT), evoked PSAs remained equal for the two genotypes. To better visualize this effect, the non-conditioned PSAs were plotted as function of the non-conditioned fEPSP amplitudes. The resulting excitability plot of the high-CARP mice displayed a rightward shift as compared to

the plot of the WT animals (Figure 6). This shows that the larger fEPSP evoked in slices of CARP mice does not result in the elicitation of a larger population spike.

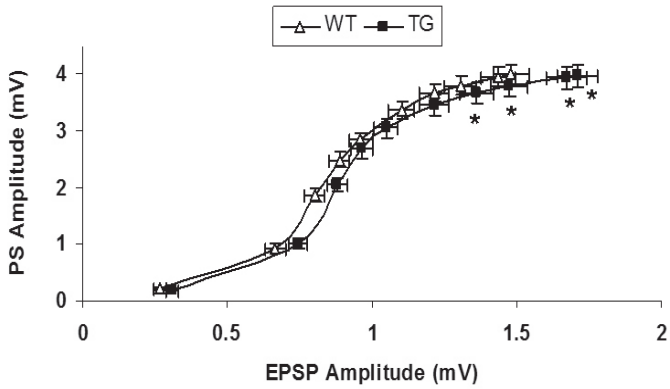

*Figure 6. Excitability plots of basal responses. Excitability plot shows a rightward shift of the curve for high-CARP mice, suggesting decreased excitability of the hippocampal network. Observations are taken from 4-6 slices per animal (n=11). * p<0.05, significantly different from wild-type control.*

Differentially expressed genes in the hippocampus of high-CARP mice

To gain an initial insight into the most prominently regulated genes in the hippocampus of high-CARP mice, a large-scale screen for overall differences of hippocampal gene expression of 6 months old high-CARP and control mice was performed in a n=2 experiment (Table 2). To validate potential differentially expressed genes, we then analyzed independent biological samples (n=5) using qPCR. In TG mice we found alterations in CA3/CA1 network functioning that consisted mostly of an increase in evoked fEPSPs. Since fEPSPs represent a glutamate-dependent response, we concluded that high-CARP mice show an overall tendency towards decreased excitability of the CA3/CA1 network (see previous section). We therefore searched for genes related to glutamatergic transmission, such as glutamate metabolism or glutamate receptor subunits. No genes related to these groups were detected by our initial large scale screen. However, we identified two genes involved in Ca^{2+} metabolism and neurotrophic factor signaling that are important for hippocampal function: Calretinin (Calbindin 2;

Calb2) and RapGef4 (also known as Epac2, Woolfrey et al., 2009), a guanine nucleotide exchange factor for the GTPase Rap1. These genes were selected for validation with qPCR and were reproducibly differentially expressed in TG mice. High-CARP mice showed a significant 1.94 fold up-regulation of Calb2 in the hippocampus ($p<0.01$; Figure 7A). The RapGef4 gene was also significantly differentially expressed in the hippocampal formation of these mice, with a fold-change of 0.70 ($p<0.05$; Figure 7B).

Gene ID	Gene name	Fold Change
mCG7015.2	Toll-like receptor 3	4.76
mCG3971.1	Voltage-dependent L-type Ca^{2+} channel α1F subunit	4.29
mCG125130	B-Galactosidase	4.06
mCG141740	Olfactory receptor	3.42
mCG1865.1	chemokine (C-C motif) ligand 24	3.20
mCG8869.1	Sodium- and chloride-dependent glycine transporter SLC6A9-related	2.64
mCG16946.2	**Calretinin (Calbindin 2)**	**1.60***
mCG142372	Myosin,heavy polypeptide 7,cardiac muscle, beta	1.48
mCG9329.2	wingless-related MMTV integration site 4	1.43
mCG119325.1	Coronin, non-motor actin binding protein 2A	1.34
mCG16698.2	Claudin 4	0.74
mCG128278.1	**cAMP-Dependent Rap1 Guanine-nucleotide exchange factor 4**	**0.70***
mCG130054	pregnancy specific glycoprotein 17	0.70
mCG54361.1	Keratin associated protein	0.65
mCG21885.2	Organic cation transporter	0.63

Table 2. Differentially expressed genes in high-CARP hippocampus, determined by micro array (n=2). Ten genes were up-regulated, while 5 genes were down-regulated. Calretinin and RapGef4 are indicated in bold and were selected for validation using qPCR.

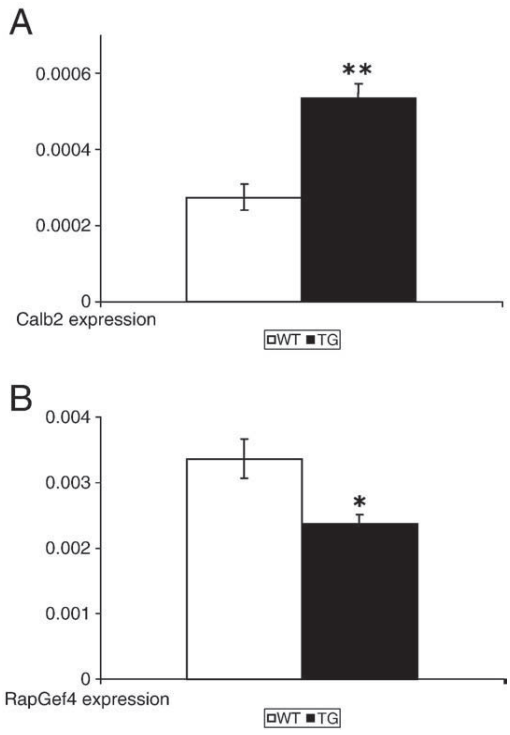

*Figure 7. qPCR quantification of differential gene expression in the hippocampus of WT mice (white bars) and high-CARP (black bars), age 6 months. Significantly different relative mRNA concentrations of Calb2 (A; ** p<0.01) and RapGef4 (B; * p<0.05) are shown. Fold changes for Calb2 and Rapgef4 expression are 1.94 and 0.70 respectively. Gene expression was normalized to expression levels of GAPDH and Cyclophillin.*

Differential expression in high-CARP mice: *in situ* hybridization

Since Calb2 was the most prominently differentially expressed gene validated with qPCR and plays an important role in Ca^{2+} metabolism, we decided to investigate the expression of Calb2 in more detail. To identify differential expression of Calb2 in hippocampal subfields we examined the contra lateral hemisphere of each brain used in the qPCR experiment by *in situ* hybridization. Calb2 expression levels were measured in the DG, CA1 and CA3 subfields of the hippocampus for each experimental group. In line with the results regarding the increase in Calb2 expression measured by qPCR in 6 months old high-CARP mice, we found an increase of Calb2 mRNA in the hippocampus in this group ($p<0.05$), but not in mice

of the other ages we investigated (Figure 8A). This increase was a consequence of Calb2 up-regulation in the DG exclusively, while no induction was observed in any of the CA subfields, when measured with *in situ* hybridization (Figure 8B). Representative autoradiograms of Calb2 mRNA hybridized WT and TG brain sections, are shown in Figure 8C.

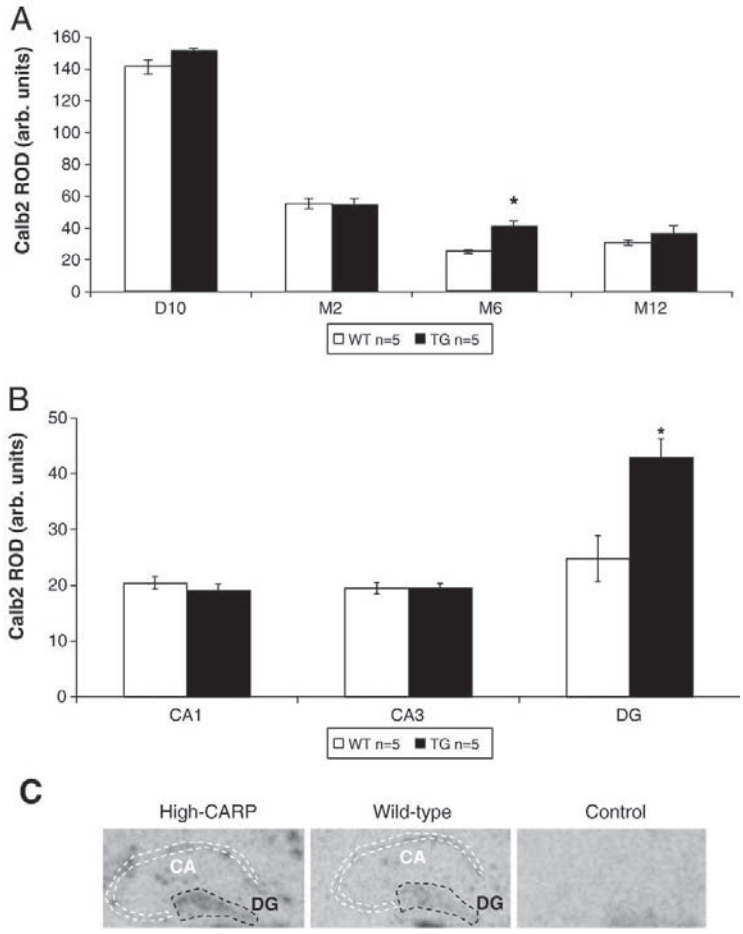

*Figure 8. Quantification of differential Calb2 gene expression in hippocampal sub regions by in situ hybridization. Calb2 is differentially expressed in the DG of M6 high-CARP mice, but not at D10 days, M2 or M12 (A). In M6 high-CARP mice, significant differential mRNA expression is exclusively a result of up-regulation in the DG (B). Representative autoradiograms of Calb2 with mismatch controls are shown in (C). * p<0.05.*

Discussion

We here report on the successful generation of a new transgenic mouse line with brain specific over-expression of the DCLK gene transcript CARP. We have characterized this transgenic strain, designated high-CARP, using an electrophysiological approach to examine the consequences of CARP over-expression on hippocampal network functioning. In addition, we have performed an initial characterization of gene expression in the hippocampus of these mice. Previously, the function of DCX and DCLK has been well studied during embryogenesis and DCX and DCLK are known to have overlapping, yet distinct functions during cortical and hippocampal development in mice (Koizumi et al, 2006; Deuel et al., 2006; Tanaka et al., 2006; Friocourt et al., 2007). The generation of this novel transgenic mouse line with brain specific over-expression of CARP may thus be of importance in unveiling the function of this non-DCX domain-containing member of the DCLK gene family during adulthood. We show that evoked fEPSPs are increased in high-CARP mice, while PSAs recorded in the stratum pyramidale are equal between CARP over-expressing mice and littermate controls. In addition, we demonstrate that Calb2 and RapGef4 are differentially expressed in the hippocampus of these mice.

CARP is highly expressed in transgenic mouse brain

By qPCR and *in situ* hybridization using a probe specific for CARP we examined the temporal and regional distribution of CARP mRNA in the brains of high-CARP mice. Investigating the expression of CARP in mice of different ages, we observed expression from postnatal day 10 onward that was still elevated in 12 months old animals, while expression levels reached a maximum at 6 months of age. CARP mRNA was localized in several brain regions, with the highest expression levels in the amygdaloid nuclei and hippocampal areas. These observations are well in line with previously described characteristics of the Thy-1.2 promotor (Vidal et al., 1990; Hirrlinger et al., 2005). Under physiological conditions CARP mRNA expression is low or even below detection levels in the adult rat brain (Vreugdenhil et al., 1999; Schenk et al., 2007). Here we demonstrate that CARP mRNA is also below detection levels under physiological circumstances in the hippocampus of

adult WT mice using qPCR and does not exceed the background signal when visualized by *in situ* hybridization.

Interestingly, specific induction of CARP mRNA has previously been associated with kainate-induced seizures in hippocampal neurons (Vreugdenhil et al., 1999), with administration of D1-agonists in striatal neurons (Berke, Paletzki et al. 1998; Glavan, Sket et al. 2002), with apoptotic cells in DG granule cells of adrenalectomized rats (Schenk et al., 2007) and with BDNF-induced LTP (Wibrand et al., 2006). High-CARP mice were found to express levels of CARP that parallel the high induction of CARP observed in the kainic acid model (Vreugdenhil et al., 1999). In these paradigms, CARP induction is probably a consequence of seizure activity and of apoptosis in the DG respectively, while our present observations in transgenic animals suggest that the over-expression of CARP by itself is not able to initiate these processes *in vivo* (Schenk et al., unpublished results). This observation is analogous to our previous *in vitro* findings in neuroblastoma cells, where CARP was able to exacerbate, but not initiate, the apoptotic process (Kruidering et al., 2001; Schenk et al., 2007).

Altered glutamatergic transmission

High levels of CARP are found in the hippocampus after kainic acid administration, a well-known model for the induction of epileptic seizures (Hellier et al., 1998; Vreugdenhil et al., 1999) and also following BDNF-LTP (Wibrand et al., 2006). Since animals from this line have high levels of CARP mRNA in the hippocampal formation we investigated CA3/CA1 network functioning in the high-CARP line. By measuring field potentials, we found that electrically evoked neuronal transmission was affected in transgenic mice. More specifically, fEPSPs recorded in the stratum radiatum of the CA1 area were much larger in amplitude in high-CARP mice, while these fEPSPs elicited a PS in the stratum pyramidale that was comparable in amplitude to those of WT mice for all test protocols used. Furthermore, with the application of paired-pulse protocols we observed that facilitation and inhibition processes in the CA3/CA1 network were similar in hippocampi of CARP and WT mice. The increased fEPSP amplitudes are likely a consequence of altered glutamatergic transmission. This apparent increase in glutamatergic transmission is possibly a consequence of alterations in vesicle release, pre- or post-synaptic

glutamate receptor expression levels and glutamate receptor affinity and subunit composition.

To visualize the efficiency of glutamate dependent synaptic transmission and excitability of the hippocampal CA3/CA1 network more clearly, excitability plots of the non-conditioned pulses were constructed. As a consequence of the increased evoked fEPSP amplitudes and the unaffected PSAs, the excitability plot was shifted to the right for TG animals (compared to WT) for all protocols used. Thus, fEPSP amplitudes were increased in the transgenic group, especially at higher stimulation intensities. Nevertheless, these increased fEPSP amplitudes did not evoke larger PSAs, indicating a reduced excitability of the network, suggesting that due to a (unknown) compensatory mechanism the increased glutamatergic neurotransmission of high-CARP animals does not translate into increased excitation of the CA1-CA3 network.

Importantly, using the described experimental approach hippocampal slices of both groups displayed equal levels of facilitation, suggesting that accumulation of Ca^{2+} and subsequent glutamate release are not responsible for the observed differences in electrophysiological phenotypes. In addition, activation of the network by first applying an 80% of maximal pre-pulse followed by a second pulse 20 ms later, yielded similar significant levels of inhibition in WT and high-CARP mice. The absence of significant differences between the two genotypes here suggests that feedback inhibition by activation of GABA-ergic interneurons synapsing on CA1 pyramidal neurons does not contribute to the transgenic phenotype of decreased excitability of the CA3/CA1 network. Follow-up studies, e.g. by measuring synaptic currents (EPSCs) in CA1 neurons may give further insight into processes that contribute to the observed alterations in synaptic transmission.

Interestingly, epileptic seizures are typically associated with elevated Ca^{2+} levels and highly increased glutamate mediated excitatory neuronal transmission (Murphy and Miller, 1988; Vreugdenhil and Wadman, 1994). Moreover, recorded evoked fEPSPs in the CA1 from kainic acid treated hippocampal slices are characterized by increased neuronal excitability. This increased neuronal excitability in kainic acid treated slices is explained partly because of increased fEPSP amplitudes and also by a decreased inhibition in the CA1 area that contributes to an increase in paired-pulse excitability (Franck and Schwartzkroin, 1985; Ashwood et al., 1986; Ashwood

and Wheal, 1986; Franck et al., 1988; Cornish and Wheal, 1989; Simpson et al., 1991; Turner and Wheal, 1991). This decrease in inhibition has been proposed to be caused by a selective loss of GABAergic, Ca^{2+}-binding proteins expressing interneurons and a decreased expression of $GABA_A$ receptors (Best et al., 1993; 1994; Tsunashima et al., 1997).

Here we found increased fEPSPs, which did not result in the elicitation of larger PSAs. This suggested decrease in neuronal excitability with equal levels of inhibition between genotypes indicates an effect that is opposite to the common effects caused by kainic acid treatment. In high-CARP mice the decreased excitability thus suggests that the high levels of CARP under basal physiological conditions might play a role in providing a negative feedback on glutamatergic excitatory synaptic transmission. Similarly, up-regulation of CARP during kainite-induced seizures may serve the same purpose. Thus functionally, the robust increase of CARP might enable the hippocampal network to cope with the aberrant glutamate release during seizures by returning hippocampal excitation to homeostatic levels. Strikingly, recent data show that knock-out rather than over-expression of two closely related members of the DCLK gene family, DCX and DCLK2, in mice results in frequent spontaneous seizures that originate in the hippocampus (Kerjan et al., 2009). Our findings suggest that the over-expression of CARP in our novel high-CARP line, and thus indirectly its endogenous up-regulation during neuronal activity, may have an effect that is opposite to the deleterious effects of DCLK gene knock-down: namely a protective role that enables the hippocampal network to adapt to physiological processes that coincide with seizure activity.

Robust CARP induction has also been associated with BDNF-LTP. Several of the up-regulated genes that accompany the induction of CARP have known functions in excitatory synaptogenesis, axon guidance and glutamate receptor clustering (Wibrand et al., 2006). Therefore, CARP may also play a role in similar processes. In this respect a study by Sahún et al. is of importance. Using mice with CNS over-expression of tropomyosin receptor kinase C (TrkC), which binds both NT-3 and BDNF, the investigators demonstrate an electrophysiological phenotype that is similar to our current observations in high-CARP mice: highly increased evoked extracellular fEPSPs at the CA3/CA1 synapse (Gruart et al., 2006; Sahún et al,

2007). Previously, CARP and DCLK-short have been associated with neurotrophic factor signaling (Schenk et al., 2007; Dijkmans et al., 2008; 2009). Given these observations a striking parallel between expression of DCLK gene family members that are expressed in response to neurological stimuli and neurotrophic factor signaling emerges.

Based on our electrophysiology data and previous associations of CARP with kainic acid induced seizures (Vreugdenhil et al., 1999) and BDNF-LTP (Wibrand et al., 2006) we performed an initial screen of hippocampal gene expression, hypothesizing that genes related to glutamatergic transmission and/or neurotrophic signaling may be affected. Although no genes directly related to glutamate signaling were identified, we spotted the Ca^{2+} binding protein Calb2 and RapGef4 as differentially expressed.

RapGef4 is down-regulated in the hippocampus of high-CARP mice

Rap guanine exchange factor 4 (Gef) is an activator of Rap1, which is highly homologous to Ras (Bourne et al., 2000). RapGef4, amongst other guanine nucleotide exchange factors, carries out the conversion of the inactive, Guanosine Diphosphate (GDP)-bound form into the active, Guanosine Triphosphate (GTP)-bound form of Rap1 (Kooistra et al., 2007; Pannekoek et al., 2009). Downstream processes resulting from this conversion include cell-cell adhesion, migration and cell polarity (Bos, 2005). Ras and Gefs are highly expressed in the CNS and have been shown to be localized at synapses (Chen et al., 1998). Importantly, Rap1 Gefs play a role in pathways leading to actin reorganization and downstream neurite and spine outgrowth (Radha et al., 2007; Woolfrey et al., 2009) and have also recently been implicated in regulating neuronal cell survival and plasticity of human neuroblastoma cells (Radha et al., 2008). Moreover, mouse embryos lacking this Gef develop a cortical neuron migration defect (Voss et al., 2008).

DCLK-long and DCL exhibit high homology with DCX and are both expressed during development, where they control neuronal migration (Deuel et al., 2006; Koizumi et al., 2006; Shu et al., 2006; Vreugdenhil et al., 2007). It is well-known that mutations in the DCX gene cause migration deficits, which often coincide with an epileptic phenotype (Francis et al., 2006; Spalice et al., 2009). In addition, it has been shown that application of Clostridium *Sordelli* Lethal Toxin-82 (LT-82), an

inhibitor of Rap activity, significantly increases the amplitude of isolated N-Methyl-D-Aspartic acid (NMDA)-EPSPs in hippocampal slices (Murray and O'Connor, 2004). NMDA receptors play a central role in plasticity and neurotoxicity in the CNS. In fact, activation of NMDA receptors and the postsynaptic influx of Ca^{2+} are necessary for the induction of LTP in the CA1 and DG (Collinridge et al., 1983; Malenka et al., 1988). Here we demonstrate a down-regulation of RapGef4 mRNA that may in turn lead to a decreased activity of Rap1. Similar to the effects found using LT-82, the observed increased amplitude of evoked fEPSPs in our study might be a consequence of Rap inhibition through reduced levels of RapGef4 mRNA. Whether the increased fEPSPs have consequences for LTP in transgenic high-CARP mice remains to be elucidated, although increased levels of LTP are associated with the larger evoked fEPSPs in mice over-expressing TrkC in the hippocampus (Sahún et al., 2007). Given aforementioned established observations and the down-regulation of RapGef4 mRNA in the CARP over-expressing hippocampus, RapGef4 may well play an important regulatory role in concert with members of the DCLK gene family during neuronal plasticity.

Calb2 is up-regulated in the DG of high-CARP mice

Calb2 is a Ca^{2+}-binding protein that is expressed throughout the neuronal cell layers of the hippocampus. Expression of Calb2 is typically found in populations of interneurons in all hippocampal subfields (Gulyás et al., 1992; Résibois and Rogers 1992; Liu et al., 1996; Fujise et al., 1998). Early post-mitotic DG neurons in mice transiently express Calb2 most prominently during the stage of axonal and dendritic targeting. During this phase, the immature neurons send axonal projections toward the CA3 pyramidal cell layer (Brandt et al. 2003; Llorens-Martin et al., 2006; Von Bohlen and Halbach, 2007). Additionally, Calb2 has been suggested to play a role as a Ca^{2+} buffer during differentiation (Nag and Wadhwa, 1999) and neuroprotection (Vogt-Weisenhorn et al., 1996; Schierle et al., 1997; Hattiangady et al., 2008). Strikingly, kainate-induced seizures have a strong effect on cell proliferation that is paralleled by an increase of Calb2-positive cells (Brandt et al., 2003; Domínguez et al., 2003). We observed a nearly 2-fold induction of Calb2 mRNA in the hippocampus of high-CARP mice using qPCR, which upon closer inspection by *in* situ hybridization, was found to be a result of an increase in

the DG specifically. Importantly, Calb2 is expressed in a widely distributed subset of GABAergic interneurons and in mossy fibre cells of the DG and it has been proposed that the expression of Calb2 contributes to the control of synaptic plasticity by regulating the activity of GABAergic interneurons (Schurmans et al., 1997; Holter et al., 2007). Interestingly, in the above mentioned DCX/DCLK2 knock-out mice a loss of interneurons is reported, where disrupted lamination of the hippocampus leads to a reduced inhibitory synaptic tone. Moreover, the authors suggest that this reduced inhibitory input underlies the epilepsy associated with lissencephaly (Kerjan et al., 2009). Conversely, grafting of GABAergic neurons that express Calb2 in rat hippocampus following kainic acid induced seizures have shown an increased inhibitory control, which plays a key role in the beneficial effects in elevating the epileptic phenotype (Hattiangady et al., 2008). CARP over-expression might induce the observed increase of Calb2 mRNA, however since the observed electrophysiological effects in high-CARP mice are localized in the CA3/CA1 network, while the deregulation of Calb2 was found in the DG, further experiments are necessary to elucidate this phenomenon and establish a potential causal relation between CARP and Calb2 expression during epileptic seizures.

Conclusion

Given the specific conditions under which CARP mRNA is induced (i.e. seizures, BDNF-LTP), we demonstrate a function for this non-DCX domain-containing splice variant of the DCLK gene that is common to the processes where CARP is induced: affecting glutamatergic neuronal transmission. The novel high-CARP line opens up new avenues in elucidating the function of DCLK gene splice variants that are prominently expressed in the brain during adulthood in response to neuronal activity. Ultimately, a better understanding of functions of the DCLK gene in epilepsy may form a new basis for therapeutic intervention.

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Chapter 4

Hippocampal CARP Over-expression Solidifies Consolidation of Contextual Fear Memories

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Abstract

The Doublecortin-Like Kinase (DCLK) gene has been shown to be involved in neuronal migration during development. Alternative splicing of the DCLK gene also produces a transcript called Ca^{2+}/calmodulin dependent protein kinase (CaMK)-related peptide (CARP) that is expressed exclusively during adulthood in response to neuronal activity. To study the function of CARP, we have generated transgenic mouse strains with over-expression of the CARP transcript in, amongst other brain areas, the hippocampus. We aimed to characterize possible behavioural adaptations of these mice by using a Pavlovian fear conditioning approach. This type of fear conditioning, in which both the hippocampus and amygdala are critically involved, allows studying the formation and extinction of fear related memories. We here report on the behavioural adaptations of two distinct transgenic lines: one with high levels of CARP in the hippocampus and amygdala, whilst the other has high levels of CARP in the hippocampal formation, but not in the amygdala. We tested both mouse lines separately by comparing them to wild-type littermate controls. We found increased freezing during memory testing in the context, but not during presentation of the cues. In addition, we provide evidence suggesting consolidation of contextual fear memories is strengthened in mice of both lines.

Introduction

The Doublecortin-Like Kinase (DCLK) gene encodes two conserved microtubule-binding domains called Doublecortin (DCX) domains, as well as a catalytic kinase domain and a serine proline (S/P)-rich domain. The DCLK gene is subject of massive alternative splicing. Major splice variants include the full length transcripts (DCLK-long), the DCX domains containing transcript doublecortin-like (DCL) and the kinase-only variants (DCLK-short) (Vreugdenhil, Engels et al. 2001; Burgess and Reiner 2002). Interestingly, several studies using knockout mice and RNAi-mediated knockdown of target genes, suggest 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; Friocourt et al., 2007; Vreugdenhil et al., 2007). Alternative splicing of the DCLK gene also produces 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 domains and therefore does not exert any microtubule binding properties or kinase activity. While DCX domain containing gene products of the DCLK gene are predominantly expressed during development, CARP is expressed exclusively during adulthood. Importantly, CARP is expressed at extremely low levels or is below detection levels under basal conditions. In contrast, in the hippocampus, CARP mRNA is highly up-regulated by kainic acid-induced seizures (Vreugdenhil et al., 1999), by adrenalectomy-induced apoptosis in the dentate gyrus (DG) (Schenk et al., 2007) and during brain derived neurotrophic factor induced longterm potentiation (BDNF-LTP; Wibrand et al., 2006). Although many studies have aimed to elucidate the function of DCX and DCX-domain containing DCLK splice variants during cortical and hippocampal development, the function of CARP during adulthood *in vivo* remains elusive.

Recently, we have established that over-expression of CARP in high-CARP mouse brain leads to alterations in hippocampal network excitability (Schenk et al., 2010). We have generated an additional transgenic mouse line with a more restricted over-expression of the CARP transcript in the brain, called low-CARP. Previous experiments using the high-CARP line strongly suggest that hippocampal network excitability is decreased (Schenk et al., 2010). Because of these alterations in

hippocampal neuronal transmission, we aimed to characterize possible behavioural adaptations of these transgenic mice by using a Pavlovian fear conditioning approach. This type of fear conditioning, in which the hippocampus is critically involved, allows studying the formation and extinction of fear related memories (Maren, 2001; Ji and Maren 2007; 2008). We here report on the behavioural adaptations of the two distinct transgenic lines with neuronal CARP over-expression and provide evidence demonstrating that consolidation of contextual fear memories is strengthened in these mice.

Materials and Methods

Generation of transgenic CARP mice

Generation of transgenic mice was performed using a Thy-1.2 expression vector as described (Schenk et al., 2010). The Thy-1.2 promotor specifically drives expression in neurons that starts at postnatal day 6, leaving embryonic development unaffected (Vidal et al., 1990; Hirrlinger et al., 2005). The CARP expression construct was microinjected into a C57BL/6j background and backcrossed to C57BL/6j to produce transgenic offspring. The presence of the transgenic CARP transcript was confirmed by PCR analysis of DNA isolated from tail biopsies. The sense (5'-GTC CAA ATC ATC CGA CGA GAG A-3') and the anti-sense (5'-GCA GTC AGC TCT CCA CTC CG-3') primers were used to amplify a 150-bp fragment of CARP DNA. All transgenic (TG) mice used in the present study were heterozygous. Non-transgenic littermates were used as wild-type (WT) controls for all experiments. The used lines (high-CARP and low-CARP) are fertile with normal frequency and size of litters and stably transmit the transgene to its offspring. All animal treatments were approved by the Leiden University Animal Care and Use Committee (Udec# 01022).

Animals

For the mapping of CARP mRNA expression in the brain, 8-12 weeks old heterozygous TG low-CARP and littermate WT male animals were used. WT (n=5) and TG low-CARP (n=5). For the fear conditioning paradigm male high-CARP, low-CARP and their WT littermates of 8-12 weeks old (n=8 per group) were used.

Sampling of tail and trunk blood was also performed in these animals. All mice were individually housed one week prior to the experiment. Animals had access to food and water *ad libitum* and were kept under standardized housing conditions with a 12h/12h dark/light cycle (lights on 8am).

***In situ* hybridization**

Tissue sample collection and processing was performed as described (Schenk et al., 2010). 40mer oligonucleotides and mismatch oligonucleotides with 6 substitutions were used as control. 5'-GCC GCC ACT GTG CTG GAT ATC TGC AGA ATT CTT ACA CTG A-3' was the perfect match recognizing CARP and 5'-GCC GCG ACT GTC CTG GAA ATC TGG AGA ATA CTT ACT CTG A-3' its mismatch control (substitutions are underlined). *In situ* hybridisation was performed as described (Meijer, Steenbergen et al. 2000). Subsequently, slides were exposed to an X-OMAT AR film (Kodak) for approximately 14 days. Films were scanned using Umax MagicScan and further processed with Adobe Photoshop 9.0.

Fear-conditioning paradigm: Apparatus

The fear conditioning chamber was made of black Plexiglas (25x 25 x 35 cm high) covered by a transparent rim (3 cm width). A speaker was fixed into one wall (25 cm high) and connected to a tone generator (70 dB). The floor consisted of stainless steel bars (5 mm in diameter, spaced 0.5 cm apart) connected to a shock generator. Hereunder was a tray with paper tissues to collect faeces and urine of the mice. A white light source (260 lux) and a camera connected to a video recorder were fixed 20 cm above the conditioning chamber. A radio produced 20 dB of background noise and the light intensity of the experimental room was 90 lux. After each animal, the chamber was cleaned with tap water and the tissues were replaced.

Fear-conditioning paradigm: Procedure

The fear conditioning paradigm allowed differentiating between context and context/cue related behavioural responses in the same setting (Brinks *et al.* 2008). Conditioning: Training (day 1) involved 3 minutes of baseline recording, followed by 6 light/tone (CS) + shock (UCS) pairings separated by a 1-min interval, i.e., the

context episode. Light and tone were paired for 20 seconds and an electric footshock was administered during the last two seconds. Mice were returned to their homecage 2 minutes after the last pairing. Memory / extinction: Fear memories, expressed as scanning and freezing behaviour were monitored 48 hrs and 72 hrs later (days 3 and 4, respectively). To this end the same experimental procedure was repeated *without shocks.* The procedure lasted 12 minutes per mouse/day. Tests were performed between 8.00 hrs and 13.00 hrs in an experimental room adjacent to the housing room.

Fear-conditioning paradigm: Behavioral assessment

During the conditioning period on day 1, freezing and scanning were measured continuously during presentation of the cues and in the context only as soon as the animal was placed in the setting (first point in Figure 2). Acquisition was assessed during the 1 min intervals in between the 20 s light+tone foot shock pairings (points 2-7 in Figure 2). Freezing is defined as total immobility of the body and is devoid of interaction with the environment. Scanning is defined as immobility of the body, while the head is moving horizontally from side to side. In this case the animal is still actively interacting with its environment. Although scanning and freezing are interdependent, they express a different quality of fear (Brinks et al., 2008). Fear memories, expressed as scanning and freezing behaviour were also assessed 48 hrs and 72 hrs later (day 3 and day 4, respectively). Behaviours were scored with a semi automatic scoring system (The Observer 4.1, Noldus, Wageningen, The Netherlands) from video tape.

Nociception

Mice were single housed. To determine possible differences in the pain threshold between TG and WT mice that might influence their responses to the electric footshock given in the fear conditioning paradigm, mice (n=5/group) were subjected to a tail flick protocol. Mice were held at the neck with one hand, while holding the tail vertically with the other hand. To test thermal pain perception the last two cm of the tail were submerged in water with a constant temperature of 55°C (Bilsky et al., 1996). The time to withdraw the tail (tailflick latency) in three subsequent trials (interval 5-7 min) was determined with a cut-off latency of 12 sec.

The experiment was performed between 09.00 and 10.00 hrs to ensure low circulating circadian levels of corticosterone.

Radio Immunoassay

Corticosterone concentrations were determined at several time points. Blood samples from each of the animals (n=8) tested in the fear conditioning experiment were obtained via tail incision as described (Dalm et al., 2005), with approximately 24 hours between blood samples. Basal corticosterone levels were measured from tail-incision blood samples taken on the day before conditioning between 9am and 10am, when circadian corticosterone levels are low. In addition, two tail-incision blood samples were obtained 30 min. after the start of conditioning on day 1 and memory testing on day 3. A final blood sample was taken by collecting trunk blood following decapitation 30 min. after the start of the memory testing on day 4. Blood was collected in potassium–EDTA coated capillaries (Sarstedt, Germany), stored on ice and centrifuged with 13,000 rpm at 4 ◦C for 10 min. Plasma was collected and kept at -20 ◦C until use. Corticosterone concentrations were analyzed using a commercially available radio immunoassay kit ^{125}I-corticosterone (MP Biomedicals, Inc., NY, USA).

Analysis and Statistics

ANOVA with repeated measures was used to test for significant progression or decrease of freezing over conditioning intervals on each day. Student's t-test was used to compare total percentage of time spent scanning or freezing for context and context+cue between TG and WT animals on days 1, 3 and 4. Corticosterone concentrations of the different strains and controls were also compared at each time point. For all tests a probability level of 5% was used as the minimal criterion of significance.

Results

CARP expression in transgenic low-CARP mice

We investigated the distribution of CARP mRNA in the brain using *in situ* hybridization. Localization was based on the mouse brain atlas by Franklin and Paxinos (Franklin and Paxinos, 1997). CARP distribution was limited in low-CARP mice, as transcripts were only found in the hippocampal formation and the subiculum (Figure 1). More specifically, within the hippocampus, CARP mRNA expression was restricted to the CA1 pyramidal cell layer (Figure 1B, E and H-J) and the granule cell layer of the DG (Figure 1E and 1J). In addition, high levels of CARP were observed in the subiculum (1D, H and J). In low-CARP mice, expression of CARP was not found in any other area of the brain.

Acquisition of Fear Memories

Previous experiments performed in high-CARP mice suggest that hippocampal network excitability is decreased in these mice (Schenk et al., 2010). Because of these alterations in hippocampal neuronal transmission, we aimed to characterize possible behavioural adaptations. Using a Pavlovian fear-conditioning paradigm we investigated the consequence of CARP over-expression in the brain of both high-CARP and low-CARP mice on fear memory related behaviour. Acquisition of fear memories was measured as the percentage of time spent freezing in context on day 1. Both high-CARP and their wild-type littermates and low-CARP and their littermates were tested (Figure 2 and 3, respectively). Freezing behaviour is shown for each of the 7 one minute intervals during which the animal was presented with the context only. During the first 3 minutes of the trial, freezing behaviour was absent, as can be appreciated from the first point in the graphs. As the number of tone+light and foot shock pairings increased (to a total of 6), the amount of time spent freezing in the context also increased. This can be seen from points 2-7 in the graphs (Figure 2A: high-CARP and Figure 3A: low-CARP). Using an ANOVA with repeated measures to test for progression of contextual freezing over conditioning intervals indeed revealed significant progression of fear related behaviour in the context. High-CARP and littermate controls $p<0.001$; low-CARP and littermate controls $p<0.001$. No significant differences in progression of

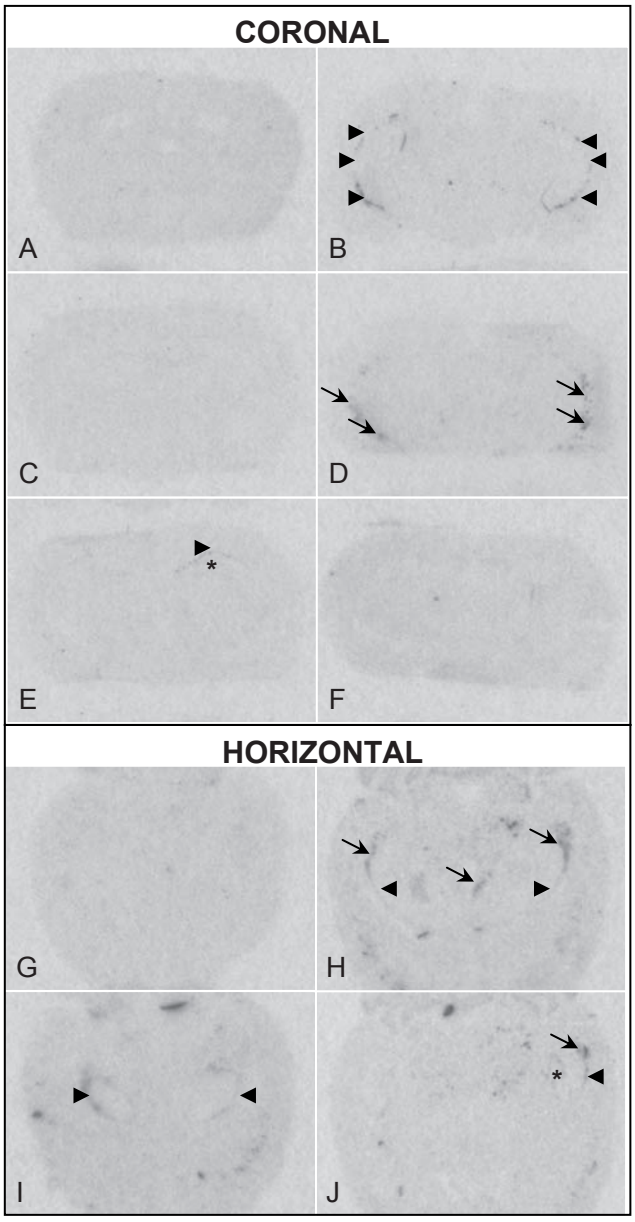
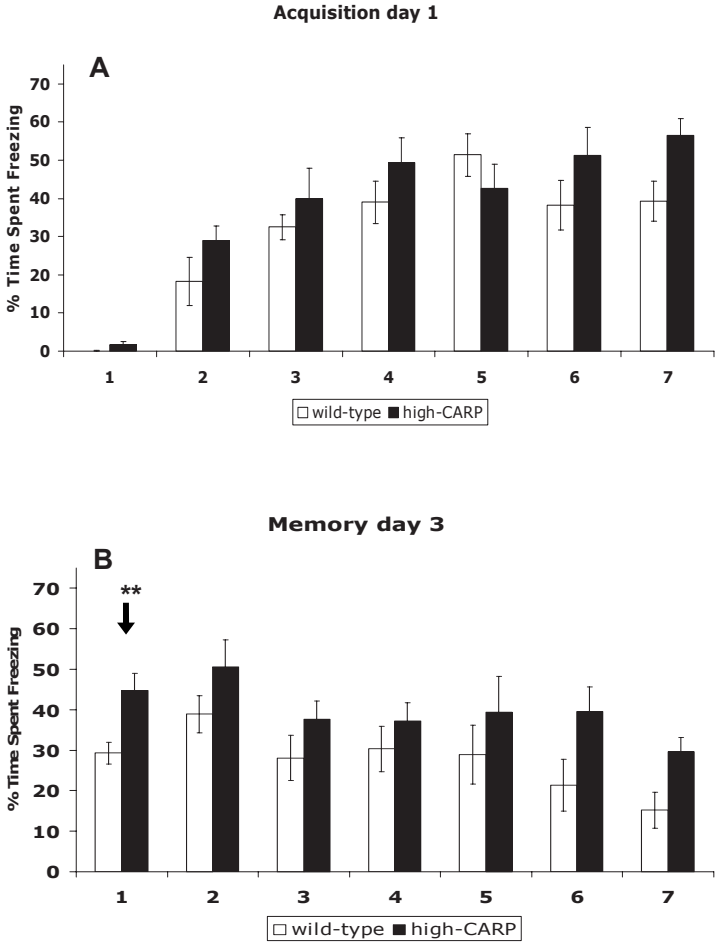


Figure 1. Mapping of CARP expression in low-CARP mouse brain using in situ hybridization. Note the highly specific distribution pattern of CARP transcripts in CA1 (indicated by arrowheads in B, E and H-J), DG (indicated by asterisks in E and J) and subiculum (indicated by arrows in D, H and J). Coronal (A-F) and horizontal (G-J) sections are shown. The mismatch control probe yields a signal that is equal to background (F).

freezing in the context were found between groups (high-CARP $p<0.101$; low-CARP $p<0.997$). To test to what extent the subjects remembered the context and the context in association with the non-aversive stimuli (the tone+light) animals were tested on days 3 and 4 using the same paradigm, but without application of a footshock.



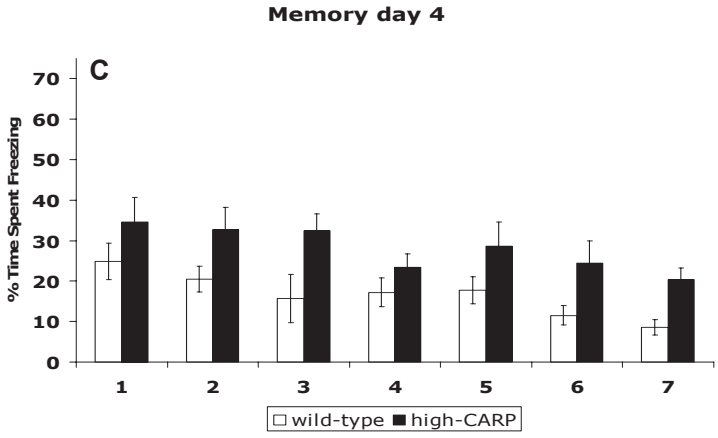
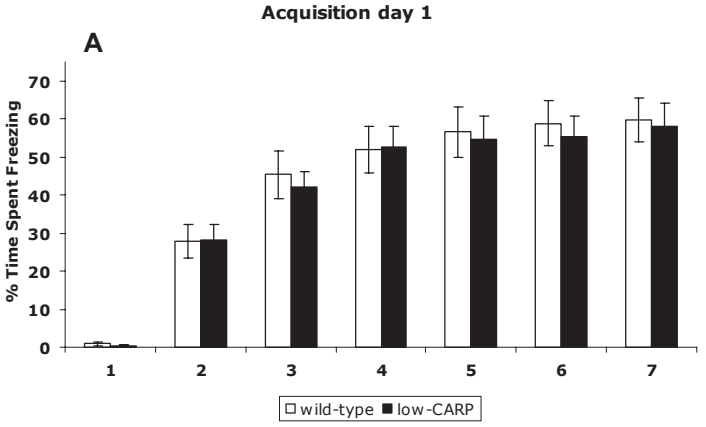


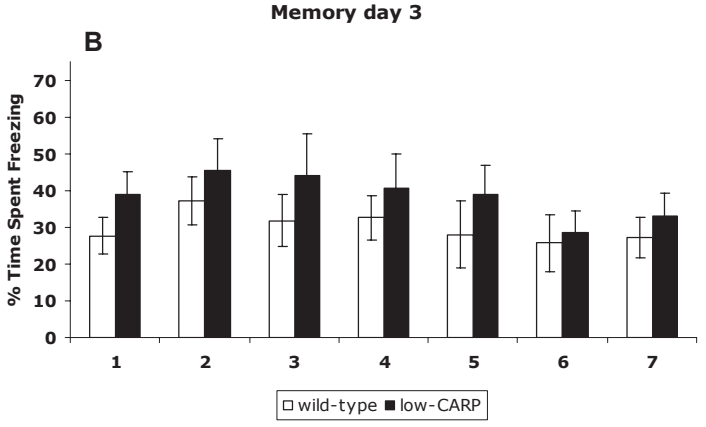
Figure 2. Acquisition and memory of fear behaviour in high-CARP mice and wild-type littermates. (A) Acquisition: 6 light/tone+shock pairings alternate with intervals of context only. Percentage of freezing in context is measured, including the initial 3 min (first point in the graph) and during the 1 min intervals between the light+tone foot shock pairings (points 2-7 in the graph). Note the lack of freezing during the first interval. Freezing significantly increases over context intervals for both groups ($p<0.001$). No significant differences were observed between high-CARP mice and their wild-type controls. (B, C) For assessment of memory of fear behaviour animals were presented with the same context and cues, but did not get a shock. Contextual freezing significantly decreased over intervals on both days in high-CARP mice (day 3 $p<0.001$ (B); day 4 $p<0.001$ (C)), but no differences were found between high-CARP and wild-type groups ($p<0.828$ (B) and $p<0.946$ (C)). Interestingly, when first exposed to the context only on the second day, high-CARP mice showed significantly increased freezing compared to wild-types. Wild-type controls: 29.31±2.71% high-CARP: 44.72±4.25% (indicated by arrow in (B)).

Memory of fear Behaviour

Memory of fear behaviour was assessed by scoring scanning and freezing behaviour on days 3 and 4 in the context only and in the context combined with the tone+light (cue) for each of the intervals of memory testing. The percentage of time animals spent freezing in context only on day 3 and day 4 is shown for each of the 7 intervals. High-CARP mice and controls showed a significant progression in the decrease of freezing during both days of re-testing (Figure 2B day 3 $p<0.001$; Figure 2C day 4 $p<0.001$). In low-CARP and control mice, no significant decrease was found although a trend towards significance was observed for the decrease in freezing (Figure 3B day 3 $p<0.054$; Figure 3C day 4 $p<0.123$). No differences

between high-CARP and wild-types in overall decrease of fear related behaviour in the context on day 3 and day 4 were observed (Figure 2B $p<0.828$ and Figure 2C $p<0.946$). A similar, non-significant pattern was found for low-CARP mice compared to wild-type controls (Figure 3B $p<0.951$ and Figure 3C $p<0.581$).





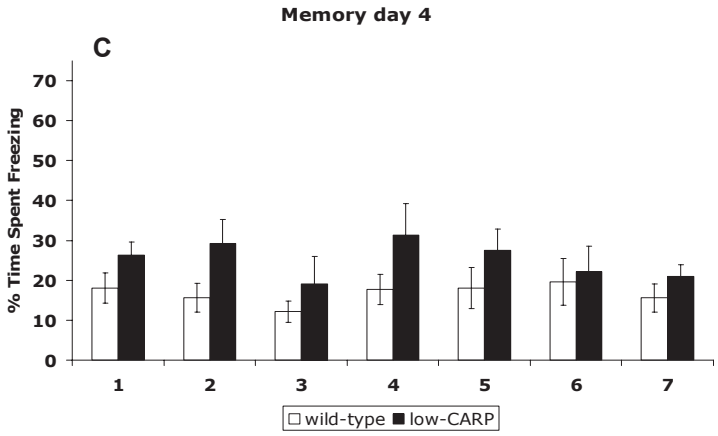


Figure 3. Acquisition and memory of fear behaviour in low-CARP mice and wild-type littermates. (A) Acquisition: 6 light/tone+shock pairings alternate with intervals of context only. Percentage of freezing in context is measured, including the initial 3 min (first point in the graph) and during the 1 min intervals between the light+tone foot shock pairings (points 2-7 in the graph). Note the lack of freezing during the first interval. Freezing significantly increases over context intervals for both groups ($p<0.001$). No significant differences were observed between low-CARP mice and their wild-type controls. (B, C) Memory of fear behaviour in low-CARP mice and littermate controls. A trend for a decrease in contextual freezing was observed, but this did not reach significance (day 3 $p<0.054$ (B); day 4 $p<0.123$ (C)). The decrease in freezing in context showed no significant differences for low-CARP mice and their controls ($p<0.951$ (B) and $p<0.581$ (C)).

When first exposed to the context only on the day 3, high-CARP mice showed significantly increased freezing compared to wild-types (first point in Figure 2B). This observation was not found for low-CARP mice (first point in Figure 3B). Moreover, although high-CARP mice showed a similar decrease in freezing behaviour in context over intervals as wild-types, their freezing behaviour is still clearly elevated at the end of each day, and in contrast to low-CARP mice, never reaches wild-type levels of freezing (last points in Figure 2B-C for high-CARP and Figure 3B-C for low-CARP). Scanning behaviour was also monitored, but no differences were observed for any of the groups, regardless of day of testing or whether the observations were taken from the context only or during presentation of the cues in the context (Supplementary Figure S1).

The total average percentage of time spent freezing in context and during cues for each day was also calculated and is indicated in Figure 4 for high-CARP mice and Figure 5 for low-CARP mice. No significant differences were seen on day 1, an observation that is in line with the similar progression of freezing in context between groups (Figures 2A and 3A). However, significant differences for total freezing behaviour in the context only were found on days 3 and 4 for both high-CARP (Figure 4 $p<0.004$ and $p<0.001$, days 3 and 4 respectively) and low-CARP (Figure 5 $p<0.033$ and $p<0.002$, days 3 and 4 respectively) when compared to wild-type controls. Thus, for both CARP over-expressing mouse lines total freezing was significantly increased on days 3 and 4.

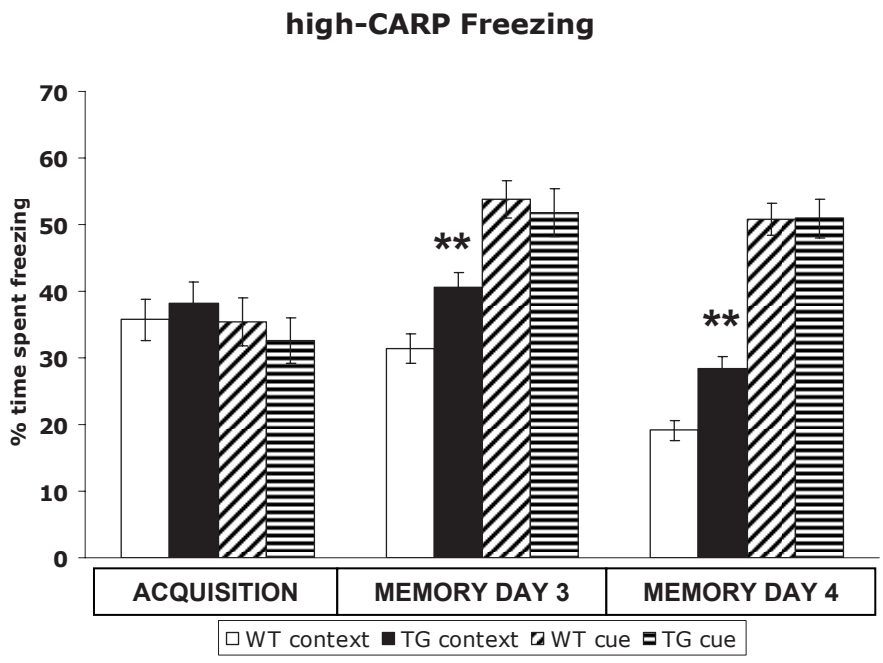


*Figure 4. The total percentage of freezing during acquisition and memory testing in high-CARP and littermate control mice. The total amount of freezing in context is significantly higher in high-CARP mice for memory testing on days 3 and 4. No significant differences were observed for freezing during the cues (striped bars). ** p<0.01.*

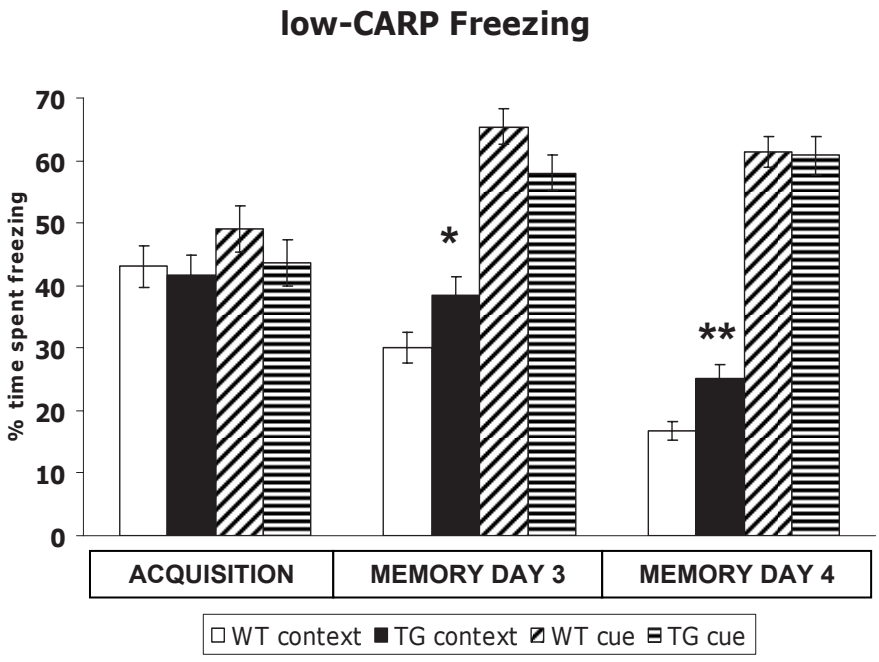


*Figure 5. The total percentage of freezing during acquisition and memory testing in low-CARP and littermate control mice. The total amount of freezing in context is significantly higher in high-CARP mice for memory testing on days 3 and 4. No significant differences were observed for freezing during the cues (striped bars). * p<0.05; ** p<0.01.*

The decrease in the total percentage freezing seen from day 3 to day 4, however, appears to be similar between CARP mice and controls, an observation that is supported by the lack of significant difference between groups when subjected to an ANOVA with repeated measures (Figure 2B-C high-CARP; Figure 3B-C low-CARP). This difference in percentage freezing from day 3 to day 4 can be estimated by subtracting the percentage of time spent freezing on day 4 from the value obtained on day 3. This difference is 12.2% for high-CARP (41.6%-28.4%) and 12.3% (31.4%-19.1%) for wild-type controls. The difference for low-CARP animals is 13.2% (38.5%-25.2%), with 13.3% (30.0%-16.7%) for wild-type controls. Using this approach, it becomes clear that the decrease in time the animals spent freezing between days 3 and 4 is indeed comparable. The seemingly different values for percentage of freezing during the tone+light observed for low-CARP

mice on day 3 was non-significant (striped bars in Figure 5; $p<0.064$). Consequentially, no significant differences for freezing during the presentation of the cues for any of the groups on the 3 days of conditioning were observed (see Supplementary Figure S2 for freezing during individual cue intervals).

Radio Immunoassay and Nociception

Fluctuations in plasma corticosterone concentrations may influence acquisition and consolidation of fear memories (Cordero et al., 2002; 2003; Cai et al., 2006; Sandi et al., 2008; McGaugh and Roozendaal 2008). Additionally, the DCLK gene has been reported as corticosteroid-responsive (Datson et al., 2001; Alfonso et al., 2004). To rule out the possibility that the observed differences between CARP over-expressing mice and controls are a result of altered levels of corticosterone, blood samples were collected at several time points. Basal corticosterone concentrations were determined from blood samples taken one day prior to the start of the experiment. Blood samples were also taken 30 minutes after the start of the experiment on each of the three days of conditioning. Plasma corticosterone levels were highly increased following conditioning on each of the days of testing and significantly differed from basal corticosterone concentrations. No significant differences in basal plasma corticosterone concentrations were found between transgenic animals and their littermate controls. Moreover, stress levels of corticosterone were not significantly different between groups (Table 1). In order to rule out potential differences in pain perception between genotypes, a test for nociception in high-CARP and wild-type mice (n=5) was performed. The tail was submerged in a 55 °C warm water bath and the latency to withdraw the tail was measured. High-CARP mice had a withdrawal latency of 4.5±0.44 (s) and littermate controls 3.6±0.32 (s) which is not significantly different, suggesting pain perception is comparable in these mice.

	Basal	Day 1*	Day 3*	Day 4*
high-CARP	5.5 ± 0.9	140.1 ± 17.2	107.6 ± 9.8	113.5 ± 6.5
wild-type	7.5 ± 1.6	127.0 ± 7.9	121.0 ± 11.5	115.7 ± 10.8
low-CARP	20.1 ± 13.0	169.3 ± 15.5	149.7 ± 9.6	160.9 ± 10.9
wild-type	12.7 ± 4.8	158.4 ± 9.3	137.4 ± 8.0	156.2 ± 13.9

Table 1. Plasma corticosterone concentrations of high-CARP, low-CARP and wild-type control mice (n=8, concentrations are in ng/ml and represented as mean±SEM). Blood samples were collected one day prior to the start of the experiment (basal) and 30 minutes after the fear conditioning sessions (days 1, 3 and 4). No significant differences were observed between CARP mice and their controls. All concentrations measured from samples taken on experimental days are significantly different from basal and correspond to stress levels of corticosterone (p<0.01).*

Discussion

We here report on the successful generation of a novel transgenic mouse line with specific over-expression of the DCLK gene transcript CARP in the brain, called low-CARP. We show highly specific expression of CARP transcripts in the hippocampus and subiculum. In addition, for the characterization of possible behavioural differences, we subjected the novel low-CARP line, as well as the recently described high-CARP line (Schenk et al., 2010), to a fear conditioning paradigm. We aimed for a characterization of both acquisition and memory of fear related behaviours and provide evidence that the hippocampus dependent processing of fear memories is altered in transgenic mice compared to wild-type littermates. Freezing, a passive type of fear behaviour was elevated in both transgenic lines compared to wild-type controls, while scanning behaviour was comparable. Taken together we conclude that hippocampal over-expression of CARP solidifies consolidation of contextual fear memories.

Low-CARP mice express CARP with high specificity

Two transgenic lines with over-expression of the DCLK gene transcript CARP were generated by pro-nuclear injection of a Thy-1.2 promoter driven expression construct and subsequent implantation. Previously, we have shown high levels of

CARP expression in a line called high-CARP. This line has a robust and widely distributed expression of the CARP transcript throughout the brain. Importantly, the neuronal cell layers of the hippocampus and the amygdala express high levels of CARP (see Table 1 and Schenk et al., 2010). We here examined another transgenic CARP over-expressing line, designated low-CARP. We performed a mapping of CARP mRNA expression in the brains of these mice. Compared to high-CARP mice, expression of CARP was much more restricted to the hippocampal formation and the subiculum. This highly specific distribution resembles the characteristic expression pattern that is commonly associated with the use of Thy-1.2 driven expression constructs (Vidal et al., 1990). Thus, the two described transgenic lines have a distinct, yet partly overlapping, distribution of CARP in the brain. The applied conditioning paradigm allowed for the observation of 'context only' and 'context and cue' related fear behaviour in the same setting. The hippocampus is of crucial importance for contextual learning, while both the amygdala and the hippocampal formation are known to play an important role in fear behaviour elicited by presentation of the cues in the context (Maren, 2001; Phelps and LeDoux 2005; Rosen and Donley 2006; Herry et al., 2008). Low-CARP mice have specific expression of CARP in the hippocampus, but not the amygdala. High-CARP mice on the other hand show high levels of CARP throughout the brain, including the hippocampus and the amygdaloid nuclei. This raises the possibility to determine the contribution of differential CARP over-expression on behavioural responses that coincide with learning and memory formation. As such, comparing these two transgenic lines may reveal whether CARP over-expression affects processing of fear memories in the amygdala.

Acquisition of Fear Memories is similar between genotypes

In high-CARP and littermate control mice, high levels of immobility were observed on the first day. The same was the case for low-CARP mice and their littermate controls. The percentage of freezing was comparable to that of earlier observations in C57/BL6j mice during fear conditioning (Brinks et al., 2008). No significant differences in progression of freezing in the context were found between groups on the first day of testing, suggesting both CARP and control animals are capable of

acquisition of fear memories. Importantly, high-CARP and low-CARP freezing behaviour in the context did not differ from their wild-type controls during the first 3 minutes of the trial, indicating that the context by itself is not experienced as an aversive stimulus. Moreover, progression of freezing in the context during the presentation of the cues did not differ significantly between groups on the first day. Thus, both transgenic strains and their littermate controls are able to learn the presented association equally well. To evaluate the memory of fear behaviour, animals were tested in the same setting 48 and 72 hours later.

Consolidation of contextual fear memories is strengthened in CARP mice

The extent to which the fear related experience is remembered is reflected by the amount of time the animal shows behaviour of immobility. This immobility is characterized by scanning and freezing behaviour. Whereas freezing can be viewed upon as an adaptive response or a passive type of fear behaviour, scanning is a more active coping style (Brinks et al., 2008). No differences in scanning were found between groups. Thus, over-expression of CARP only affected freezing but not scanning behaviour in transgenic mice. Strikingly, when first exposed to the context only, high-CARP mice showed significantly increased freezing compared to wild-types. This observation was not found for low-CARP mice. However, high-CARP mice and wild-type controls and low-CARP mice and their controls did not differ significantly in the decrease of freezing behaviour over intervals, suggesting extinction of the conditioned response follows a similar trajectory in transgenic and wild-type mice. Given these observations, the actual increase that is found concerning total freezing behaviour on day 3 and 4 in CARP mice is probably a consequence of the consolidation process. As such, we propose that CARP mice display a more robust consolidation that is reflected by increased freezing in reaction to the context, whilst overall progression of extinction remained comparable to that of wild-type littermates. Since the hippocampus is of crucial importance for this type of contextual memory (Maren and Holt, 2000; Ji and Maren, 2007; 2008), the observed effects are likely a consequence of the hippocampal over-expression of CARP.

Glucocorticoids have been shown to affect hippocampus and amygdala mediated memory formation in these types of fear-related behavioural tasks (Cordero et al., 2002; 2003; Cai et al., 2006; Brinks et al., 2008; Sandi et al., 2008; McGaugh and Roozendaal 2008). In addition, the DCLK gene has been found in large scale screening experiments as being down-regulated by chronic stress (Alfonso et al., 2004) and by high glucocorticoid-replacement in adrenalectomized animals (Datson et al., 2001). We here collected blood samples at a single fixed time point following the start of each conditioning trail and did not thoroughly characterize the stress-response of CARP over-expressing transgenic mice. Nevertheless, neither basal nor stress levels of corticosterone differed significantly between high-CARP mice and controls or low-CARP mice and their controls. Moreover, pain perception in high-CARP and control mice was not significantly different. Together, these observations suggest that the strengthened consolidation in CARP mice is indeed a consequence of CARP over-expression in the hippocampus, as opposed to a centrally acting corticosterone effect or a nociception effect.

Strengthened consolidation is independent of CARP in the Amygdala

The suggestion that the observed phenotype is a consequence of CARP over-expression in the hippocampal formation is also supported by the fact that both high-CARP and low-CARP mice displayed more total freezing behaviour to the context when compared with littermate controls on days 3 and 4, whilst freezing behaviour of wild-type and transgenic animals was comparable during cue intervals. The amygdala is crucially involved in these cue-related fear memories (Maren, 2001; Goosens and Maren, 2001; Kim and Jung, 2006). Interestingly, previously described associations of CARP expression include the hippocampal formation (Vreugdenhil et al., 1999; Wibrand et al., 2006; Schenk et al., 2007) and the striatum (Berke et al., 1998; Glavan et al., 2002), but not the amygdaloid nuclei. In fact, two paradigms that are capable of inducing high levels of CARP transcripts in the hippocampus are kainic acid induced seizures (Vreugdenhil et al., 1999) and adrenalectomy-induced apoptosis in the DG (Schenk et al., 2007). Both seizures and adrenalectomy are known to have deleterious effects on hippocampal neuronal viability and hippocampus dependent learning (De Kloet et al., 1986;

1998; Parent et al., 1997; Brown-Croyts et al., 2000; Mikati et al., 2001; Ben-Ari, 2001; Parent and Lowenstein 2002; Jessberger et al., 2007; Brinks et al., 2007). As such, the lack of significant differences between groups for freezing and scanning during cues suggests that the additional over-expression of CARP in the amygdaloid nuclei of high-CARP mice does not contribute to the acquisition or the consolidation process in this paradigm. This suggests that CARP does not play a role in the amygdala during fear conditioning and has a more restricted area of functionality within the brain that includes the hippocampus.

Possible physiological basis for strengthened Consolidation

Recently, electrophysiological examination of the hippocampus of high-CARP mice has revealed that field excitatory post synaptic potentials (fEPSPs) of the CA3/CA1 network are highly increased (Schenk et al., 2010). Here we demonstrate increased freezing during memory testing of high-CARP and low-CARP mice and suggest that the consolidation process is more robust in CARP over-expressing mice. Possibly, a parallel between the increased fEPSPs and strengthened consolidation exists. Changes of long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus are amongst the best described physiological bases of learning and memory formation (Bliss and Collingridge, 1993; Lynch, 2004). During Pavlovian fear conditioning, contextual (but not cued fear) memory is dependent on the hippocampal formation (Kim and Fanselow, 1992; Phillips and LeDoux, 1992) and fear conditioning itself is known to affect LTP and LTD in CA1 pyramidal neurons (Li et al., 2005). The responsiveness of CA3/CA1 synaptic transmission is known to be modulated during associative learning, and this is prevented by inhibition of LTP and NMDA-receptor activity (Gruart et al., 2006). Interestingly, a robust induction of endogenous CARP has previously been associated with BDNF-LTP (Wibrand et al., 2006). Moreover, improvement of contextual fear conditioning has been associated with excitatory neuronal transmission in the hippocampus, through elevated presynaptic glutamate release (Liu et al., 2004; 2007; Wu et al., 2008). High-CARP mice show elevated evoked fEPSPs, albeit without increased population spike amplitudes (Schenk et al., 2010). This does not rule out the possibility that our observations regarding the

hippocampus-dependent processing of fear memories may be a consequence of altered neuronal excitability in CA3/CA1 network. Thus, increased fEPSPs in high-CARP mice might lead to a strengthened consolidation of contextual fear memories.

Possible molecular basis for Strengthened Consolidation

Several publications show the importance of cytoskeletal changes implicated in the plasticity of neuronal cells, synapses, LTP and learning and memory formation (Krucker et al., 2000; Ressler et al., 2002; Meng et al., 2002; Fischer et al., 2004; Rabenstein 2005; Mantzur et al., 2008; Ploski et al., 2008; Dijkmans et al., 2009). From this perspective it is interesting to note that we recently demonstrated that CARP increases DCL-induced polymerisation of tubulin. Moreover, CARP interacts with the adapter protein Growth factor receptor-bound 2 (Grb2) *in vitro* (Schenk et al., 2007). Grb2 plays a role in the formation of dendritic spines, a critical aspect of synaptic development (Moeller et al., 2006) and is also implicated in the regulation of the actin cytoskeleton (Buday et al., 2002). In addition, Grb2 is involved in Ras-ERK kinase activation (Katz and McCormick, 1997). The ERK/MAPK pathway is well-known to be activated during fear conditioning (Schafe et al., 2000; 2008; Herry et al., 2006; Trifilieff et al., 2006; Chwang et al., 2006; Lewis and Gould, 2007; Sindreu et al., 2007). More specifically, MAPK activation is required for the formation of hippocampus-dependent memory (Atkins et al., 1998; Blum et al., 1999). Strikingly, ERK-dependent phosporylation of DCLK-short occurs at a specific serine residue that lies within the S/P-rich domain, which is also present in CARP, and is crucial for the process of neuritogenesis *in vitro* (Dijkmans et al., 2009). The potential effect of CARP action through cytoskeletal changes is also supported by the observation that CARP is concomitantly up-regulated with several genes involved in excitatory synaptogenesis and axon guidance following BDNF-LTP (Wibrand et al., 2006). Given these observations, it is plausible that the over-expression of CARP affects the consolidation of fear memories through interaction with Grb2, leading to alterations in ERK/MAPK pathway activity and subsequent molecular and cellular changes that are crucial for memory formation. It is of importance to point out that while high-CARP mice did show a significant decrease

of freezing during memory testing on days 3 and 4 and displayed highly increased freezing during the first interval of the third and fourth day, low-CARP mice did not. Although a trend towards significance was observed concerning the decrease over intervals, low-CARP mice did not show the same characteristics of freezing as high-CARP mice. A dose dependent effect of CARP on the polymerization of tubulin and on the interaction with the adapter protein Grb2 *in vitro* exists (Schenk et al., 2007). Since expression levels of CARP in the hippocampus of low-CARP mice are much lower and more restricted, the possibility arises that the effects of CARP in the hippocampus *in vivo* are also dose and/or distribution dependent.

Conclusion

In recent years it has become clear that unveiling the molecular and cellular mechanisms associated with learning and memory is a crucial step in understanding the interplay between genetics and behavioural adaptations. Since CARP is highly regulated in response to neuronal activity and the DCLK gene is likely involved in cytoskeletal plasticity through the ERK/MAPK pathway, these newly generated transgenic mice are a useful tool in studying those processes in which members of the DCLK gene family are involved, such as neuronal plasticity and migration.

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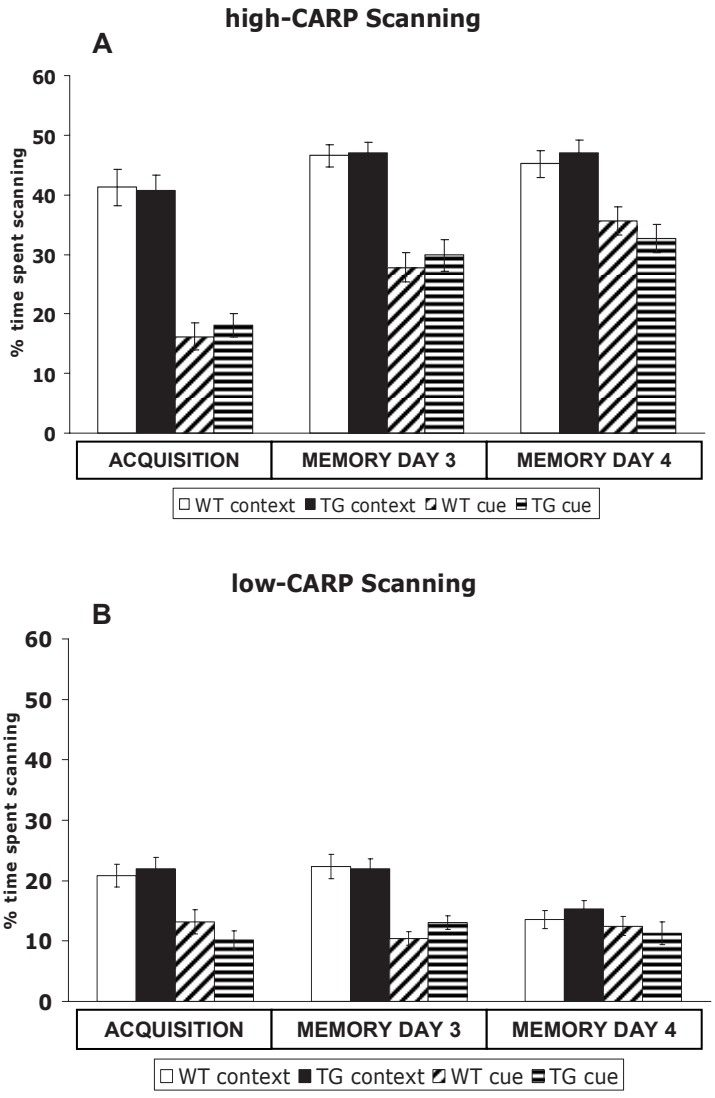
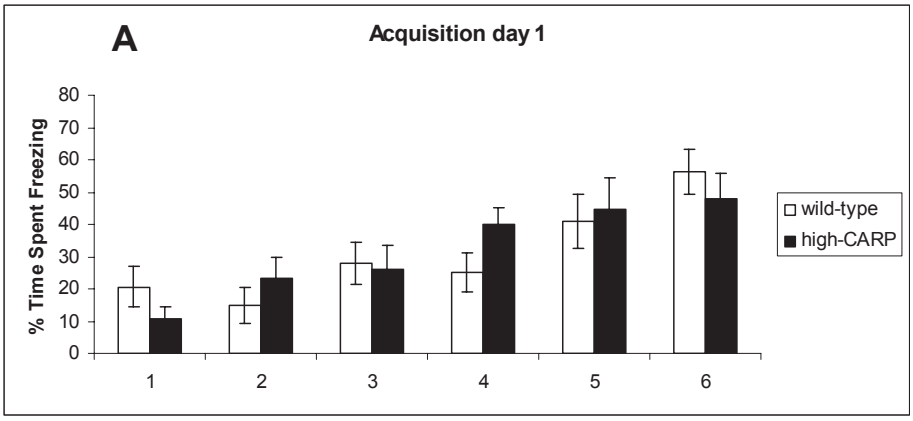
Supplementary Material



Supplementary Figure S1. Total amount of scanning in the context or during cues is not significantly different in high-CARP (A) or low-CARP (B) mice on any of the conditioning days.


A
Acquisition day 1
% Time Spent Freezing
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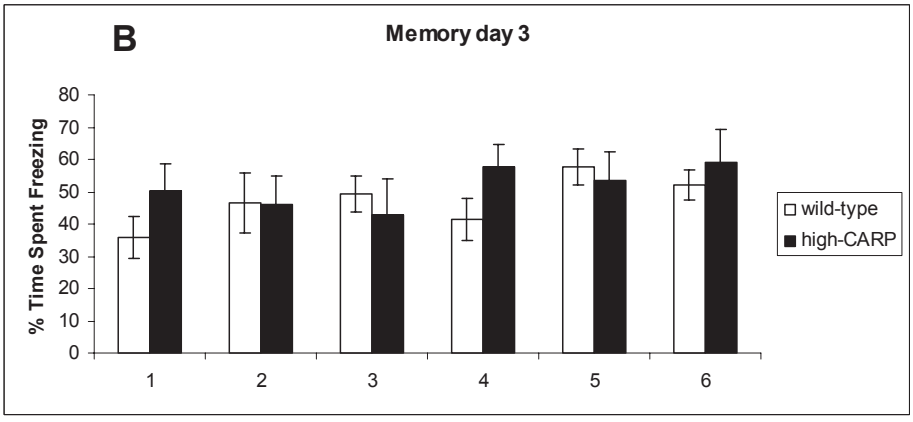
wild-type
high-CARP


B
Memory day 3
% Time Spent Freezing
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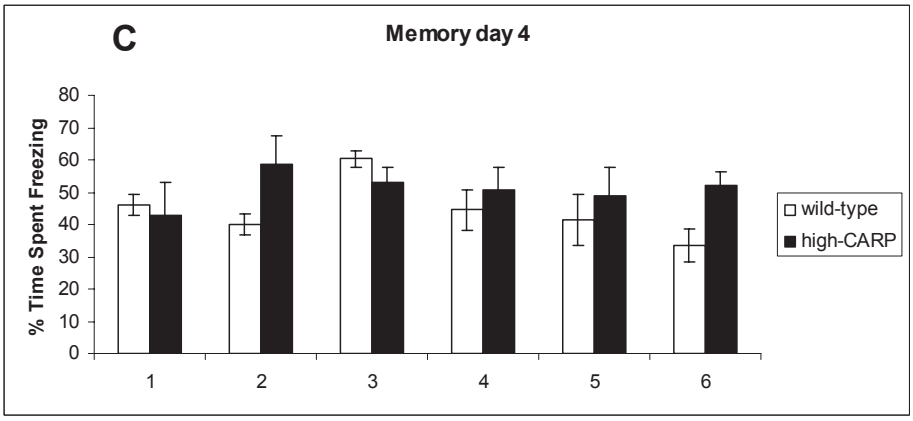
wild-type
high-CARP


C
Memory day 4
% Time Spent Freezing
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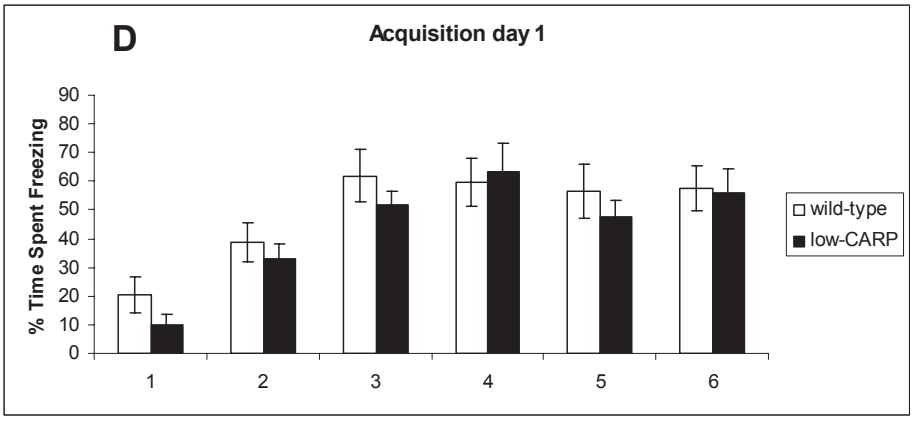

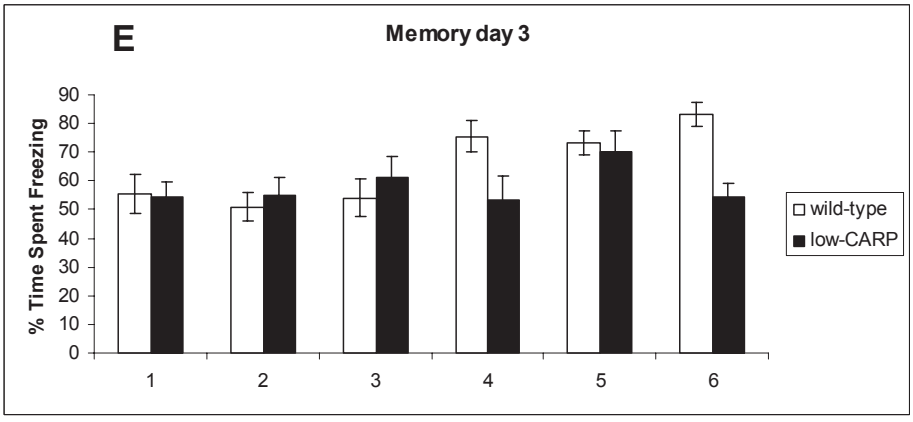

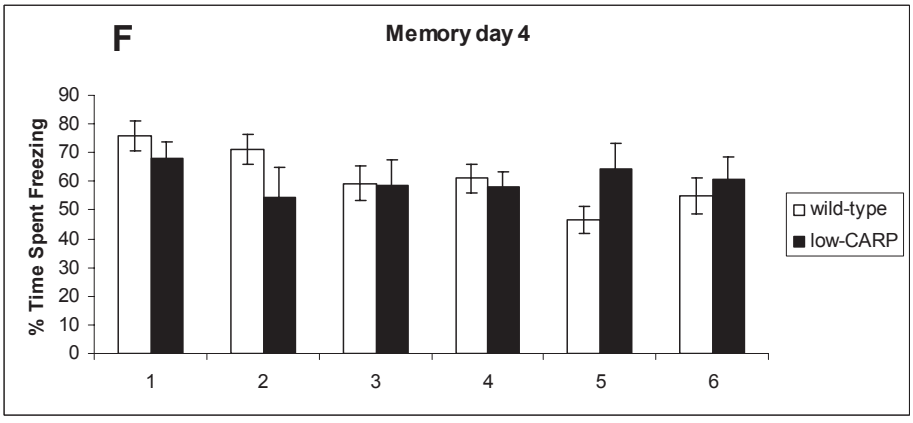

Supplementary Figure S2. Total amount of freezing is not significantly different between CARP mice and their littermate controls during presentation of the cues (Figures 4 and 5). Here freezing during cues is indicated for individual episodes. Acquisition: high-CARP (A) and low-CARP (D). Memory testing days 3 and 4: high-CARP (B and C, respectively) and low-CARP (E and F, respectively).

Chapter 5

Over-expression of δC-DCLK-short in Mouse Brain Results in a More Anxious Behavioral Phenotype

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Abstract

Products of the Doublecortin-Like Kinase (DCLK) gene are associated with cortical migration and hippocampal maturation during embryogenesis. However, the functions of those DCLK gene transcripts that encode kinases and are expressed during adulthood are incompletely understood. To elucidate potential functions of these DCLK gene splice variants we have generated and analyzed transgenic mice with neuronal over-expression of a truncated, constitutively active form of DCLK-short, designated δC-DCLK-short. Previously, we have performed an extensive molecular characterization of these transgenic δC-DCLK-short mice and established that a specific sub-unit of the $GABA_A$ receptor, which is involved in anxiety-related GABA-ergic neurotransmission, is down-regulated in the hippocampus. Here we show that δC-DCLK-short mRNA is highly expressed in the hippocampus, cortex and amygdala of transgenic mice. We provide evidence that the δC-DCLK-short protein is expressed and functional. In addition, we examined anxiety-related behavior in δC-DCLK-short mice in the elevated plus maze. Interestingly, δC-DCLK-short mice spend less time, move less in the open arms of the maze and show a reduction in the number of rim dips. These behaviors indicate that δC-DCLK-short mice display a more anxious behavioral phenotype.

Introduction

The Doublecortin-Like Kinase (DCLK) gene is expressed during neuronal development and has high homology to the neurogenesis-related gene doublecortin (DCX); it encodes two conserved microtubule-binding DCX domains as well as a serine/proline (SP) rich and a kinase domain, and is subject of massive alternative splicing. Splice variants include the full length transcripts DCLK-long (Burgess, Martinez et al. 1999); (Lin, Gleeson et al. 2000), the DCX domains containing transcript doublecortin-like (DCL; (Vreugdenhil, Kolk et al. 2007)) and a 55-amino-acid SP-rich peptide, CaMK-related peptide (CARP; (Schenk, Engels et al. 2007); (Vreugdenhil, Datson et al. 1999)). The DCLK gene also produces a transcript comprising the SP-rich N-terminal domain corresponding to CARP and the C-terminal catalytic domain, but not the microtubule binding domains, called DCLK-short (Vreugdenhil, Kolk et al. 2007); (Burgess and Reiner 2002); (Friocourt, Koulakoff et al. 2003); (Vreugdenhil, Engels et al. 2001); (Kruidering, Schouten et al. 2001). Interestingly, several studies using knockout mice and RNAi-mediated knockdown, show that DCX and DCLK have overlapping functions during cortical and hippocampal development in mice (Vreugdenhil, Kolk et al. 2007); (Koizumi, Tanaka et al. 2006); (Deuel, Liu et al. 2006); (Tanaka, Koizumi et al. 2006); (Tuy, Saillour et al. 2008). These functions, however, are mostly attributed to the microtubule binding domains (Lin, Gleeson et al. 2000); (Sapir, Horesh et al. 2000). Since DCLK-short is expressed in the adult brain and lacks the microtubule binding DCX domains, the DCLK gene may have additional functions beyond neurogenesis and neuronal development (Burgess, Martinez et al. 1999); (Vreugdenhil, Datson et al. 1999); (Burgess and Reiner 2002); (Hevroni, Rattner et al. 1998); (Silverman, Benard et al. 1999). Although DCLK-short has recently been implicated in neuritogenesis *in vitro* (Dijkmans, van Hooijdonk et al. 2009); (Dijkmans, van Hooijdonk et al.), its function remains largely unclear. To elucidate the function of DCLK-short *in vivo*, we have generated transgenic mice with brain specific over-expression of the DCLK-short transcript. We aimed to produce a constitutively active form of transgenic DCLK-short by omitting 44 amino acids from its C-terminus. Truncation of this auto-inhibitory domain has been associated with increased kinase activity (Engels, Schouten et al. 2004); (Ohmae, Takemoto-Kimura et al. 2006). Because of its C-terminal truncation, this novel

transgenic mouse line was designated δC-DCLK-short. Previously, we performed a thorough large scale screen spanning several platforms to examine hippocampal gene expression in these mice. This large scale screen revealed differential gene expression covering several relevant biological pathways, including calmodulin-dependent protein kinase activity, microtubule associated vesicle transport and GABAergic neurotransmission (Pedotti, t Hoen et al. 2008); (t Hoen, Ariyurek et al. 2008). Here, we describe the expression of δC-DCLK-short at the mRNA and protein levels in the adult hippocampus and demonstrate that the transgenic kinase is active. Additionally, since interference with GABAergic neurotransmission is associated with anxiety-related behaviors (Crestani, Lorez et al. 1999); (Rudolph, Crestani et al. 1999); (Low, Crestani et al. 2000); (Atack 2005); (Rudolph and Mohler 2006), we subjected mice from this novel transgenic line to the elevated plus maze (EPM) paradigm, a well-validated test for anxiety-related behaviors (Hogg 1996); (Rodgers and Dalvi 1997); (Rodgers, Cao et al. 1997). We provide evidence that δC-DCLK-short mice have a significantly more anxious behavioral phenotype.

Materials and Methods

Generation of transgenic δC-DCLK-short mice

A cDNA construct containing the DCLK gene kozac sequence and the sequence encoding the DCLK-short transcript was generated. The C-terminal domain of the DCLK-short transcript, comprising 44 amino acids encoding the auto-inhibitory domain, was omitted from the cDNA sequence, rendering this truncated form of DCLK-short constitutively active (Engels, Schouten et al. 2004). In addition, a FLAG-tag was added to the construct at the C-terminal end, for easy detection of the transgenic δC-DCLK-short protein. A pTSC expression construct was used; this vector contained an 8.1 kb EcoR1 fragment comprising the mouse Thy-1.2 gene. A 1.5 kb Ban1/Xho1 fragment (located in exon 2 and exon 4, respectively) was replaced by the δC-DCLK-short cDNA (Vidal, Morris et al. 1990); (Moechars, Lorent et al. 1996). The Thy-1.2 promotor specifically drives expression in neurons and starts at postnatal day 6, leaving embryonic development unaffected (Vidal, Morris et al. 1990). Subsequently, transgenic offspring was generated by

microinjection of the DCLK expression construct into a C57BL/6j background and backcrossed to C57BL/6j for at least 10 generations to produce transgenic offspring. The presence of the transgenic DCLK transcript was confirmed by PCR analysis of DNA isolated from tail biopsies. The sense (5'-AAGAAGAGTCCGACGAAGGT-3') and the anti-sense (5'-AGGTATTTAATGGCACTGGC-3') primers were used to amplify a 350-bp fragment of DCLK-short DNA. All transgenic (TG) mice used were heterozygous. Non-transgenic littermates were used as wild-type (WT) controls for all experiments. All animal treatments were in accordance with the Leiden University Animal Care and Use Committee (DEC#01022).

Animals

δC-DCLK-short and littermate control WT young adult (8-10 weeks old) mice were used for all experimental procedures. Male animals were used to rule out any hormonal and physiological fluctuations that normally occur during oestrous cycle in female animals. For all experimental procedures, heterozygous δC-DCLK-short mice were used to ensure the availability of negative wild-type littermate controls. For behavioral experiments mice (n=10 per group) were individually housed one week prior to the experiment. Animals had access to food and water *ad libitum* and were kept under standardized housing conditions with a 12h/12h dark/light cycle (lights on 8am). Animals were tested between 9am and noon to ensure low circulating corticosterone levels. For *in situ* hybridization, western blot and immunoprecipitation experiments mice were housed under similar conditions (n=3 per group for each experiment).

***In Situ* Hybridization**

Brain tissue samples were collected and processed as described (Schenk, Engels et al. 2007). δC-DCLK-short mRNA was detected using 40mers. Mismatch oligonucleotides with 6 substitutions were used as control. 5'-CCGCCACTGTGCTGGATATCTGCAGAATTCCTACTTGTCA-3' is the perfect match recognizing δC-DCLK-short with 5'-

CCGCCTCTGTGGTGGATTTCTGCTGAATTGCTACTTCTCA-3' as its mismatch control (substitutions are underlined). *In situ* hybridization was performed as described (Meijer, Steenbergen et al. 2000). Subsequently, slides were exposed to an X-OMAT AR film (Kodak) for approximately 10 days. Films were scanned (at 1200 dpi) using Umax MagicScan. Brain regions with expression of δC-DCLK-short transcripts or lack thereof were identified using the mouse brain atlas by Franklin and Paxinos (Franklin 1997).

Western Blot analysis

For western blot experiments TG and WT hippocampus and cerebral cortex were dissected quickly at 4 °C and transferred directly to a tube containing ice cold lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Triton) and total protein was extracted. Western blotting was performed as described (Schenk, Engels et al. 2007). A mouse monoclonal anti-FLAG primary anti-body was used for detection of transgenic δC-DCLK-short protein (M2; Sigma-Aldrich, Co. St. Louis, MO, U.S.A.). In addition, endogenous DCLK-short and δC-DCLK-short were detected using a previously described antibody recognizing the SP-rich N-terminus of DCLK-short (Schenk, Engels et al. 2007); (Vreugdenhil, Datson et al. 1999); (Boekhoorn, Sarabdjitsingh et al. 2008). Horseradish peroxidase (HPA)-conjugated secondary antibodies (used at 1:5000) were from Santa Cruz. Detection with an anti-tubulin primary antibody served as a loading control (Santa Cruz biotechnology, Inc. Santa Cruz, CA, U.S.A.). For semi-quantification of protein expression, relative optical densities (R.O.D.'s) of the bands were measured using Image-J.

Immunoprecipitation

Total protein content was obtained from hippocampus in a mild lysis buffer, containing 50μM β gly-P; 15μM EGTA; 10μM EDTA; 10μM DTT; 2x Na_3VO_4; 50μM NaF; and one complete proteinase inhibitor pill (Roche). Lysis was performed and lysates were centrifuged at 15,000 rpm for 30 minutes at 4°C. The supernatant was retained. Protein-G beads, normal mouse IgG and PBS were added and mixed for one hour. Samples were pre-cleared using this IgG/beads complex and washed

with PBS and lysis buffer. All samples underwent the pre-clearing stage with the IgG-beads complex. Protein-G beads and M2 anti-flag antibody were coupled, centrifuged and washed with PBS and lysis buffer. Normal mouse IgG was also coupled to protein-G beads and served as a negative control. Immunoprecipitation was performed by diluting the lysates to a total volume of 500 μL with PBS. The protein-G beads/M2 anti-flag antibody complex was added to each positive sample. The protein-G beads/IgG complex was added to each negative control. After precipitation for at least 4 hours at 4°C, samples were washed with PBS and washing buffer, containing 300μg β-gly-P; 2.25μM EGTA; 1μM EDTA; 1μM DTT; 0.25 Na_3VO_4. The precipitate was stored at -80 °C until use. Subsequently, precipitated samples were western blotted as described above. δC-DCLK-short was detected using a previously described antibody recognizing the SP-rich N-terminus of DCLK-short (Schenk, Engels et al. 2007); (Vreugdenhil, Datson et al. 1999); (Boekhoorn, Sarabdjitsingh et al. 2008).

δC-DCLK-short Kinase Activity.

In addition to western blot analysis, precipitated proteins were examined for kinase activity. For kinase assays, a reaction mixture was prepared containing 25mM HEPES, 10mM $MgCl_2$, 50μM ATP, 4μCi γ-[^{32}P] ATP, and 5mM EGTA. 20μM autocamtide-2 (Bachem, Bubendorf, Switzerland) was used as a substrate for δC-DCLK-short. 10μl of the reaction mixture was incubated with 10μl of sample for 10 minutes at 23˚C. 20μl H_3PO_4 was added to stop the reaction and centrifugation took place at 10,000 rpm for 1 minute. 20μl of the top layer of each sample was spotted onto P81 filters (Whatman) and air dried for 10 minutes. The filters were washed 3x5 minutes with 1 ml 75mM H_3PO_4 and 1x with 1 ml 70% acetone. Filters were air-dried and disintegrations per minute (DPM) were counted for 2 minutes per sample using a β-counter. Data were analyzed using ANOVA and Tukey's tests, accepting significance at $p<0.05$.

Elevated Plus Maze: Apparatus

The Elevated Plus Maze (EPM) was made of grey PVC and consisted of four arms, forming a 'plus' shape, elevated by four extendible metal rods, 100 cm above ground level. The rods were supporting the ends of the four arms. The arms were 28 cm long and 6 cm in width. Two opposite arms were surrounded by transparent Plexiglas walls of 15 cm in height (the 'closed' arms); the other two opposite arms did not have surrounding walls (the 'open' arms). The center, where the four arms connect, consisted of a square area measuring 6x6 cm. Behavioral parameters were analyzed by digitizing film material using a computer program. To this end, a camera hanging above the maze filmed the EPM during the entire experiment. Spatial cues were present in the testing room (i.e. posters on the walls). Light intensity was set at 80 Lux and 20 dB background noise was present in the testing room. The setup was cleaned with water after each mouse as described previously (Brinks, van der Mark et al. 2007).

Elevated Plus Maze: Behavioral Analyses

WT and δC-DCLK-short mice (n=10 per group) were placed onto the center of the EPM, head facing one of the closed arms. During the following 5 minutes animals were allowed to walk around and explore the maze freely. Mice were tested in alternating fashion and their genotypes were unknown to the observer during assessment of behaviour. Behavioral parameters were adopted from Brinks et al., 2007 (Brinks, van der Mark et al. 2007). 1) The number of defecations was counted as a measure for arousal. 2) Locomotor activity and location of the mouse were analyzed using Ethovision (Noldus Information Technology, Wageningen, The Netherlands). 3) The following behavioral parameters were scored using a semi-automatic scoring system Observer Psion Workabout and analyzed using the matching software program Observer (Noldus Information Technology, Wageningen, The Netherlands): number of rearings, rim dips, and stretched attends, duration of grooming, sitting and walking. 4) Time spent in different zones of the maze (center, proximal and distal parts of the arms), frequency of entries into open and closed arms, total distance moved and general velocity were determined. Entries into a specific zone were only counted when all four paws of the mouse

were positioned across the pre-defined boundary from one area to the next. Independent t-tests were used to determine significant differences in behavior between WT and δC-DCLK-short mice. For all tests a probability level of 5% was used as the minimal criterion of significance.

Results

Mapping of δC-DCLK-short mRNA expression in transgenic mice

A transgenic line with over-expression of a modified DCLK gene transcript was generated by injection of a Thy-1.2 promoter driven expression construct into fertilized eggs and subsequent *in utero* implantation. The transgenic line, designated δC-DCLK-short, is fertile with normal frequency and size of litters and stably transmits the transgene to the offspring. A schematic representation of the expression construct is shown (Figure 1). The δC-DCLK-short line was characterized by high expression of the transgenic transcript throughout the neuronal cell layers of the hippocampus and subiculum. Within the hippocampus expression levels were found in the dentate gyrus (DG) and cornu ammonis (CA; Figure 1E-G). Expression of δC-DCLK-short was not limited to the hippocampal formation as it was also found in other limbic areas, such as the amygdala and thalamic nuclei (Figure 1D-G). In addition, transcripts were found in several cortical area's, including the infralimbic, prelimbic, cingulate and periform cortices (Figure 1A-E). Specific expression of δC-DCLK-short mRNA was also found in other regions (Table 1). Typically, no expression of δC-DCLK-short mRNA was observed in the corpus callosum, caudate putamen and nucleus accumbens (Figure 1A-D). In addition, areas lacking δC-DCLK-short mRNA expression are indicated (Table 1). In WT control subjects using the same *in situ* hybridization probe, δC-DCLK-short expression was undetectable (data not shown). The control probe with several substituted nucleotides yielded a signal that did not exceed background levels (Figure 1H). It is also of importance to note that we did not observe any gross changes in anatomy in δC-DCLK-short mouse brain (data not shown). Next, we aimed to verify the presence of transgenic protein in δC-DCLK-short brain.

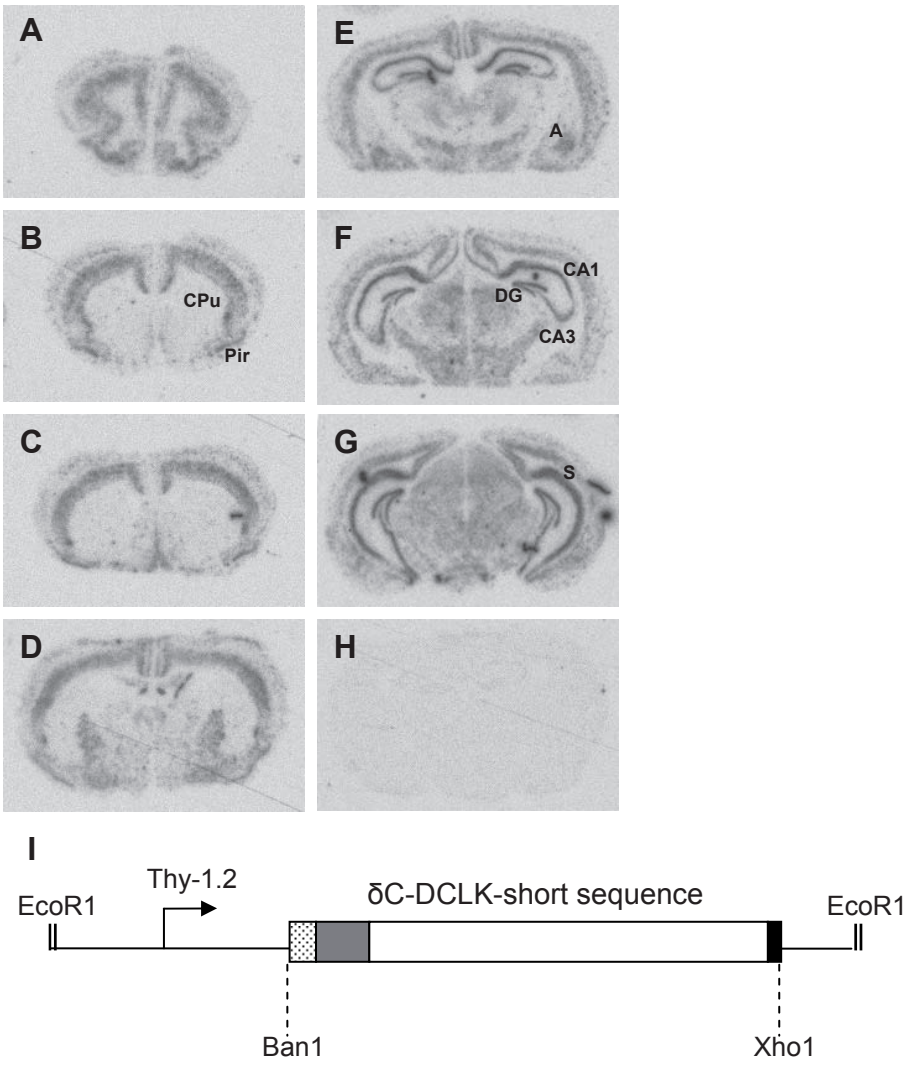

Figure 1. δC-DCLK-short mRNA expression in transgenic mouse brain. A coronal overview from rostral (A) to caudal (G) of δC-DCLK-short expression is shown. Note the high expression in several cortical areas (A-E), hippocampus (E-G), amygdaloid and thalamic nuclei (D-G), which is characteristic for the Thy-1.2 promotor (Vidal, Morris et al. 1990). (H) shows the autoradiogram of a section hybridized with the mismatch control. Several relevant brain regions are indicated: A (Amygdala), CA1/CA3 (Cornu Ammonis 1/3), DG (Dentate Gyrus), S (Subiculum), Pir (Piriform Cortex) and CPu (Caudate Putamen). The Thy-1.2 driven pTSC expression construct is shown in (I). From left to right: EcoR1 restriction site, Thy-1.2 promotor with DCLK gene kozac sequence, Ban1 restriction site, domain unique for DCLK-short and CARP (black dots), SP-rich domain (grey), catalytic domain excluding auto-inhibitory C-terminus (white), FLAG-tag (black), Xho1 and EcoR1 restriction sites.

Brain area	DCLK Expression
Infralimbic cortex	+
Prelimbic cortex	+
Cingulate cortex	+
Piriform cortex	+
Anterior olfactory nucleus	+
Anterior commisure, anterior	-
Forceps minor corpus callosum	-
Dorsal peduncular cortex	-
Caudate putamen	-
External capsule	-
Corpus callosum	-
Olfactory tubercule	+
Accumbens nucleus, core	-
Accumbens nucleus, shell	-
Claustrum	+
Dorsal endopiriform nucleus	+
Medial septal nucleus	+
Nucleus vertical limb diagonal band	+
Lateral septal nucleus, dorsal	+
Paraventricular thalamic nucleus, anterior	+
Lateral globus pallidus	+
Ventral pallidum	+
Cerebral cortex	+
Hippocampus	+
Cornu ammonis 1	+
Cornu ammonis 2	+
Cornu ammonis 3	+
Dentate gyrus	+
Subiculum	+
Amygdaloid nucleus	+
Anterior cortical amygdaloid nucleus	+
Basolateral amygdaloid nucleus, anterior	+
Basolateral amygdaloid nucleus, posterior	+
Basomedial amygdaloid nucleus, posterior	+
Zona incerta	+
Parafascicular thalamic nucleus	+
Subparafascicular thalamic nucleus	+
Cerebral peduncle, basal	-
Posterior thalamic nuclear group	+
Posterior hypothalamic area	+
Ventral tegmental area	+
Red nucleus, parvocellular	+
Anterior pretectal nucleus	+

Table 1. Overview of δC-DCLK-short mRNA expression in transgenic δC-DCLK-short mouse brain. Expression of δC-DCLK-short mRNA (+) or lack thereof (-) is indicated for several brain structures. Localization is based on the mouse brain atlas by Franklin and Paxinos (Franklin 1997). Semi-quantification is based on the in situ hybridization images shown in Figure 1.

δC-DCLK-short Protein Expression in the Brain

We dissected and prepared protein extracts from cerebral cortex and the hippocampus from WT and δC-DCLK-short animals. Western blotting was performed using an anti-FLAG antibody to demonstrate expression of the transgenic protein. As expected, WT controls did not show any bands corresponding to a FLAG-tagged protein. In contrast, a predicted band around 45 kD demonstrated the presence of the FLAG-tagged protein in δC-DCLK-short animals (Figure 2A). By using an antibody recognizing both endogenous DCLK-short and δC-DCLK-short (Schenk, Engels et al. 2007); (Vreugdenhil, Datson et al. 1999) we demonstrate the presence of endogenous DCLK-short next to δC-DCLK-short (Figure 2B). To visualize the magnitude of the over-expression, we have also measured relative optical densities of the bands corresponding to the endogenous DCLK-short and δC-DCLK-short in WT and TG hippocampus (Figure 2C). Expression of endogenous DCLK-short protein is comparable between WT and TG animals. Moreover, expression of δC-DCLK-short protein in TG hippocampus is comparable to the expression of endogenous DCLK-short, while the signal of δC-DCLK-short protein in WT hippocampus was equal to background levels. Thus, expression of the δC-DCLK-short protein was confirmed, with the highest level of expression in the transgenic hippocampus.

δC-DCLK-short Kinase Activity in Hippocampus

Since C-terminal truncation of DCLK-short has previously been associated with an increase in kinase activity (Engels, Schouten et al. 2004), we here focused on determining δC-DCLK-short's activity. Using the anti-FLAG antibody coupled to protein-G beads we aimed to immunoprecipitate the transgenic protein from hippocampal lysates and monitor its functionality. Immunoprecipitation with normal mouse IgG coupled to beads served as a control. Figure 2D shows a single band detected by western blotting using a rabbit antibody recognizing the SP-rich domain of DCLK (Schenk, Engels et al. 2007); (Vreugdenhil, Datson et al. 1999). This band corresponded to the 45 kD size of the δC-DCLK-short protein. No protein was precipitated from WT hippocampus or when using the non-specific normal mouse IgG/beads complex. Subsequently, we used the obtained immunoprecipitates and tested their phophorylation activity towards a substrate

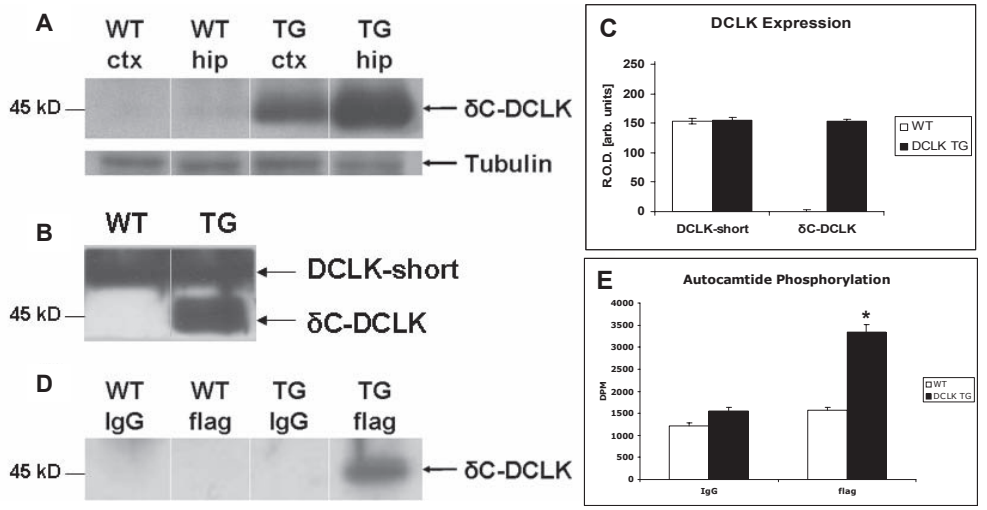

*Figure 2. δC-DCLK-short protein expression in cortex and hippocampus (A). Western blotting was performed using an anti-FLAG antibody to specifically recognize the transgenic protein. A specific band at 45 kD demonstrates the presence of the FLAG-tagged δC-DCLK-short protein in TG animals. WT controls do not show any bands corresponding to a FLAG-tagged protein. Tubulin was used as a loading control. A single band is detected by western blotting in WT hippocampus using the rabbit antibody recognizing both endogenous and transgenic DCLK-short (Schenk, Engels et al. 2007); (Vreugdenhil, Datson et al. 1999). Importantly, two specific bands are detected in TG hippocampus; the upper band is endogenous DCLK-short, whilst the lower band corresponds to the 45 kD band of δC-DCLK-short (B). By measuring relative optical densities (R.O.D.'s) of the bands, hippocampal DCLK-short protein expression was compared to visualize the magnitude of over-expression (C). Expression of endogenous DCLK-short is comparable between genotypes. In addition, expression of δC-DCLK-short protein is equal to background levels in WT hippocampus, while it is comparable to endogenous levels of DCLK-short in TG hippocampus. Immunoprecipitation of δC-DCLK-short protein from hippocampus (D). Immunoprecipitation was performed using an anti-FLAG antibody. A single band is detected by western blotting using the rabbit antibody recognizing the CARP-domain of DCLK (Schenk, Engels et al. 2007); (Vreugdenhil, Datson et al. 1999). This band corresponds to the 45 kD size of the δC-DCLK-short protein. Immunoprecipitation with normal mouse IgG coupled to beads served as a control. No protein was precipitated from WT hippocampus or by using the non-specific normal mouse IgG/beads complex. Kinase activity of δC-DCLK-short (E). Incorporation of radioactively labelled phosphate groups was investigated by adding autocamtide-2 as a substrate to immunoprecipitates (Engels, Schouten et al. 2004). A significantly increased number of disintegrations per minute (DPM) is found δC-DCLK-short mouse hippocampus using the anti-FLAG antibody. Samples obtained from WT hippocampus did not show any differences in kinase activity, regardless of the used antibody/beads complex. *p<0.01, significantly different from wild-type and IgG immunoprecipitates. TG=transgenic, WT=wild-type, ctx=cortex, hip=hippocampus, R.O.D.=relative optical density, DPM=disintegrations per minute.*

that is highly specific for DCLK-short: autocamtide-2 (Engels, Schouten et al. 2004). Incorporation of radioactively labelled phosphate groups was investigated by performing kinase assays. Samples obtained from WT hippocampus did not show any differences in kinase activity, regardless of the used antibody/beads complex. However, immunoprecipitation using the antibody against the FLAG-tag resulted in an increased number of disintegrations per minute (DPM) of more than 200% the background signal in samples obtained from δC-DCLK-short mice (Figure 2E). This suggests that δC-DCLK-short protein, precipitated from the transgenic hippocampus, displays kinase activity and is in fact functional.

Behavioral Characterization of δC-DCLK-short Mice

For analysis purposes the Elevated plus Maze (EPM) was divided into zones, comprising a center area, two open and two closed arms and the proximal and distal parts of these arms (Figure 3A). The distances moved in each zone were determined. When examining the way mice spent their time by dividing it into either walking or sitting, no significant differences were found between genotypes. In addition, general velocity and the total distance moved were comparable between groups (Table 2), indicating locomotor activity was not significantly different between groups. Also, the number of entries into each zone was comparable between δC-DCLK-short and WT mice. However, significant differences between groups were found as δC-DCLK-short mice moved less distance in the center area (Figure 3B, $p=0.014$; $t(17)=2.726$) and the distal portions of the open arms compared to WT mice (Figure 3B, $p=0.0019$; $t(17)=3.659$). The amount of time spent in each of the defined zones was also calculated as a percentage of the total time. δC-DCLK-short mice spent less time in the outer part of the open arms compared to control mice (Figure 3C; $p=0.0048$; $t(17)=3.241$). To clearly visualize the differential open versus closed arm activity between genotypes, open/closed arm ratios are indicated for time spent and distance moved (Figure 3D).

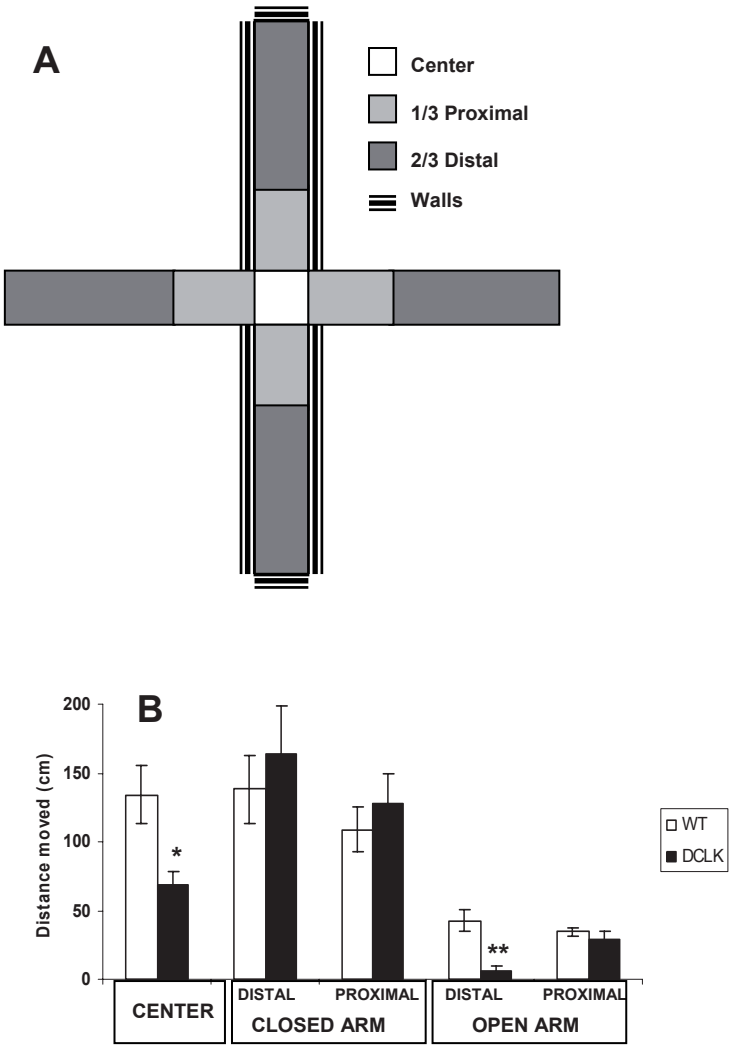

Figure 3. Spatiotemporal parameters of the EPM. The EPM is divided into five zones (A), comprising a center area, two open arms and two closed arms and the subdivision in the proximal and distal parts of these arms. The distances moved (cm) in each zone are shown (B). Significant differences between δC-DCLK-short mice and wild-type controls are indicated (p<0.05; **p<0.01) and include a reduction in distance moved in the center area and the distal portions of the open arms.*

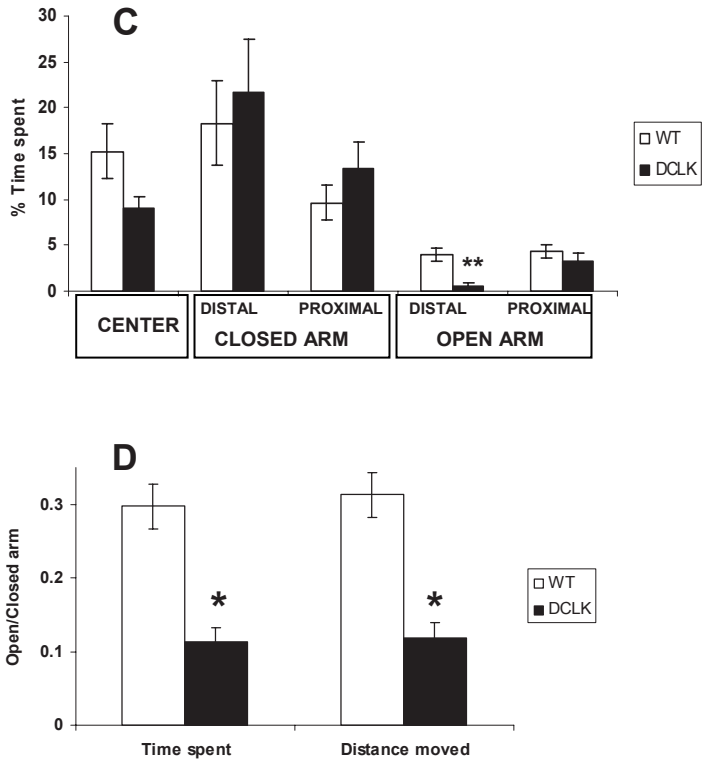

*Figure 3. (Continued) The percentage of time spent in each of the defined zones is also shown (C). δC-DCLK-short mice spend less time in the outer part of the open arms compared to wild-type mice (** p<0.01). To clearly visualize differential open versus closed arm activity between genotypes, ratios are indicated for time spent and distance moved (D; * p<0.05). Also see Table 2 for an overview of additional behaviors.*

In addition to these spatiotemporal parameters, other relevant behaviors were scored and analyzed (Table 2). The number of rearings and stretched attends was not significantly different between the groups. However, δC-DCLK-short mice looked over the rim of the maze significantly less than wild-type mice, i.e. the number of rim dips was significantly lower in transgenic mice ($p=0.021$; $t(16)=2.561$). The duration of grooming was also differed between groups; δC-DCLK-short mice groomed themselves significantly more than WT mice ($p=0.008$; $t(17)=3.000$). In addition, the number of defecations was significantly decreased ($p=0.0004$; $t(17)=4.441$).

	Wild-type	δC-DCLK-short
Defecation (#)	2.89 ± 0.51	***0.40 ± 0.22 *****
Rearings (#)	5.1 ± 1.37	7.67 ± 0.95
Stretched attends (#)	8.13 ± 0.84	7.10 ± 1.20
Rim dips (#)	8.11 ± 1.3	***3.89 ± 0.87 ****
Grooming (s)	6.69 ± 1.57	***14.53 ± 1.98** *
Closed arm entries (#)	3.60 ± 0.48	3.05 ± 0.49
Open arm entries (#)	2.30 ± 0.34	1.15 ± 0.24
Sitting (s)	112.49 ± 23.17	98.9 ± 17.10
Walking (s)	181.85 ± 23.46	187.91 ± 17.39
Total distance moved (cm)	803.27 ± 104.01	777.10 ± 63.14
Velocity (cm/s)	2.66 ± 0.34	2.60 ± 0.21

*Table 2. EPM behavioral parameters. #=number, s=seconds, cm=centimetres. * p<0.05; ** p<0.001. Values are represented as mean ± SEM.*

Discussion

We here report on the successful generation of a novel transgenic mouse line with brain specific over-expression of a constitutively active form of the DCLK gene transcript DCLK-short. Previously, the function of DCX and DCLK family members has been studied during embryogenesis and DCX and DCLK are known to have overlapping, yet distinct functions during cortical and hippocampal development (Koizumi, Tanaka et al. 2006); (Deuel, Liu et al. 2006); (Tanaka, Koizumi et al. 2006); (Tuy, Saillour et al. 2008); (Friocourt, Liu et al. 2007). DCLK-short is a splice product that lacks the DCX domains and is expressed only in mature neurons in the adult brain. The function of DCLK-short remains elusive. Here, we have characterized expression of δC-DCLK-short at the mRNA and protein levels in the hippocampus of adult mice and demonstrate that the transgenic kinase is active. In addition, we have subjected δC-DCLK-short mice to the EPM test and provide evidence that these animals show significantly more anxiety-like behaviors than wild-type controls.

δC-DCLK-short expression in transgenic mouse brain

By *in situ* hybridization using a probe specific for δC-DCLK-short we examined the regional distribution of δC-DCLK-short mRNA in the brains of transgenic mice. δC-DCLK-short transcripts were localized in several brain regions, with high expression levels in the hippocampus, cortical areas and amygdaloid nuclei. This is in accordance with previous observations regarding the Thy-1.2 promotor (Vidal, Morris et al. 1990). The δC-DCLK-short mRNA expression pattern reported here overlaps with previously observed endogenous DCLK-short mRNA localization under physiological conditions, which includes expression in the hippocampus, amygdala, piriform cortex and zona incerta (Vreugdenhil, Engels et al. 2001); (Engels, Schouten et al. 2004), whilst its expression is absent in the nucleus accumbens (Engels, Schouten et al. 2004). Based on this mapping study of DCLK-short mRNA in the brain, we dissected the hippocampus and cerebral cortex to investigate expression of the transgenic protein. We found high levels of transgenic δC-DCLK-short protein in cortical and hippocampal lysates, with the highest expression in the hippocampal formation. In addition, immunoprecipitated δC-

DCLK-short protein was capable of recognizing and phosphorylating autocamtide-2, indicating that the transgenic protein is active. Extracellular signal-regulated kinase (Erk)1/2 dependent phosphorylation of the SP-rich domain of DCLK-short is crucial for its activation and downstream effects like neuritogenesis *in vitro* (Dijkmans, van Hooijdonk et al. 2009); (Dijkmans, van Hooijdonk et al.). Previously, ablation of the C-terminal domain has been demonstrated to increase DCLK-short kinase activity in cell lines (Engels, Schouten et al. 2004). Thus, activation of DCLK-short is important in generating its putative downstream effects. Since the δC-DCLK-short expression pattern partly overlaps with endogenous DCLK-short localization in the brain and the δC-DCLK-short protein is functional, these transgenic mice may serve as a basis to study the functions of the activated state of DCLK-short *in vivo* in the adult mouse brain.

δC-DCLK-short mice show more anxiety-like behaviors

Previously, we have shown that hippocampal over-expression of δC-DCLK-short in mice leads to highly significant regulation of numerous gene sets involved in GABA-related processes (Pedotti, t Hoen et al. 2008). The regulation of GABAergic neurotransmission, together with the observation that the transgenic δC-DCLK-short protein is produced and functional within the hippocampal formation, has led us to investigate whether behavioral differences may occur in this mouse line. Since aberrances in GABAergic neurotransmission are associated with anxiety-related behaviors (Crestani, Lorez et al. 1999); (Rudolph, Crestani et al. 1999); (Low, Crestani et al. 2000); (Atack 2005); (Rudolph and Mohler 2006); (Whiting 2006); (Mohler, Fritschy et al. 2002) and the hippocampus is crucially and directly involved in the mediation of unconditioned anxiety reactions in rodents (Engin and Treit 2007), we subjected δC-DCLK-short mice and littermate controls to the EPM paradigm. The EPM is a validated model for the assessment of anxiety-related behaviors in rodents (Hogg 1996); (Rodgers and Dalvi 1997); (Rodgers, Cao et al. 1997). We demonstrate significant differences between genotypes for several behaviors that are relevant for anxiety, including a decrease in distance moved and time spent in the distal parts of the open arms, a decreased number of rim dips and an increase in the time spent grooming in δC-DCLK-short mice. Importantly, these

behavioral alterations were observed in the absence of changes to rearing, number of arm entries, sitting, walking, total distance moved or average velocity, strongly suggesting that the observed changes to anxiety-related measures were behaviorally selective.

The reduction in distance moved and time spent in the open areas is indicative of a more anxious behavioral phenotype. This is underscored by a lower number of rim dips in δC-DCLK-short mice (Brinks, van der Mark et al. 2007). The duration of grooming is known to increase both during stress and comfort, although the pattern of grooming changes as well as the frequency and the average duration per grooming session (Kalueff and Tuohimaa 2004). Given the aforementioned significantly different anxiety-related parameters pointing towards a more anxious phenotype, the increased levels of grooming are probably a form of displacement behavior as a consequence of discomfort. This suggests that transgenic δC-DCLK-short mice have a different coping style in response to unconditioned anxiety. It is of importance to mention that we observed a lack of change in stretched attend postures between genotypes, suggesting risk assessment is equal between genotypes, although C57/BL6 mice have been shown to display very low basal levels of risk assessment behavior (Augustsson and Meyerson 2004). Also, given the other measures that point to a more anxious behavioral phenotype, defecation is typically expected to increase (Flint, Corley et al. 1995); in contrast, we observed decreased defecation. Although this observation is associated with a less anxious behavioral phenotype, the explorative component of the anxiety-related behavior is clearly affected in δC-DCLK-short mice. Altogether, we conclude that δC-DCLK-short mice are more anxious when tested in the EPM paradigm. However, in order to firmly establish the observed anxious phenotype of these mice, future research should be aimed at performing additional tests for anxiety.

Possible mechanisms underlying increased anxiety in δC-DCLK-short mice

Given the molecular background of these transgenic animals, the observed effects at the behavioral level may well be a consequence of deregulations of specific genes and biological pathways. In this respect GABAergic signalling is of importance. GABA is the main inhibitory neurotransmitter in the brain. Several

classes of GABA interneurons are present in the brain and they mainly function in feedforward and feedback circuits. Enhancement of GABAergic neuronal inhibition underlies the therapeutic action of the classical benzodiazepine drugs in the treatment of anxiety disorders and epilepsy (Rudolph and Mohler 2006). The anxiolytic effect is achieved through an agonistic effect at the level of $GABA_A$ receptors, which are highly expressed in the limbic system (Low, Crestani et al. 2000); (Mohler, Fritschy et al. 2002); (Dawson, Collinson et al. 2005); (Papadopoulos, Korte et al. 2007). $GABA_A$ receptors are membrane proteins consisting of several classes of subunits and are sensitive to benzodiazepines depending on their subunit composition (Low, Crestani et al. 2000); (Atack 2005); (Rudolph and Mohler 2006); (Mohler, Fritschy et al. 2005). Pharmacological findings suggest that agonists selective for α2- and/or α3-containing $GABA_A$ receptors provide anxiolysis (Rudolph and Mohler 2006). Interestingly, from the behavioral assessment of α2 sub-unit point-mutated mice in the EPM, it seems that the anxiolytic effect of benzodiazepines is mediated by α2-containing $GABA_A$ receptors (Low, Crestani et al. 2000); (Atack 2005). Importantly, in δC-DCLK-short mice $GABA_A$ receptor subunit α2 is significantly down-regulated (Pedotti, t Hoen et al. 2008). Interestingly, excitatory rather than inhibitory GABAergic signaling is known to control neurogenesis, neuronal migration and differentiation (Tozuka, Fukuda et al. 2005); (Ge, Pradhan et al. 2007), functions that are also ascribed to the DCLK gene. Endogenous DCLK-short expression starts postnatally from day 6 onward, a time point that is marked by a switch from excitatory to inhibitory GABAergic neurotransmission (Ganguly, Schinder et al. 2001); (Ben-Ari 2002). The Thy-1.2 promotor drives expression of transgenic δC-DCLK-short from postnatal day 6 onward (Vidal, Morris et al. 1990), opening up the possibility to investigate the interplay between GABAergic signaling and the active state of DCLK-short during this crucial time of postnatal neuronal development. Our findings suggest that down regulation of the $GABA_A$ receptor α2 subunit in δC-DCLK-short mice contributes to their more anxious behavioral phenotype.

Recently performed large scale screens in δC-DCLK-short mice also revealed differential gene expression in biological pathways involved in calmodulin-dependent protein kinase activity (t Hoen, Ariyurek et al. 2008). Interestingly, functional studies show that DCLK-short has CaMK-like properties (Engels,

Schouten et al. 2004); (Shang, Kwon et al. 2003). Since regulation of CaMKs has previously been implicated in cognition and fear-related behaviors (Izquierdo and Medina 1997); (Kouzu, Moriya et al. 2000); (Ahi, Radulovic et al. 2004); (Blaeser, Sanders et al. 2006); (Papaleo, Crawley et al. 2008), the deregulation of calmodulin-dependent protein kinase signalling may play a role in addition to GABAergic neurotransmission in determining the behavioral phenotype of δC-DCLK-short mice.

Involvement of the hippocampus in δC-DCLK-short mice

The hippocampus is crucially and directly involved in the mediation of unconditioned anxiety reactions in rodents, such as those tested in the EPM paradigm (Engin and Treit 2007). More specifically, when subdividing the hippocampus along the septotemporal axis into dorsal and ventral regions, the ventral hippocampus is commonly associated with fear and anxiety processes, whereas the dorsal hippocampus is believed to play a preferential role in the temporal and contextual aspects of learning and memory formation (Moser and Moser 1998); (Bannerman, Rawlins et al. 2004); (McHugh, Deacon et al. 2004); (Trivedi and Coover 2004); (Esclassan, Coutureau et al. 2009). Whether the expression of δC-DCLK-short mRNA in the amygdala, a brain structure well-known for its implications in fear and anxiety-related emotions, contributes significantly to the increased anxiety in δC-DCLK-short mice can not be concluded from these experiments. However, amygdaloid lesions do not influence exploration of the open arms of the elevated plus-maze (Treit, Pesold et al. 1993); (Sommer, Moller et al. 2001) and in fact many of the anxiolytic effects of benzodiazepine drugs are not mediated by the amygdala (Treit, Pesold et al. 1993). The behavioral dissociations inferred from lesion studies suggest that limbic structures such as the amygdala and hippocampus exert parallel but distinct control over different fear reactions (Treit and Menard 1997). Thus, based on our findings, the molecular basis for the observed behavioral phenotype of δC-DCLK-short mice probably resides in the ventral hippocampus.

Conclusion

The DCLK gene has been implicated in cortical and hippocampal development (Vreugdenhil, Kolk et al. 2007); (Koizumi, Tanaka et al. 2006); (Deuel, Liu et al. 2006); (Tanaka, Koizumi et al. 2006); (Tuy, Saillour et al. 2008). These functions of the DCLK gene, however, are mostly attributed to the microtubule binding domains (Lin, Gleeson et al. 2000); (Sapir, Horesh et al. 2000). Since DCLK-short is expressed in mature neurons in the adult brain and is lacking the microtubule binding domain, the DCLK gene may encode proteins with functions beyond the embryonic stage. Our novel δC-DCLK-short mouse line opens up new avenues in elucidating the function of the activated state of the DCLK gene splice variant DCLK-short, which is prominently expressed during adulthood. Based on our current findings, GABAergic neurotransmission appears of crucial importance in attributing potential functions to DCLK-short.

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Chapter 6

General discussion outline

General discussion

1. Introduction

The functions of DCX-domain containing DCLK gene splice variants are best described during neuronal development. In contrast, the potential roles of splice variants that do not contain the microtubule binding DCX-domains and function during adulthood, DCLK-short and CARP, remain elusive. Therefore, we have analysed CARP expression in the adult brain deprived from the input of glucocorticoids. In addition, two transgenic mouse lines were generated with over-expression of CARP and a line with over-expression of a constitutively active form of DCLK-short in the brain and examined the consequences of neuronal over-expression of these DCLK gene transcripts at different functional levels.

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 endogenous CARP in apoptosis in the DG following corticosteroid depletion by adrenalectomy (ADX) and indeed show that CARP is associated with apoptosis. **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. We demonstrate that network excitability is decreased in high-CARP animals and suggest that this may be a consequence of deregulation of specific genes that are important for neuronal viability and network functioning. 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. We show that consolidation of contextual fear memories is strengthened in these mice. **Chapter 5** is dedicated to characterization of the third transgenic line; 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). Previously, a large scale genomics screen comprising multiple microarray platforms and deep sequencing has been performed, demonstrating differential expression of several relevant biological pathways in the

hippocampus (Pedotti et al., 2008; ‘t Hoen et al., 2008). Using the elevated plus maze test we show that δC-DCLK-short mice are more anxious and propose this is likely a consequence of deregulation of GABA-related gene expression. Next, we will discuss potential functions and involvements of the DCLK gene splice variants CARP and DCLK-short in the adult brain, based on previous research and the work performed here.

2. Functions of the DCLK gene

The observations described in this thesis indicate that several biological processes are connected to CARP and DCLK-short function: The DCLK gene may have a function in neuronal apoptosis; network excitability in the hippocampus is affected by over-expression of DCLK gene transcripts and hippocampus-dependent behavioural phenotypes were established for all of the investigated transgenic lines. These observations are tied together by the occurrence of several important biological and physiological phenomena. These phenomena include 1) neuronal viability, 2) neuronal transmission, 3) neurotrophic factor signalling and 4) regulation of cytoskeleton dynamics. These processes may also affect each other, making their interactions and the potential roles of CARP and DCLK-short therein a rather complicated matter. To clarify this, the next sections will discuss the roles of CARP and DCLK in relation to these four processes separately (Figure 1).

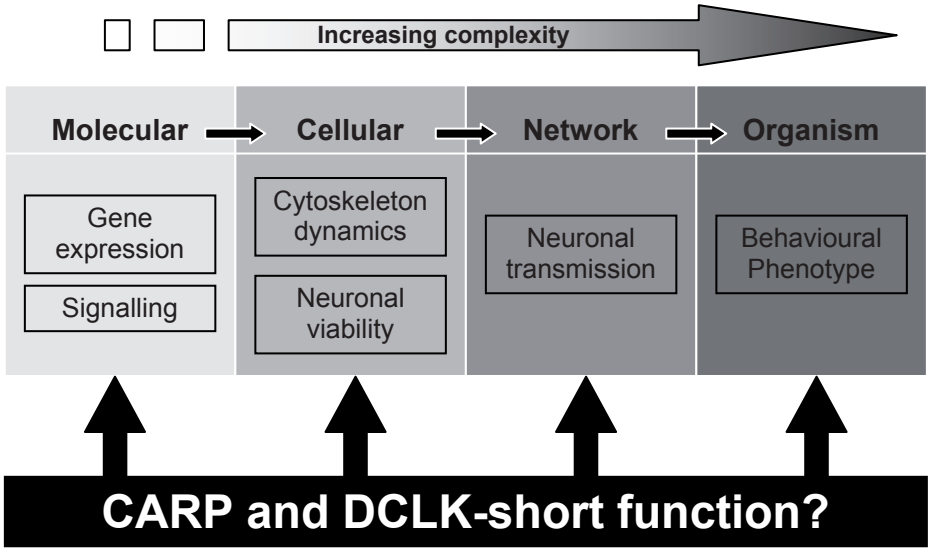

Figure 1. Different functional levels ranging from molecular to the entire organism have increasing complexity. CARP and DCLK-short may play a role on each of these functional levels and affect crucial biological phenomena such as neuronal viability, neuronal transmission, signalling and regulation of cytoskeleton dynamics. Note that these processes may also influence each other and provide feedback circuits.

2.1 The DCLK gene and neuronal viability

In Chapter 2 we demonstrated a significant and positive correlation between CARP mRNA expression and ADX-induced apoptosis in DG granule cells *in vivo*. Healthy cells did not express CARP, suggesting that CARP has a function in an apoptotic context. In line with this hypothesis is the finding that CARP over-expression in healthy neuroblastoma cells did not influence the number of apoptotic cells. In contrast, CARP over-expression in neuroblastoma cells challenged by serum deprivation exacerbated apoptosis *in vitro*. Importantly, CARP expression in the brains of high-CARP mice did not affect the number of apoptotic or necrotic cells in the hippocampus during basal conditions *in vivo*. This underscores once more that the potential pro-apoptotic properties of CARP are evident in a specific cellular context only: that of an apoptotic cell.

DCLK-short may also have a function related to the apoptotic process. Interestingly, DCLK-short is homologous to CaMK family members. For instance, DCLK-short and CaMK-IV are both able to phosphorylate myelin basic protein (MBP; Lin et al., 2000; Silverman et al., 1999) and syntide and autocamtide, two highly specific CaMK substrates (Engels et al., 2004; Ohmae et al., 2006) and they have a similar distribution pattern in the brain (Engels et al., 1999). In neuroblastoma cells, CaMK-IV is cleaved during apoptosis challenged with pro-apoptotic agents, suggesting this is a survival signal which needs to be shut off during the execution of a cell death program (McGinnis et al., 1998). Both DCLK-short and CaMK-IV contain an extended S/P-rich N-terminal domain of approximately 60 amino acids, which is lacking in other CaMKs. The observation that calpain, a protease with a wider variety of substrates than caspases, is capable of breaking down DCLK proteins also suggests a role for the DCLK gene in the apoptotic process (McGinnis et al., 1998; Burgess and Reiner, 2001). In line with a role in cell survival, a study in the zebrafish demonstrates that knockdown of DCLK (zDCLK) induces a significant increase of apoptotic cells in the central nervous system (Shimomura et al., 2007). The expression profiles and molecular similarities between DCLK-short and CaMK-IV suggest involvement, albeit probably through different mechanisms, of DCLK-short in processes similar to those in which CaMK-IV is implicated, namely apoptosis and cell survival.

In fact, DCLK-short has been reported as a substrate for caspases *in vitro* and *in vivo* and DCLK-short cleavage by caspases is necessary for apoptosis to proceed (Kruidering et al., 2001). Cleavage of DCLK-short by caspases generates a N-terminal fragment that overlaps largely with CARP and has similar pro-apoptotic properties as CARP when studied during serum-deprived apoptosis (Kruidering et al., 2001). This suggests that CARP and the N-terminal part of DCLK-short share a common motif that is responsible for the observed pro-apoptotic properties (Figure 2). However, full-length DCLK-short does not exacerbate apoptosis, suggesting release of the S/P-rich CARP-like N-terminal domain of DCLK-short is essential for unveiling its role during apoptosis. Altogether CARP appears to have pro-apoptotic properties exclusively in neuronal cells that have already received the proper signals to die through programmed cell death, while CARP by itself is incapable of

inducing apoptosis. DCLK-short on the other hand must be broken down before apoptosis can take place and as such rather appears to prevent the apoptotic process.

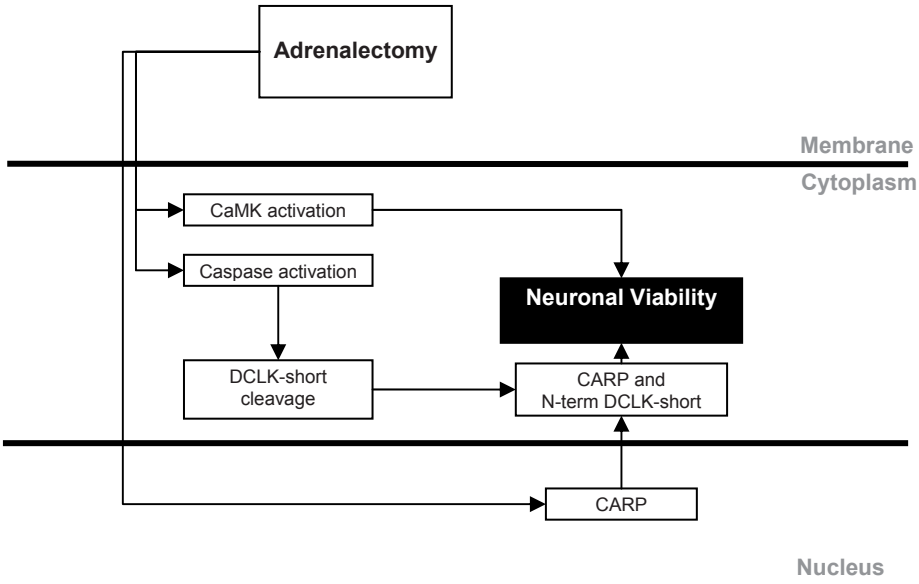

Figure 2. Schematic representation of events involving DCLK-short and CARP, ultimately leading to an effect on neuronal viability through apoptosis. For details see section 2.1.

The precise mechanism by which CARP actually affects neuronal viability is presently unknown. Currently the CARP peptide has not been identified, although the predicted CARP structure lacks microtubule binding domains and the kinase domain. This suggests that the S/P-rich domain, through protein-protein interactions, is likely crucially involved. Based on this assumption I hypothesize that CARP interferes with neurotrophic factor signalling and cytoskeleton dynamics. Importantly, neurons, and the networks they support, 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. neurotrophic factor signal transduction and cytoskeletal architecture. These cellular alterations ultimately lead to changes in network functioning and behavioural output. This plasticity of neurons is crucial for brain function during basal conditions, but even

more so for the individual's ability to make adaptive changes when faced with a challenge. Thus, both neurotrophic factor signalling and the regulation of cytoskeleton dynamics have important consequences for neuronal transmission. The potential the roles of CARP and DCLK-short therein will be discussed in the next section.

2.2 The DCLK gene and neuronal transmission

2.2.1 CARP and neuronal transmission

In chapter 3 we showed that electrically evoked neuronal transmission is highly affected in high-CARP mice. More specifically, fEPSPs recorded in the stratum radiatum of the CA1 area are much more pronounced in high-CARP mice, whilst the same stimulation paradigm elicits a PS in the stratum pyramidale that is equal in amplitude to those of control mice. This decreased excitability suggests that the efficiency of glutamatergic transmission in the hippocampal network is diminished. At the same time both wild-type and high-CARP mice displayed comparable levels of facilitation and inhibition. Facilitation of synaptic vesicle release is a result of build-up of free pre-synaptic Ca^{2+} during repetitive arrivals of action potentials (Zucker, 1999), whereas feedback inhibition is defined as GABAergic interneurons receiving input from CA1 pyramidal cells that were previously activated by the Schaffer collaterals (Kandel et al., 1961). This suggests that neither Ca^{2+} levels nor GABA-mediated feedback inhibition behave differently in the CARP over-expressing hippocampus. So high-CARP mice seem to have an electrophysiological phenotype, but how is this phenotype best explained and what does this imply for hippocampal functioning?

Interestingly, robust CARP induction has previously been associated with BDNF-induced LTP. Amongst the genes induced by BDNF-LTP is neuronal activity-regulated pentraxin (Narp; Wibrand et al., 2006), a gene that was first identified in a screen for seizure-induced genes (Tsui et al., 1996). Narp is enriched at excitatory synapses where it associates with α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)-type glutamate receptors. In addition, Narp causes clustering of AMPA receptors and is crucially involved in synaptogenesis of excitatory synapses

(O'Brien et al., 1999, 2002; Xu et al., 2003). The concomitant induction of CARP and Narp implies a role for CARP in similar processes and may partly explain the observed decreased network excitability. Additionally, high levels of endogenous CARP were, like Narp, first discovered in the hippocampus after kainic acid administration, a well-known model for the induction of epileptic seizures (Hellier et al., 1998; Vreugdenhil et al., 1999). Seizures are typically associated with elevated Ca^{2+} levels and increased glutamate mediated excitatory neuronal transmission (Murphy and Miller, 1988; Vreugdenhil and Wadman, 1994). Moreover, recorded evoked fEPSPs in the CA1 from kainic acid treated rat hippocampal slices are characterized by increased neuronal excitability 2-4 weeks post kainic acid treatment (Franck and Schwartzkroin, 1985; Ashwood and Wheal, 1986). In contrast, neuronal excitability that is observed in high-CARP hippocampal slices is robustly reduced. This decreased excitability may indicate that the high levels of CARP found during kainate-induced seizures serve as a negative feedback on aberrant excitatory neuronal transmission, albeit without affecting Ca^{2+} or GABA metabolism.

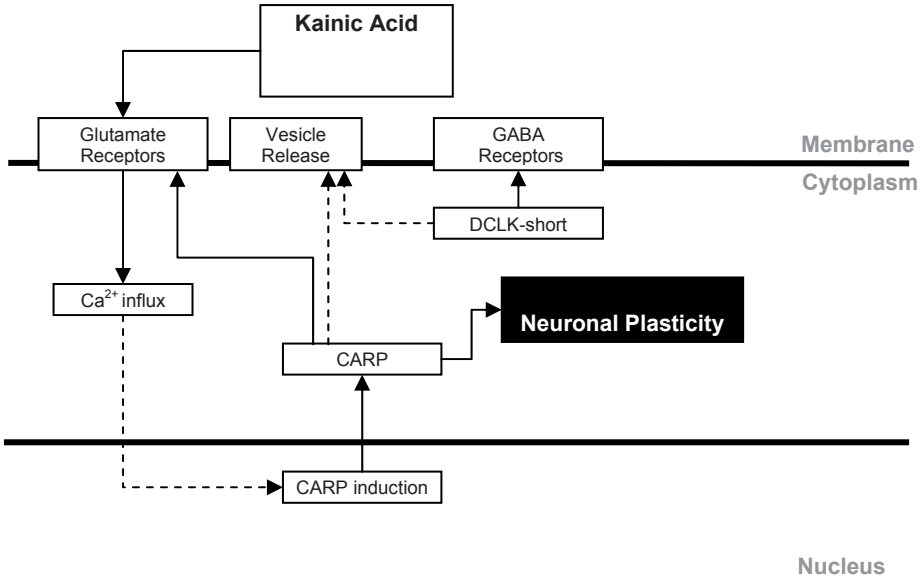

Figure 3. Kainic acid stimulation result in Ca^{2+} influx and induction of CARP expression. Based on electrophysiological data, CARP may affect glutamate receptor composition, affinity and vesicle release. DCLK-short over-expression leads to deregulation of GABA-mediated neurotransmission and may also affect vesicle release through regulation of SNARE proteins. For details see section 2.2.

Note that this leaves open the possibility that CARP up-regulation is triggered by increased Ca^{2+} influx, while CARP itself does not influence Ca^{2+} metabolism. Importantly, Ca^{2+}-regulated signalling pathways play a crucial role as mediators of synaptic plasticity, including LTP, and subsequent behavioural responses (Blaeser et al., 2006). CARPs potential position down-stream of Ca^{2+} influx implies that it may have a role in regulating excitatory synaptic plasticity (Figure 3).

Thus, CARP may return hippocampal excitation to homeostatic levels, thereby counteracting the increased excitatory transmission during seizures. How this is achieved precisely remains elusive, but given the expected involvement of a shift in glutamate release it seems plausible that the decreased excitability is a consequence of differences in vesicle release, pre- or post-synaptic glutamate receptor expression levels, glutamate receptor affinity and subunit composition or a combination of these factors, this however, cannot be concluded from the currently available data.

2.2.2. DCLK-short and neuronal transmission

The evidence linking DCLK-short function to neuronal transmission is mostly inferred from microarray studies revealing regulation of biological pathways that are relevant for neuronal transmission (Pedotti et al., 2008; 't Hoen et al., 2008) Deregulation of gene sets in the δC-DCLK-short mouse hippocampus representing biological functions is found across several microarray platforms and includes neurotransmitter receptor activity, neurotransmitter binding and $GABA_A$ receptor activity (Pedotti et al., 2008). Importantly, the anxiolytic effect of classic benzodiazepines is mediated by α2-containing $GABA_A$ receptors (Löw et al., 2000; Atack, 2005; Möhler et al., 2005; Rudolph and Möhler, 2006). Strikingly, in δC-DCLK-short mice, $GABA_A$ receptor subunit α2 is highly significantly down-regulated (Pedotti et al., 2008), potentially posing a neurotransmission-dependent basis for the more anxious behavioural phenotype of these mice.

Other significantly regulated pathways include, calmodulin-dependent protein kinase activity, microtubule associated vesicle transport and SNARE binding ('t

Hoen et al., 2008). Interestingly, the DCLK gene has been implicated in microtubule binding (Lin et al., 2000; Sapir et al., 2000) and has more recently been shown to play a role in microtubule guided transport of SNARE-protein containing synaptic vesicles (Deuel et al., 2006). Importantly, SNAREs play a role in vesicle fusion and synaptic vesicle release (Fasshauer et al., 1998; Sutton et al., 1998). This suggests synaptic vesicle transport may be disturbed in δC-DCLK-short mice, thereby possibly affecting neurotransmitter release. GABAergic neurotransmission is critically involved in anxiety disorders (Rudolph and Möhler, 2006) and epilepsy (Brook-Kayal et al., 2009; Thompson, 2009), as such the DCLK gene potentially (dis)functions in neuronal transmission during these diseases. Thus, based on our current findings, deregulation of GABAergic neurotransmission, potentially through altered synaptic vesicle release and disturbances in receptor subunit composition, appears of crucial importance in attributing functions to DCLK-short (Figure 3).

2.3 The DCLK gene and neurotrophic factor signalling

Neuronal viability and synaptic transmission are highly dependent on the availability and actions of neurotrophic factors in the brain; therefore they will be discussed in relation to the DCLK-gene. Several lines of evidence link neurotrophic factor signalling to DCLK gene function. The sequence GKSPSPSPTSPGSLR of CARP is predicted to interact with Growth receptor bound 2 (Grb2), an intracellular adapter protein containing a SH3-SH2-SH3 configuration that has been implicated in Tropomyosin related kinase (Trk)-receptor signalling (Tari and Lopez-Berestein, 2001). In fact, we confirmed interaction of CARP with Grb2 *in vitro* (Schenk et al., 2007). Grb2 is part of the Trk-receptor complex where it tranduces neurotropic factor binding to activation of the Ras GTP-exchange factor Son of sevenless (Sos) (Egan et al., 1993) and becomes internalized by transport vesicles (Howe and Mobley, 2005), ultimately leading to activation of the Ras-extracellular signal-regulated kinase (ERK1/2) kinase cascade (Katz and McCormick, 1997). In turn, ERK1/2 signalling is of importance for nerve growth factor (NGF)-induced neuronal differentiation and is also involved, as are DCX and DCLK-long, in the development of cortical and hippocampal neurons (Barnabe-Heider and Miller, 2003; Thomson

et al., 2007; Yan et al., 2007). Importantly, the viability of neuronal cells is linked to a change in the availability of neurotrophic factors and their receptors (Nichols et al., 2005; Schaaf et al., 2000). Thus, through interaction with Grb2, CARP may interfere with this cascade, thereby affecting the viability and morphology of neurons.

As described above, CARP is highly induced by kainate-induced seizures (Vreugdenhil et al., 1999) and this is also a time during which several neurotropic factors are induced (Gall and Lauterborn, 1992; Lindvall et al., 1994). Interestingly, another neurotrophic factor, BDNF, is a major regulator of LTP induced by high-frequency stimulation of excitatory synapses (Bramham and Messaoudi, 2005). BDNF is released from glutamatergic synapses following high-frequency stimulation and performs its intracellular signaling via TrkB receptors (Nawa and Takei, 2001; Balkowiec and Katz, 2002). In fact, BDNF stimulation itself induces LTP and this phenomenon is accompanied by a robust increase of CARP expression (Wibrand et al., 2006). Strikingly, mice with brain specific over-expression of TrkC, which is capable of binding both Neurotrophin (NT)-3 and BDNF, develop a phenotype that is similar to the electrophysiological phenotype of high-CARP mice: highly increased evoked extracellular fEPSPs at the CA3/CA1 synapse (Schenk et al., 2010; Sahún et al, 2007). Collectively, these data underscore CARPs potential to intervene with neurotrophic factor signalling.

Evidence for involvement in neurotrophic factor signalling not only exists for CARP, but also for DCLK-short. Recently, nerve growth factor (NGF) stimulation of Neuroscreen-1 PC12 cells has been shown to highly induce the expression of DCLK-short (Dijkmans et al., 2008; 2009). Moreover, activation of DCLK-short *in vitro* depends critically on ERK1/2 activation, leading to phosphorylation of a specific serine residue situated within DCLK-short's S/P-rich domain (Dijkmans et al., 2009). Given the abundant examples of processes in which concomitant activity and regulation of DCLK gene expression and neurotrophic factors is evident, we propose that CARP and DCLK-short exert their functions, at least partly, through interference with neurotrophic signal transduction and that interaction with Grb2 is highly probable (Figure 4).

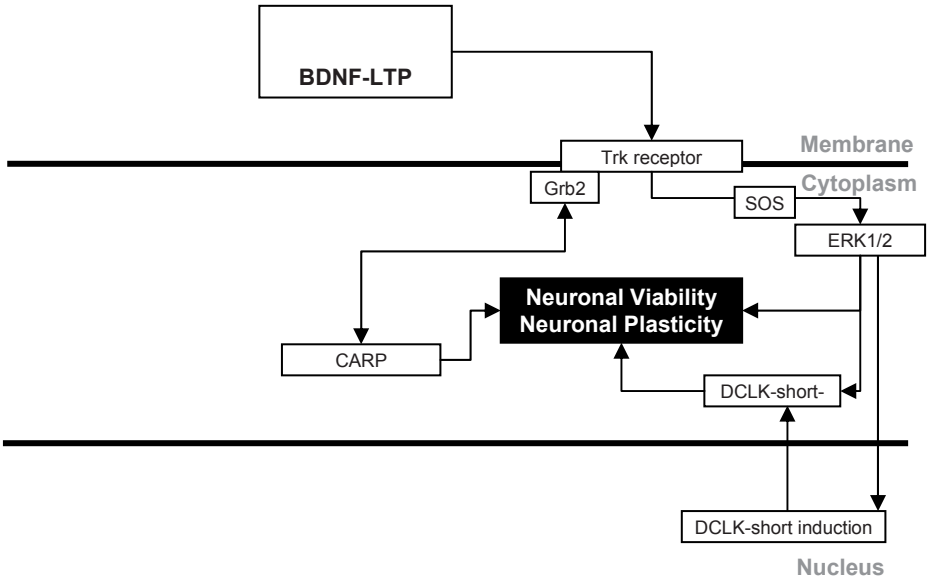

Figure 4. Cartoon showing DCLK-short and CARP in relation to neurotrophic factor signaling. For details see section 2.3.

2.4 The DCLK gene and cytoskeleton dynamics

Since both neuronal viability and synaptic transmission are highly dependent on the architecture of the microtubule and actin cytoskeletons, their dynamics will be discussed in more detail here. Importantly, cytoskeleton dynamics largely determine the morphological constraints within which cells and synapses may function physiologically. Members of the doublecortin family are known to induce microtubule polymerization and stabilization and to interact with F-actin (Francis et al., 1999; Lin et al., 2000; Edelman et al., 2005; Coquelle et al., 2006; Vreugdenhil et al., 2007). CARP is highly homologous to the S/P-rich C-terminal parts of both DCX and DCLK, a domain that is implicated in protein interactions (Friocourt et al., 2001; Moores et al., 2004). As mentioned above, CARP was found to interact with Grb2, most likely through SH-3 domain binding. As Grb2 has been implicated in regulation of the actin cytoskeleton (Buday et al., 2002), this then suggests CARP indirectly plays a role in regulation of the actin cytoskeleton.

Previously, the DCX domain containing isoforms of the DCLK gene have been held responsible for the role of DCLK in dynamic rearrangements of the microtubule cytoskeleton. In fact, DCL plays a pivotal role in determining neuronal fate by regulating mitotic spindle positioning and stability (Shu et al., 2006; Vreugdenhil et al., 2007; Boekhoorn et al., 2008). Moreover, DCL over-expression results in robust formation of microtubule bundles. (Vreugdenhil et al., 2007; Fitzsimons et al., 2008). Using a tubulin polymerization assay I demonstrated that CARP increases DCL-induced polymerization of tubulin in a dose-dependent fashion (Schenk et al., 2007). Through a similar mechanism, CARP may exert its pro-apoptotic properties through arrest of the microtubule skeleton.

CARP up-regulation has been found following kainate-induced seizures (Vreugdenhil et al., 1999), BDNF-LTP (Wibrand et al., 2006), D1-receptor stimulation (Berke et al., 1998; Glavan et al., 2002), and in apoptotic cells (Schenk et al., 2007). A common feature of these processes is the requirement of cytoskeleton rearrangements underlying the plasticity of specific neuronal circuits (Reviewed in: Cai and Sheng, 2009; Conde and Cáceres, 2009; Kueh and Mitchison, 2009). Another observation that makes a potential role for the CARP domain in cytoskeleton regulation possible is that several of the co-regulated genes that accompany the induction of CARP following BDNF-LTP have known functions in excitatory synaptogenesis and axon guidance (Wibrand et al., 2006). More specifically, BDNF-LTP is associated with the induction and dendritic transport of transcripts of the immediate early gene activity-regulated cytoskeleton associated protein Arc (Lyford et al., 1995; Ying et al., 2002). BDNF activates a process of synaptic consolidation that dependents critically on Arc transcription and translation (Bramham and Messaoudi, 2005). During this process formation of stable LTP is associated with insertion of glutamate receptors at cell membranes and structural remodeling of spines (Geinisman, 2000; Harris et al., 2003). Importantly, these changes are closely related to regulation of actin dynamics (Okamoto et al., 2004; Zito et al., 2004; Oertner and Matus, 2005). These findings, in combination with the structural overlap between DCX and DCLK on the one hand and CARP on the other hand, raises the possibility that CARP affects cytoskeleton stability. Thus by affecting cytoskeleton stability, CARP may, together

with other players, influence neuronal viability.

Evidence suggests that DCLK-short also interacts with the cytoskeleton as it co-localizes with F-actin in growth cones of neurites and may regulate neuritogenesis in a phophorylation dependent fashion (Dijkmans et al., 2009). CaMKs are highly homologous to DCLK-short and from this perspective it is of interest to note that CaMKI and CaMKII also play regulatory roles in actin dynamics, thereby affecting neuronal morphology (Suizu et al., 2002; Okamoto et al., 2007; Penzes et al., 2008). CaMKI regulates actin rearrangements in growth cones and affects neurite outgrowth of granule cells in the hippocampus (Wayman et al., 2004; 2008), whereas CaMKII interacts with F-actin directly and regulates synaptic strength and dendritic morphology in hippocampal neurons (Shen and Meyer, 1999; Okamoto et al., 2007). Interestingly, putative substrates of CaMKs that are implicated in actin dynamics include guanine exchange factors (Gefs; Wayman et al., 2004). Deregulation of Rap1Gef was observed in the hippocampus of high-CARP mice, underscoring the possible involvement of the CARP domain in cytoskeleton rearrangements and its regulatory role in processes that are similar to those where CaMKs are of importance.

Our observations and those of others strongly suggest a role for the DCLK gene in cytoskeleton dynamics, potentially through interference with neurotrophic factor signalling. Altogether, this suggests that members of the DCLK gene family lacking DCX domains, i.e. CARP and DCLK-short, also have relevant biological functions related to the state of the cytoskeleton.

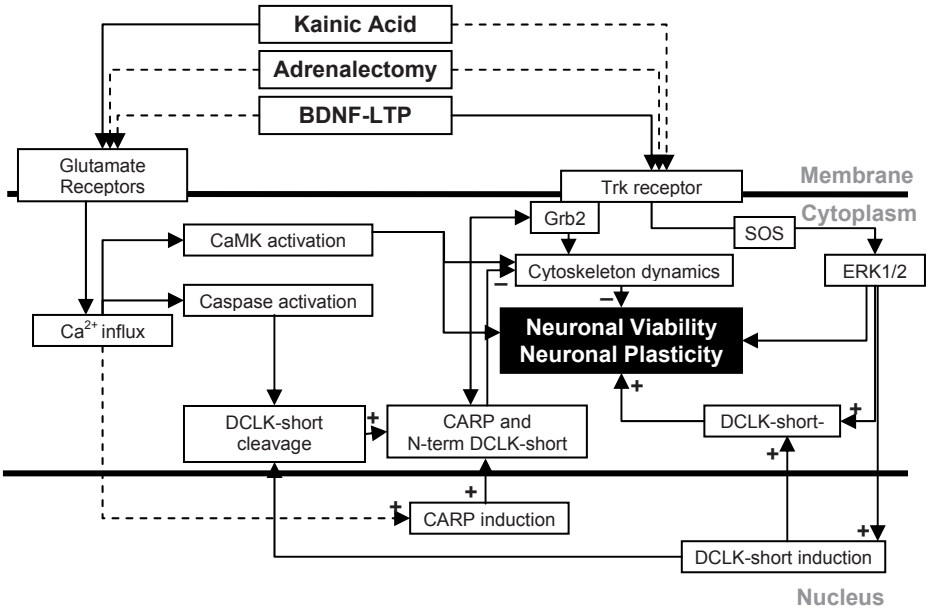

Figure 5. Neuronal viability, neuronal plasticity, neurotrophic factor signalling and regulation of cytoskeleton dynamics are processes which are of crucial importance for DCLK-gene function. Induction of DCLK-short and CARP and their reinforcing (+) and inhibiting effects (-) on neuronal viability and plasticity are indicated.

3. DCLK-short and CARP: A balancing act?

In section 2 of this chapter we discussed several possible processes in which CARP and DCLK-short may play a role. All of the described processes are interdependent, making the meaning of the results highly dependent on the chosen angle of interpretation (for a compilation see Figure 5).

Firstly, while CARP has *pro-apoptotic* properties under challenging conditions, DCLK-short may act as a *survival factor*. Secondly, effects on neuronal transmission seem evident in high-CARP mice, where *excitatory* glutamatergic signal transduction is most likely affected (Chapter 3). In contrast, regulation of genes involved in *inhibitory* GABAergic transmission, and subsequent behavioural adaptations, were observed in δC-DCLK-short mice (Chapter 5). Thirdly, we demonstrated that DCL-induced polymerization of tubulin is enhanced by the presence of CARP and propose that *arrest* of the cytoskeleton underlies the pro-apoptotic effect of CARP, while DCLK-short plays a role in actin cytoskeleton

dynamics during neuritogenesis (Dijkmans et al., 2009). Thus, CARP and DCLK-short may have partly opposing, yet complementary functions. In this light, regulation of DCLK gene expression is of importance.

The transcriptional regulation of the DCLK gene is incompletely understood, although previous results point towards involvement of cyclic AMP mediated transcriptional activity (Silverman et al., 1999). DCLK-short and CaMK-IV are highly homologous and both are able to phosphorylate cAMP responsive element binding protein (CREB) and activate CREB-dependent transcription. A major mechanism by which Ca^{2+} regulates neuronal functions involves activation of CaMK cascades (Soderling, 1999; Corcoran and Means 2001). Increases in cytosolic Ca^{2+} levels activate Ca^{2+}-dependent kinases including CaMKs. Effectively, Ca^{2+} influx can stimulate CaMKs to phosphorylate transcription factors such as CREB. However, Ca^{2+}/calmodulin, an activator of CaMKs, is not required for DCLK-short activation (Silverman et al., 1999; Shang et al., 2003). More recent data, however, suggest that DCLK-short is unable to significantly phosphorylate CREB and rather inhibits CREB-dependent gene expression by a mechanism that bypasses CREB through phosphorylation of transducer of regulated CREB activity (TORC2; Ohmae et al., 2006).

Interestingly, DCLK is also identified in a screen for corticosteroid-responsive genes in the hippocampus of ADX adult rats (Vreugdenhil et al., 1996a, 1996b). ADX is a well established model for the induction of apoptosis of granule cells in the rat DG (Sloviter et al., 1993; Hu et al., 1997). In addition, the DCLK gene has been found in large scale screening experiments as being down-regulated by chronic stress (Alfonso et al., 2004) and by high glucocorticoid-replacement in ADX animals (Datson et al., 2001). Although the DCLK gene has been identified as corticosteroid-responsive (Vreugdenhil et al., 1996a, 1996b; Datson et al., 2001; Alfonso et al., 2004), there is no evidence supporting the idea that the DLCK gene is a direct target of activated glucocorticoid receptors through transactivation. The GR and the MR are expressed in particular in the hippocampus (de Kloet et al., 1998; Nichols et al., 2001). The putative promoter sequences of the DCLK gene do not reveal any classical glucocorticoid-response elements (GREs) or GRE halfsites (Vreugdenhil et al., 2001). However, the possibility that the DCLK gene is affected

by high amounts of glucocorticoids, through a GR-mediated transrepression mechanism is still open. Several lines of evidence indicate that this is indeed the case: firstly, CARP and DCLK-short were identified in a transrepression model (Vreugdenhil et al., 1996a, 1999) and a model involving excitatory amino acids (Nedivi et al., 1993). Secondly, the putative promoter of the DCLK gene contains multiple AP-1, CREB and NFκ-B sites which are known to be subject of transrepression by activated GRs but not MRs (Pearce and Yamamoto, 1993). Thirdly, two studies show down-regulation of DCLK after administration of high levels of corticosteroids or after chronic stress (Datson et al., 2001; Alfonso et al., 2004). Thus, the DCLK gene is probably not a primary target of adrenal glucocorticoids by transactivation, but its expression may be affected by interaction of ligand bound GRs with AP-1, CREB or NFκ-B via a transrepression mechanism. Based on the findings described in this thesis CARP and DCLK-short may indeed have partly opposing, yet complementary functions, influencing the balance of several crucial biological processes, such as neuronal viability and neuronal transmission.

4. Behavioural phenotypes of transgenic mice

4.1 high-CARP; low-CARP mice

Ultimately, neuronal viability and neuronal transmission determine functioning of the neuronal network and amount to a certain behavioural output. In both transgenic CARP strains we monitored fear conditioned behaviour. Both transgenic lines were equally capable as controls of learning the association between the conditioned stimulus (CS) and an unconditioned stimulus (UCS). The memory of the association of these stimuli in the context in which the animals had previously had the learning experience was strengthened in both high- and low-CARP mice, as freezing behaviour was higher on the 2 days of retention testing in these mice.

The conditioned response can be extinguished by repeatedly presenting the CS in the absence of the UCS (Pavlov, 1927). Therefore, a gradual decrease in freezing behaviour is expected, as animals will learn that the previous association of cues and UCS is no longer relevant, as is the freezing and scanning behaviour in

response to the context and UCS. This fear extinction does not eliminate fear memories, but is a form of new learning that results in the inhibition of the conditioned fear behaviour (Bouton et al., 2006). The hippocampus is critically involved in this type of contextual memory (Corcoran et al., 2005; Ji and Maren, 2007; 2008). In fact we observed levels of extinction that were not significantly different between genotypes, even though total freezing in the context was significantly elevated in both transgenic lines. This suggests a causal relationship between strengthened consolidation and the observed increased freezing behaviour in CARP mice. Consequently, the inhibition of the conditioned fear behaviour during fear extinction appears impaired, since the memory trace of the association between CS and UCS is stored more solidly. Long-term memories are laid down when the short-term modifications in synaptic plasticity are consolidated by more permanent changes brought about through gene transcription and protein synthesis.

How this strengthened consolidation is achieved exactly in high-CARP mice remains unclear, however, changes of synaptic plasticity, such as LTP, in the hippocampus are well-described as potential molecular routes to learning and memory formation (Bliss and Collingridge, 1993; Lynch, 2004). NMDA subtype glutamate receptors at excitatory neuronal synapses play a key role in this form of synaptic plasticity and the ERK1/2 pathway is an essential component of NMDA receptor signal transduction controlling the plasticity underlying memory processes and refinement of synaptic connections (Thiels and Klann, 2006; Yashiro and Philpot, 2008). An increase in cytoplasmic Ca^{2+} in neurons plays an important role in the regulation of LTP in the hippocampus (Krapivinsky et al., 2003; MacDonald et al., 2006; Gruart et al., 2006). From this perspective, the alterations in neuronal network excitability in high-CARP mice and the potential interference of CARP with ERK signalling cascades may explain the solidified consolidation as a consequence of hippocampal CARP over-expression. Taken together, these data suggest that the expression of CARP in the hippocampus may indeed strengthen the consolidation of fear related memories.

4.1.1 CARP Mice as a Potential Model for investigating Epilepsy

The question arises whether these novel transgenic mouse lines are a useful model for studying processes in which CARP induction is implicated. Strikingly, in the adult wild-type brain CARP expression is very low under physiological conditions. The induction of CARP has been associated with kainate-induced seizures, BDNF-LTP and ADX in hippocampal neurons (Vreugdenhil et al., 1999; Wibrand et al., 2006; Schenk et al., 2007) and with administration of D1-agonists in striatal neurons (Berke et al.,1998; Glavan et al., 2002). The highest expression of CARP in transgenic mice is found in the limbic system, most notably the hippocampus, and is absent in the striatum. In addition, the distribution is not characterized by the granular distribution pattern that is typically associated with the expression of CARP following ADX in the DG (Schenk et al., 2007).

Interestingly, epileptic seizures are typically associated with elevated Ca^{2+} levels, highly increased glutamate mediated excitatory neuronal transmission (Murphy and Miller, 1988; Vreugdenhil and Wadman, 1994) and increased BDNF expression (Brooks-Kayal et al., 2009). Moreover, recorded evoked fEPSPs in the CA1 from kainic acid treated hippocampal slices are characterized by increased neuronal excitability. This *increased* neuronal excitability is explained partly because of increased fEPSPs and also by a decreased inhibition in the CA1 area that contributes to an increase in paired-pulse excitability (Reviewed in Wheal, 1989). This decrease in inhibition may be caused by a selective loss of GABAergic, Ca^{2+}-binding proteins expressing interneurons and a decreased expression of $GABA_A$ receptors (Best et al., 1993; 1994; Tsunashima et al., 1997).

High-CARP mice showed *decreased* neuronal excitability, indicating an effect that is opposite to the effects caused by kainic acid treatment. Up-regulation of CARP during kainate-induced seizures may therefore serve in providing a negative feedback on glutamatergic excitatory synaptic transmission. Thus functionally, the increase of CARP may enable the hippocampal network to adjust to the aberrant glutamate release during and after seizures and enable the hippocampal network to adapt to physiological processes that coincide with seizure behaviour. Interestingly, contextual fear conditioning has been shown to be impaired following

kainic acid administration (Yin et al., 2002; Kemppainen et al., 2006), i.e. animals show *decreased* freezing in context. We here describe *increased* contextual freezing, suggesting CARP up-regulation may be a molecular response that is beneficial for hippocampus dependent learning of conditioned fear. This underscores that the robust induction of CARP following kainic acid may form a protective mechanism to counteract the malignant effects of these processes on hippocampal functioning.

Given these observations high- and low-CARP mice might be of relevance in examining the function of this DCLK gene splice variant in the 'epileptic hippocampus'. As memory formation has been shown to be impaired following kainic acid administration (Yin et al., 2002; Kemppainen et al., 2006), it seems relevant to investigate if CARP up-regulation is indeed a molecular mechanism that is beneficial for hippocampus dependent learning in response to epileptic activity. In addition, a better characterization of the electrophysiological phenotype of high-CARP mice may aid in gaining a better understanding of the function of CARP during synaptic plasticity-related processes requiring cytoskeletal rearrangements, including LTP.

4.2 δC-DCLK-short mice

We subjected δC-DCLK-mice to the Elvated Plus Maze (EPM) and demonstrated that these mice have a more anxious behavioural phenotype. The EPM is suited for studying unconditioned fear behaviours, in contrast to fear conditioning, where the animal is trained to learn a specific association between stimuli and the context of events. We proposed that regulation of genes involved in GABAergic neurotransmission and CaMK-regulated activity contribute to the observed phenotype. As outlined above, DCLK-short has CaMK-like properties (Shang et al., 2003; Engels et al., 2004). Since regulation of CaMKs, in particular CaMKII, has previously been implicated in cognition and fear-related behaviours (Ahi et al., 2004; Blaeser et al., 2006), the deregulation of protein kinase signalling may play a role in addition to GABAergic neurotransmission in determining the behavioural phenotype of δC-DCLK-short mice. Interestingly, adult neurogenesis is also under

GABAergic control and reduced $GABA_A$ receptor function in immature neurons of the adult brain may serve as a common molecular substrate for deficits in adult neurogenesis and behaviors related to anxiety (Earnheart et al., 2007). Given these observations and the finding that anxiety-related behaviours are more pronounced in δC-DCLK-short mice, examination of potential alleviation of the more anxious behavioural phenotype of these mice by treatment with anxiolytic drugs may form an interesting approach in determining the contribution of DCLK-short to processes underlying anxiety, such as inhibitory GABAergic transmission and synaptic plasticity.

5. Future prospects

We reported on the generation of three novel transgenic mouse lines: two with different, yet overlapping expression patterns of CARP and one with over-expression of δC-DCLK-short, a constitutively active form of DCLK-short in the brain. The experiments performed in an attempt to characterize functions of CARP and DCLK-short span different relevant functional levels, including genetics, protein interactions, neuronal morphology, electrophysiology and behaviour. Several relevant findings were described, some of which raised more questions about putative DCLK gene functions. The newly generated transgenic lines provide an excellent opportunity to explore previously uncharted territory in investigating the DCLK gene. Based on the work performed in this thesis several potentially fruitful lines of future research come to mind.

Firstly, since CARP expression is associated with apoptotic cells in the DG of ADX rats (Chapter 2), examination of the prevalence of apoptotic cells in the hippocampus of high-CARP and low-CARP mice in response to an apoptotic stimulus, such as corticosteroid depletion (Sloviter et al., 1993; Karssen et al., 2005) or middle cerebral artery occlusion (Belayev et al., 1999), may help to reveal whether CARP has pro-apoptotic properties in an *in vivo* setting. Investigating the number of apoptotic cells in CARP mice and controls after such a challenge will unveil if this is the case. By subjecting δC-DCLK-short mice to the same paradigm, the nature of DCLK-short as a proposed survival factor may become clearer.

Secondly, We demonstrated highly increased evoked fEPSPs in the CA3/CA1 network of high-CARP mice, but without elevation of subsequent PSAs (Chapter 3). How this characteristic electrophysiological phenotype is realized within the hippocampal formation remains elusive. Measuring mEPSPs in hippocampal slices of high-CARP mice will shed more light on this matter. Given the strong up-regulation of Calretinin (CR) in the DG specifically, it may also be of relevance to study DG/CA3 network functioning in these mice. In addition, whether the increased fEPSPs has consequences for LTP in transgenic high-CARP (and low-CARP) mice remains to be clarified, although the previously described up-regulation of CARP following BDNF-LTP is encouraging in pursuing this train of thought.

Thirdly, we investigated fear conditioning i.e. memories of an aversive event and the association of two stimuli in a specific context (Chapter 4). We showed intensified consolidation in high- and low-CARP mice. It is of interest to also focus on other behavioural tests that are more dependent on spatial navigation, such as the Morris water maze or the circular hole board. The hippocampus is crucially involved in these types of behavioural paradigms and given the potential effects of CARP over-expression on synaptic plasticity this approach may provide additional clues of CARP function within the hippocampal network.

Finally, establishing the roles of CARP and DCLK-short in the epileptic hippocampus is of interest and may reveal new points of application for therapeutic intervention. This was already discussed more thoroughly in section 4.1.1. On a more general note, the transcriptional regulation of the DCLK gene is a relatively underexposed subject. A better understanding of signal transduction pathways and specific transcription factors that contribute to the regulation of DCLK gene expression is of utmost importance. In addition, identification of endogenous substrates of DCLK-short and interaction partners of CARP is crucial in assigning potential functions to these DCLK gene splice products.

Together the observations described in this thesis show that despite the previously established association of DCX domain-containing DCLK gene family members and functions during cortical and hippocampal development the DCLK gene also has functions beyond development. Moreover, although the presence of at least

one DCX domain has long been deemed necessary for attributing any functionality to DCLK gene family members, we here demonstrate that splice variants lacking this domain, i.e. CARP and DCLK-short, also play important roles in cognitive and cellular processes, including neuronal apoptosis and neuronal transmission.

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Chapter 7

Summary

Doublecortin (DCX) and DCX-domain containing Doublecortin-Like Kinase (DCLK) gene splice variants function during embryonic development, where they play a role in microtubule binding. Although a role for the DCLK gene during embryogenesis is clearly established, it encodes multiple, different transcripts, some of which are expressed in the adult brain or in response to neuronal activity. This suggests that the DCLK gene may have additional functions beyond neuronal development. Strikingly, the roles of two DCLK gene products, DCLK-short and CARP, remain largely elusive. 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 brain. This has opened up the possibility to study the effect of over-expression of these DCLK transcripts in the brain during adulthood. To gain more insight in DCLK gene function in the adult brain we aimed to study and describe the phenotypes of these transgenic mice at different functional levels, such as the genetic, network and behavioural level.

Firstly, in **Chapter 2,** we set out to determine the involvement of endogenous CARP in apoptosis in the DG following corticosteroid depletion by adrenalectomy and indeed show that CARP is associated with apoptosis. **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. We demonstrate that network excitability is decreased in high-CARP animals and suggest that this may be a consequence of deregulation of specific genes that play a role in neuronal viability and transmission. In **Chapter 4,** high-CARP mice and a second transgenic line with a more restricted neuronal expression profile, designated low-CARP, are characterized at the behavioural level by fear conditioning. We show that consolidation of contextual fear memories is strengthened in these mice. **Chapter 5** is dedicated to characterization of a third transgenic line; δC-DCLK-short. Mice from this background have brain specific expression of a truncated form of DCLK-short, making this kinase constitutively active. Previously, a large scale genomics screen has been performed, demonstrating differential expression of several relevant biological pathways in the hippocampus. Using the elevated plus maze test we show that δC-DCLK-short

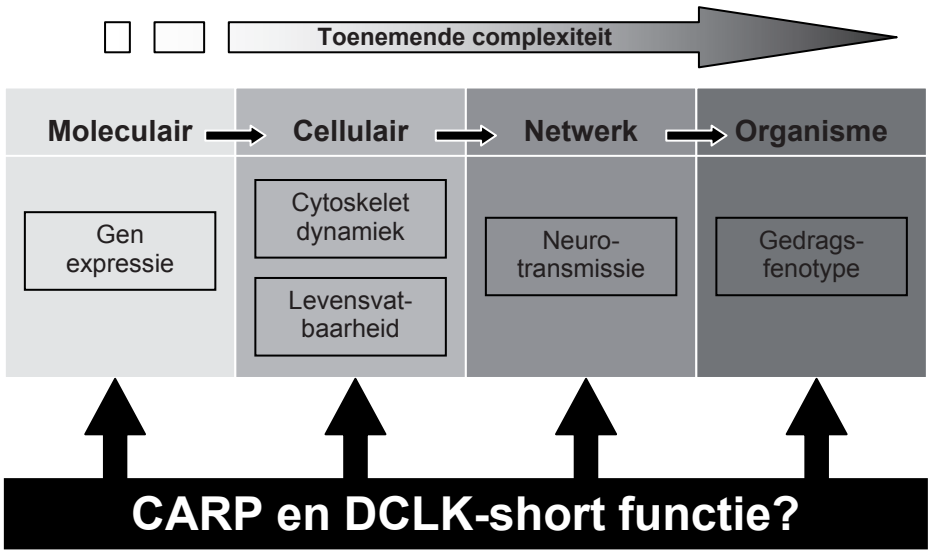
mice are more anxious and propose this is likely a consequence of deregulation of GABA-related gene expression. Our findings are discussed more thoroughly in **Chapter 6**.

In conclusion, we have successfully generated 3 novel transgenic mouse lines with over-expression of DCLK gene products that are prominently expressed during adulthood in response to neurological stimuli. We provide data describing the phenotypes of these transgenic mice at different functional levels, including the genetic, network and behavioural level, and demonstrate that the DCLK gene likely has functions independent of the conserved DCX domains. These transgenic lines may help to further our understanding of DCLK gene function during in the adult hippocampus.

Nederlandse samenvatting

Het brein bestaat uit vele miljarden zenuwcellen en het aantal onderlinge connecties tussen deze cellen is zelfs nog groter. Zenuwcellen, of neuronen, hebben een complexe morfologie die bepaald wordt door de aanwezigheid van eiwitten. Zij vormen het zogenaamde 'cytoskelet' van de cel. Cytoskelet eiwitten zijn dus de bouwstenen die neuronen hun vorm geven. De morfologische kenmerken van neuronen bepalen voor een belangrijk deel de specifieke functies van en de onderlinge connecties tussen deze cellen. De communicatie tussen zenuwcellen wordt ook wel neurotransmissie genoemd. Neuronen en de netwerken die zij samen vormen zijn niet statisch. Ze zijn juist sterk onderhevig aan veranderingen door interne en externe invloeden. Dit zijn bijvoorbeeld veranderingen in het functioneren en de beschikbaarheid van eiwitten, die betrokken zijn bij de architectuur van het cytoskelet en neurotransmissie.

Het aanpassende vermogen van neuronen wordt ook wel plasticiteit genoemd. Deze plasticiteit van het brein is van cruciaal belang, niet alleen gedurende het normale functioneren, maar vooral ook voor het maken van adaptaties wanneer het normale functioneren van neuronen wordt bedreigd. Het kunnen bieden van weerstand aan bijvoorbeeld fysiologische of farmacologische invloeden is bepalend voor de overlevingskansen van neuronen. Neuronale plasticiteit is op deze wijze dus essentieel voor de levensvatbaarheid van neuronen. Het is dus ook mogelijk dat veranderingen in de beschikbaarheid van bepaalde eiwitten verschuivingen teweeg brengen die cruciaal zijn voor de functies en levensvatbaarheid van neuronen en de netwerken die zij vormgeven. Dit kan uiteindelijk leiden tot een veranderde uitgaande informatiestroom vanuit het centrale zenuwstelsel, zoals geheugenvorming, gedrags- en emotionele reacties. Dit proefschrift gaat over de gevolgen van over-expressie van het gen 'Doublecortin-Like Kinase' (DCLK) in het muizenbrein, op het niveau van genetica, neuronale netwerken en gedrag (Figuur 1.).



Figuur 1. De complexiteit neemt toe over verschillende functionele niveaus van moleculaire processen tot het volledige organisme. Mogelijk spelen CARP en DCLK-short een rol bij elk van deze functionele niveaus, waarbij zij invloed hebben op biologische fenomenen zoals levensvatbaarheid van neuronen, neurotransmissie en regulatie van cytoskeletdynamiek. Deze biologische processen monden uiteindelijk uit in een bepaald gedragsfenotype. Overigens kunnen deze processen ook elkaar beïnvloeden en zorgen voor feedback regulatie.

Het DCLK-gen is belangrijk voor neuronale plasticiteit. Het staat erom bekend tijdens de embryonale ontwikkeling van het brein een rol te spelen bij de migratie van neuronen. Een belangrijke functie van het DCLK-eiwit tijdens de ontwikkeling van het zenuwstelsel is het binden aan en het beïnvloeden van de stabiliteit van het cytoskelet. Het is het zogenaamde 'Doublecortin' (DCX) domein van het eiwit dat hierbij van belang is. Het DCLK-gen codeert echter ook voor een aantal eiwitten zonder dit DCX-domein. In dit proefschrift kijken we in meer detail naar de mogelijke functies van deze producten van het DCLK-gen gedurende het volwassen leven. De functies van twee DCLK-genproducten in het bijzonder zullen beschreven worden. Het gaat hier om 'DCLK-short' en 'Calcium/calmodulin dependent protein kinase related peptide' (CARP). Omdat de potentiële functies van deze producten van het DCLK-gen nog niet opgehelderd zijn, hebben we transgene muizen met over-expressie van DCLK-short en CARP in de hersenen

gecreëerd. Op deze manier kunnen we de potentiële functies van deze twee producten van het DCLK-gen gedurende het volwassen stadium bestuderen.

Hoofdstuk 1 vormt de algemene introductie van dit proefschrift. In **hoofdstuk 2** wordt allereerst de rol van CARP zoals dat van nature voorkomt in het brein belicht. We beschrijven de relatie tussen CARP-expressie en geprogrammeerde celdood (apoptose) in de hersenen. We bestuderen ook hoe CARP hierbij een rol speelt via eiwit-eiwit interacties. **Hoofdstuk 3** beschrijft de eerste van de drie transgene muizenstammen die we gecreëerd en onderzocht hebben. De hier beschreven transgene lijn heeft een hoge CARP-expressie in veel verschillende hersengebieden en wordt daarom 'high-CARP' genoemd. We laten bij deze dieren zien dat neurotransmissie veranderd is ten opzichte van normale, zogenaamde 'wild-type' muizen. CARP is voor het eerst ontdekt in het epileptische brein. In dit hoofdstuk beschrijven we aanwijzingen dat de expressie van CARP de hersenen mogelijk in staat stelt om weerstand te bieden aan de invloeden die epilepsie op neuronale netwerken kan hebben. **Hoofdstuk 4** gaat niet alleen over 'high-CARP' muizen, maar ook over een tweede transgene stam, de zogenaamde 'low-CARP' dieren, die een aanzienlijk beperktere expressie van CARP in het brein hebben. In dit hoofdstuk bestuderen we mogelijke veranderingen in de uitgaande informatiestroom van het centrale zenuwstelsel als gevolg van de aanwezigheid van CARP, of anders gezegd, kijken we naar het gedrag van deze dieren. We laten zien dat de werking van het geheugen in beide muizenstammen veranderd is ten opzichte van wild-type dieren. In **hoofdstuk 5** beschrijven we de derde transgene muizenstam. Deze heeft over-expressie van DCLK-short in de hersenen en we tonen aan dat deze dieren meer angstgerelateerd gedrag vertonen en dus 'angstiger' zijn, waarschijnlijk als gevolg van veranderde genexpressie en neurotransmissie. De bevindingen in dit hoofdstuk suggereren dat DCLK-short een rol zou kunnen spelen bij de formatie van emotionele reacties en angstigheid. De resultaten beschreven in hoofdstuk twee tot en met vijf worden grondig bediscussieerd en met elkaar in verband gebracht in **hoofdstuk 6**.

Samenvattend kunnen we zeggen dat we succesvol zijn geweest in het genereren van drie nieuwe transgene muizenlijnen met over-expressie van verschillende producten van het DCLK-gen. We laten voor elk van deze lijnen relevante data zien die wijzen op veranderingen op moleculair, netwerk- en gedragsniveau. Dit is enerzijds belangrijk omdat er nog niet veel bekend was over de functie van het DCLK-gen in het brein gedurende het volwassen stadium en anderzijds omdat het DCLK-gen klaarblijkelijk ook onafhankelijk van het befaamde DCX-domein functionele producten kan voortbrengen. Door deze transgene dieren beter te bestuderen kunnen we niet alleen ons basale begrip van het DCLK-gen vergroten, maar vooral ook de relatie tot ziekten waarbij het DCLK-gen een rol lijkt te spelen, zoals bij epilepsie en angststoornissen, beter in beeld brengen. Door onze kennis hierover verder uit te breiden, kunnen we uiteindelijk tot een potentieel betere therapeutische benadering voor deze aandoeningen komen.

Curriculum Vitae

Geert Johannes Schenk was born on September 14 1979 in Hillegom, The Netherlands. In 1997 he received his athenaeum diploma from the Herbert Vissers College, Nieuw-Vennep, The Netherlands. From September 1997 till February 2002 he studied Biology with a specialization in Medical Biology at the faculty of mathematics and natural sciences of Leiden University. During this period he completed an internship at the Department of Medical Pharmacology of the Leiden/Amsterdam Center for Drug Research (LACDR) at Leiden University on a project entitled: '*Effects of corticosterone on the expression of Doublecortin-Like Kinase (DCLK) gene splice variants*' (supervisors Dr. Bart Engels and Dr. Erno Vreugdenhil). In addition, he performed research at the Department of Reproductive Physiology at Kent State University, Ohio, USA on '*Localization of TNFα receptors and gelatinolytic activity in rat ovary*' (Dr. Jennifer Marcinkiewicz). He obtained his Biology masters degree with a specialization in Neurosciences in February 2002. From April 2002 till June 2002 he was employed as 'Jr. Reseacher' at the Department of Medical Pharmacology, LACDR, Leiden University. Subsequently, he received his PhD-training at this department, investigating '*The functions of the DCLK gene through transgenesis*', from September 2002 till May 2007 (supervision Dr. E. Vreugdenhil and Prof. Dr. E.R. de Kloet). During this period he received the '*Hamilton Kinder tuition scholarship award of the University of Tennessee health science centre*' and was granted funding for a project entitled '*Functional consequences of over-expression of DCLK gene products in the hippocampus: Implications for epilepsy*' by Stichting Epilepsie Instellingen Nederland (SEIN; in cooperation with Dr. Rob Voskuyl and Dr. Erno Vreugdenhil). Currently, he is working on a STW-funded project entitled '*Advanced treatment of HIV infections*' under the supervision of Dr. Bert de Boer at the division of Pharmacology, LACDR, Leiden University, in cooperation with Prof. Dr. Ben Berkhout and Dr. Joost Haasnoot (Division of Virology, Amsterdam Medical Center, The Netherlands).

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