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\textsuperscript{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 glutamergic 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 Ca²⁺/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
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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), NaH2PO4 (1.25) NaHCO3 (25), CaCl2·2H2O (2.5), MgSO4·7H2O (1.3), glucose (10)) gassed with 95% O2 and 5% CO2. 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 °C) for at least
1 h, one slice at a time was transferred to the recording chamber and submerged in ACSF (32°C; perfusion rate was 2-3 ml/min) and gassed (95% O₂ and 5% CO₂). 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 separately 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 Abarang, 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 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 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 GTG CTG GAT ATC TGG AGA ATC TTT CCC TCC-3'
3) 5'-AGG CAA GAG TCT AGA CAT CTC TGA GAG ACC CAA TTT GCC ATC TCC-3'
4) 5'-AGG CAA GAG TCT AGA CAT CTC TGA GAG ACC CAA 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.
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<table>
<thead>
<tr>
<th>Brain area</th>
<th>CARP Expression</th>
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<tbody>
<tr>
<td>Anterior commissure</td>
<td>-</td>
</tr>
<tr>
<td>Orbital cortex</td>
<td>-</td>
</tr>
<tr>
<td>Anterior olfactory nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Periform cortex</td>
<td>+</td>
</tr>
<tr>
<td>Motor cortex</td>
<td>+</td>
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<tr>
<td>Dorsal endoperiform nucleus</td>
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</tr>
<tr>
<td>Corpus callosum</td>
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</tr>
<tr>
<td>Medial septal nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>-</td>
</tr>
<tr>
<td>Amygdaloid nucleus</td>
<td>+</td>
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<tr>
<td>Basolateral amygdaloid nucleus, anterior</td>
<td>+</td>
</tr>
<tr>
<td>Basolateral amygdaloid nucleus, posterior</td>
<td>+</td>
</tr>
<tr>
<td>Basomedial amygdaloid nucleus, posterior</td>
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</tr>
<tr>
<td>Hippocampus</td>
<td>+</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>+</td>
</tr>
<tr>
<td>Cornu ammonis 1</td>
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<tr>
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<td>+</td>
</tr>
<tr>
<td>Cornu ammonis 3</td>
<td>+</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Ventromedial thalamic nucleus</td>
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<tr>
<td>Parafascicular thalamic nucleus</td>
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</tr>
<tr>
<td>Lateral hypothalamic area</td>
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</tr>
<tr>
<td>Lateral globus pallidus</td>
<td>+</td>
</tr>
<tr>
<td>External capsule</td>
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<tr>
<td>Internal capsule</td>
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<tr>
<td>Fimbria hippocampus</td>
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<td>Stria terminalis</td>
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<tr>
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<td>Dorsal lateral geniculate nucleus</td>
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<tr>
<td>Red nucleus, parvocellular</td>
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<tr>
<td>Reticulotegmental nucleus pons</td>
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</tr>
<tr>
<td>Medial longitudinal fasciculus</td>
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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 Cyclophilin. 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\textsubscript{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 $22.1\pm1.3$ µA and $18.4\pm0.8$ µA; WT and high-CARP, respectively) to maximal ($235.1\pm10.4$ µA and $218.6\pm9.8$ 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.
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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

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.
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.

![Excitability plots of basal responses.](image)

**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).

<table>
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<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Fold Change</th>
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<tr>
<td>mCG3971.1</td>
<td>Voltage-dependent L-type Ca(^{2+}) channel α1F subunit</td>
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<td>Olfactory receptor</td>
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<td>mCG1865.1</td>
<td>Chemokine (C-C motif) ligand 24</td>
<td>3.20</td>
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<tr>
<td>mCG8869.1</td>
<td>Sodium- and chloride-dependent glycine transporter SLC6A9-related</td>
<td>2.64</td>
</tr>
<tr>
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<td><strong>Calretinin (Calbindin 2)</strong></td>
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</tr>
<tr>
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<tr>
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<tr>
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<td>Claudin 4</td>
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<tr>
<td>mCG128278.1</td>
<td><strong>CAMP-Dependent Rap1 Guanine-nucleotide exchange factor 4</strong></td>
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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 Cyclophilin.

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
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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,
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; Woelfrey 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-Martín 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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References


Chapter 4

Hippocampal CARP Over-expression Solidifies Consolidation of Contextual Fear Memories


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