Understanding doublecortin-like kinase gene function through transgenesis

Schenk, G.J.

Chapter 5

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

Geert J. Schenk, Barbera Veldhuisen, Olga Wedemeier, Caroline C. McGown, Theo G. Schouten, Melly Oitzl, E. Ron de Kloet and Erno Vreugdenhil.

Division of Medical Pharmacology, Leiden/Amsterdam Centre for Drug Research, Leiden university Medical Centre, Einsteinweg 55, 2300 RA Leiden, The Netherlands.

Physiology & Behavior. 2010. In Press.

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.

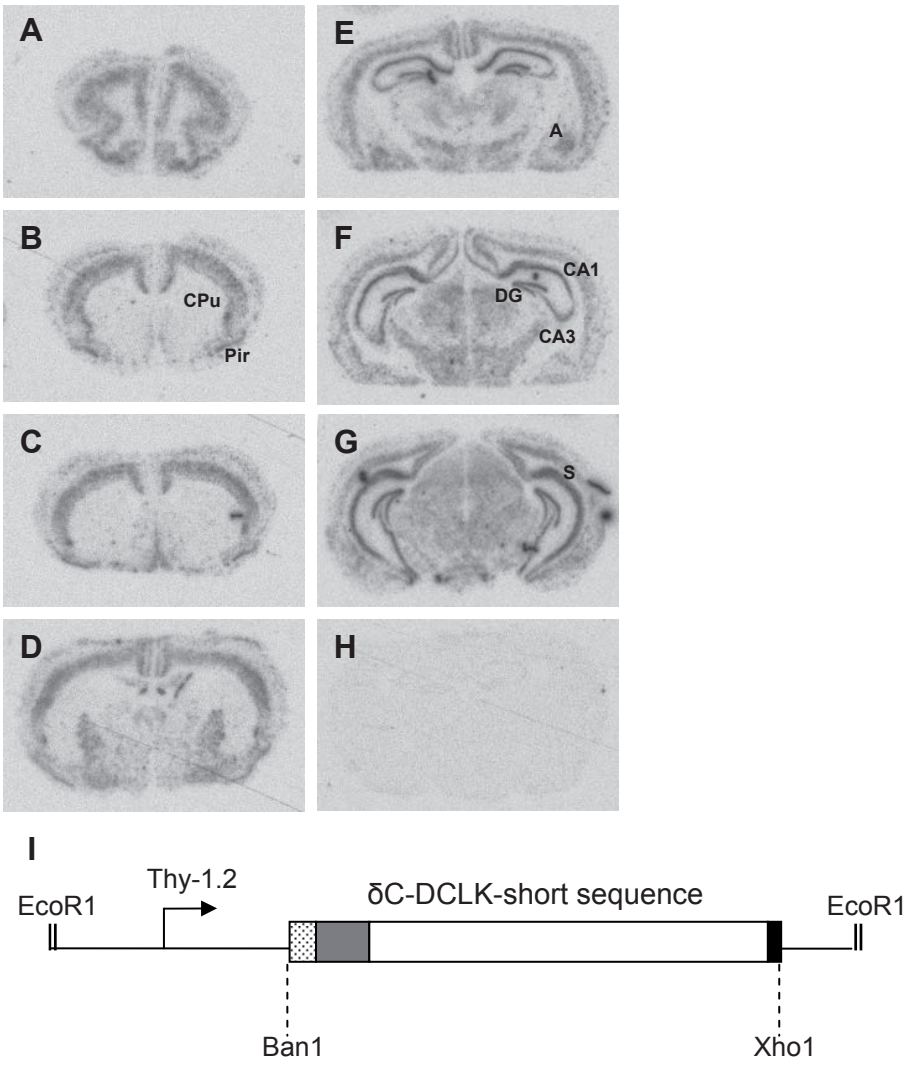

Figure 1. δC-DCLK-short mRNA expression in transgenic mouse brain. A coronal overview from rostral (A) to caudal (G) of δC-DCLK-short expression is shown. Note the high expression in several cortical areas (A-E), hippocampus (E-G), amygdaloid and thalamic nuclei (D-G), which is characteristic for the 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

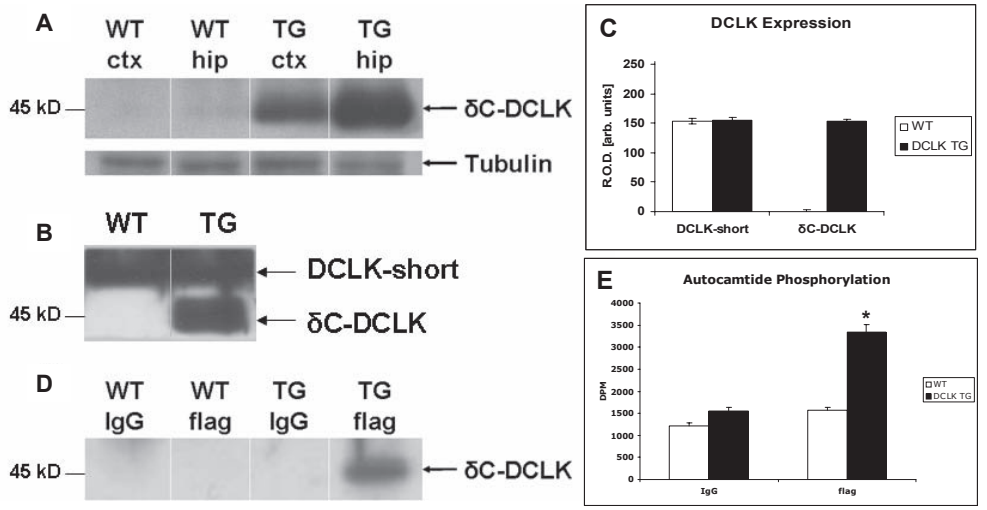

*Figure 2. δC-DCLK-short protein expression in cortex and hippocampus (A). Western blotting was performed using an anti-FLAG antibody to specifically recognize the transgenic protein. A specific band at 45 kD demonstrates the presence of the FLAG-tagged δC-DCLK-short protein in TG animals. WT controls 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).

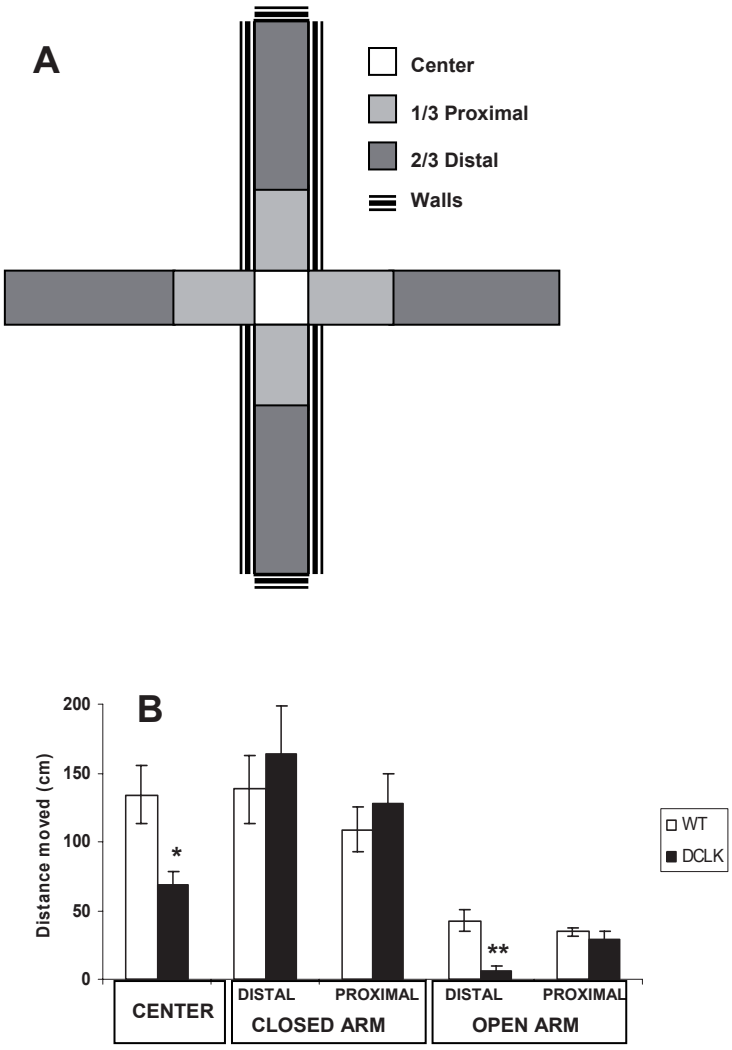

Figure 3. Spatiotemporal parameters of the EPM. The EPM is divided into five zones (A), comprising a center area, two open arms and two closed arms and the subdivision in the proximal and distal parts of these arms. The distances moved (cm) in each zone are shown (B). Significant differences between 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.*

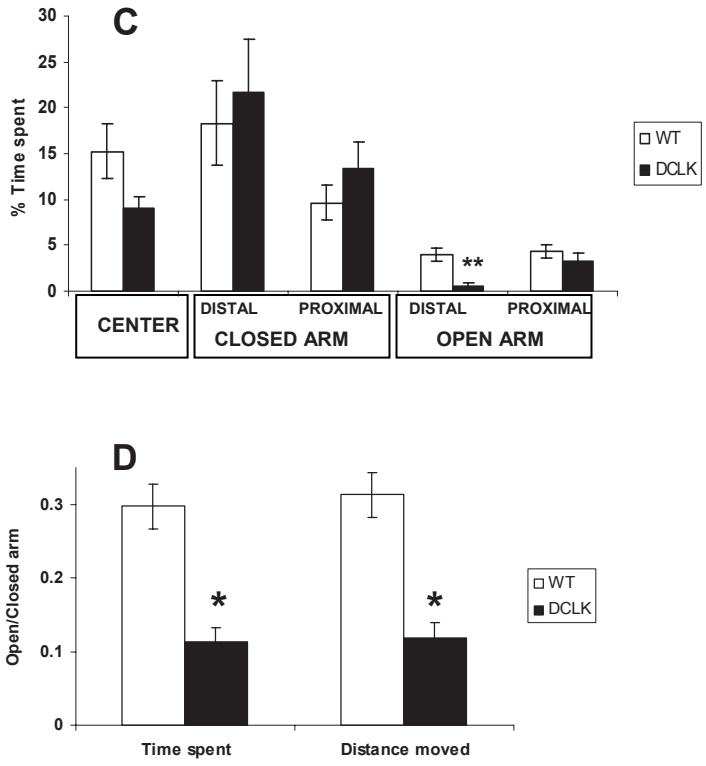

*Figure 3. (Continued) The percentage of time spent in each of the defined zones is also shown (C). δC-DCLK-short mice spend less time in the outer part of the open arms compared to wild-type mice (** p<0.01). To clearly visualize differential open versus closed arm activity between genotypes, ratios 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.

References

Ahi J, Radulovic J, Spiess J. 2004. The role of hippocampal signaling cascades in consolidation of fear memory. *Behav Brain Res.* 149(1):17-31.

Atack JR. 2005. The benzodiazepine binding site of GABA(A) receptors as a target for the development of novel anxiolytics. *Expert Opin Investig Drugs.* 14(5):601-18.

Augustsson H, Meyerson BJ. 2004. Exploration and risk assessment: a comparative study of male house mice (Mus musculus musculus) and two laboratory strains. Physiol Behav. 81(4):685-98.

Bannerman DM, Grubb M, Deacon RM, Yee BK, Feldon J, Rawlins JN. 2003. Ventral hippocampal lesions affect anxiety but not spatial learning. *Behav Brain Res.* 139(1-2):197-213.

Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, Zhang WN, Pothuizen HH, Feldon J. 2004. Regional dissociations within the hippocampus--memory and anxiety. *Neurosci Biobehav Rev.* 28(3):273-83.

Ben-Ari Y. 2002. Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci.* 3(9):728-39.

Blaeser F, Sanders MJ, Truong N, Ko S, Wu LJ, Wozniak DF, Fanselow MS, Zhuo M, Chatila TA. 2006. Long-term memory deficits in Pavlovian fear conditioning in Ca2+/calmodulin kinase kinase alpha-deficient mice. *Mol Cell Biol.* 26(23):9105-15.

Boekhoorn K, Sarabdjitsingh A, Kommerie H, de Punder K, Schouten T, Lucassen PJ, Vreugdenhil E. 2008. Doublecortin (DCX) and doublecortin-like (DCL) are differentially expressed in the early but not late stages of murine neocortical development. *J Comp Neurol.* 507(4):1639-52.

Burgess H.A., Martinez S., Reiner O. 1999. KIAA0369, doublecortin-like kinase, is expressed during brain development. *J Neurosci Res*. 15;58(4):567-75.

Burgess, H.A., and O. Reiner. 2002. Alternative splice variants of doublecortin-like kinase are differentially expressed and have different kinase activities. *J Biol Chem*. 277:17696-705.

Crestani F, Lorez M, Baer K, Essrich C, Benke D, Laurent JP, Belzung C, Fritschy JM, Lüscher B, Mohler H. 1999. Decreased GABAA-receptor clustering results in enhanced anxiety and a bias for threat cues. *Nat Neurosci.* 2(9):833-9.

Dawson GR, Collinson N, Atack JR. 2005. Development of subtype selective GABAA modulators. *CNS Spectr.* 10(1):21-7.

Deuel, T.A., J.S. Liu, J.C. Corbo, S.Y. Yoo, L.B. Rorke-Adams, and C.A. Walsh. 2006. Genetic Interactions between Doublecortin and Doublecortin-like Kinase in Neuronal Migration and Axon Outgrowth. *Neuron*. 49:41-53.

Dijkmans TF, van Hooijdonk LW, Schouten TG, Kamphorst JT, Fitzsimons CP, Vreugdenhil E. 2009. Identification of new Nerve Growth Factor-responsive immediate-early genes. *Brain Res.* 16;1249:19-33.

Earnheart JC, Schweizer C, Crestani F, Iwasato T, Itohara S, Mohler H, Lüscher B. 2007. GABAergic control of adult hippocampal neurogenesis in relation to behavior indicative of trait anxiety and depression states. *J Neurosci.* 27(14):3845-54.

Engels, B.M., T.G. Schouten, J. van Dullemen, I. Gosens, and E. Vreugdenhil. 2004. Functional differences between two DCLK splice variants. *Brain Res Mol Brain Res.* 120:103-14.

Esclassan F, Coutureau E, Di Scala G, Marchand AR. 2009. Differential contribution of dorsal and ventral hippocampus to trace and delay fear conditioning. *Hippocampus*. 19(1):33-44.

Franklin, K.B.J. and Paxinos G. 1997. The mouse brain in stereotaxic coordinates. *Academic Press.*

Friocourt, G., Koulakoff A., Chafey P., Boucher D., Fauchereau F., Chelly J., Francis F. 2003. Doublecortin functions at the extremities of growing neuronal processes. *Cereb Cortex.* 13(6):620-6.

Friocourt, G., Liu J.S., Antypa M., Rakic S., Walsh C.A., Parnavelas J.G. 2007. Both doublecortin and doublecortin-like kinase play a role in cortical interneuron migration. *J. Neurosci.* 4;27(14):3875-83.

Ganguly K, Schinder AF, Wong ST, Poo M. 2001. GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition.*Cell.* 105(4):521-32.

Ge S, Pradhan DA, Ming GL, Song H. 2007. GABA sets the tempo for activity-dependent adult neurogenesis. *Trends Neurosci.* 30(1):1-8.

Hevroni D., Rattner A., Bundman M., Lederfein D., Gabarah A., Mangelus M., Silverman M.A., Kedar H., Naor C., Kornuc M., Hanoch T., Seger R., Theill L.E., Nedivi E., Richter-Levin G., Citri Y. 1998. Hippocampal plasticity involves extensive gene induction and multiple cellular mechanisms. *J Mol Neurosci.* 10:75-98.

't Hoen PA, Ariyurek Y, Thygesen HH, Vreugdenhil E, Vossen RH, de Menezes RX, Boer JM, van Ommen GJ, den Dunnen JT. 2008. Deep sequencing-based expression analysis shows major advances in robustness, resolution and inter-lab portability over five microarray platforms. *Nucleic Acids Res.* 36(21):e141.

Hogg S. 1996. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacol Biochem Behav.* 54(1):21-30.

Izquierdo I, Medina JH. 1997. Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiol Learn Mem.* 68(3):285-316.

Kalueff AV, Tuohimaa P. 2004. Grooming analysis algorithm for neurobehavioural stress research. *Brain Res Brain Res Protoc.* 13(3):151-8.

Kerjan G, Koizumi H, Han EB, Dubé CM, Djakovic SN, Patrick GN, Baram TZ, Heinemann SF, Gleeson JG. 2009. Mice lacking doublecortin and doublecortin-like kinase 2 display altered hippocampal neuronal maturation and spontaneous seizures. *Proc Natl Acad Sci U S A*. 106(16):6766-71.

Koizumi, H., T. Tanaka, and J.G. Gleeson. 2006. doublecortin-like kinase Functions with doublecortin to Mediate Fiber Tract Decussation and Neuronal Migration. *Neuron*. 49:55-66.

Kouzu Y, Moriya T, Takeshima H, Yoshioka T, Shibata S. 2000. Mutant mice lacking ryanodine receptor type 3 exhibit deficits of contextual fear conditioning and activation of calcium/calmodulin-dependent protein kinase II in the hippocampus. *Brain Res Mol Brain Res.* 76(1):142-50.

Kruidering, M., T. Schouten, G.I. Evan, and E. Vreugdenhil. 2001. Caspase-mediated cleavage of the Ca2+/calmodulin-dependent protein kinase-like kinase facilitates neuronal apoptosis. *J Biol Chem*. 276:38417-25.

Lin, P.T., J.G. Gleeson, J.C. Corbo, L. Flanagan, and C.A. Walsh. 2000. DCAMKL1 encodes a protein kinase with homology to doublecortin that regulates microtubule polymerization. *J Neurosci*. 20:9152-61.

Löw K, Crestani F, Keist R, Benke D, Brünig I, Benson JA, Fritschy JM, Rülicke T, Bluethmann H, Möhler H, Rudolph U. 2000. Molecular and neuronal substrate for the selective attenuation of anxiety. *Science.* 290(5489):131-4.

McHugh SB, Deacon RM, Rawlins JN, Bannerman DM. 2004. Amygdala and ventral hippocampus contribute differentially to mechanisms of fear and anxiety. *Behav Neurosci.* 118(1):63-78.

Meijer OC, Steenbergen PJ, De Kloet ER. 2000. Differential expression and regional distribution of steroid receptor coactivators SRC-1 and SRC-2 in brain and pituitary. *Endocrinology*. 141(6):2192-9.

Möhler H, Fritschy JM, Rudolph U. 2002. A new benzodiazepine Pharmacology. *J Pharmacol Exp Ther.* 300(1):2-8.

Möhler H, Fritschy JM, Vogt K, Crestani F, Rudolph U. Pathophysiology and pharmacology of GABA(A) receptors. 2005. *Handb Exp Pharmacol.* (169):225-47.

Moser MB, Moser EI. 1998. Functional differentiation in the hippocampus. *Hippocampus.*8(6):608-19.

Ohmae S, Takemoto-Kimura S, Okamura M, Adachi-Morishima A, Nonaka M, Fuse T, Kida S, Tanji M, Furuyashiki T, Arakawa Y, Narumiya S, Okuno H, Bito H. 2006. Molecular identification and characterization of a family of kinases with homology to Ca2+/calmodulin-dependent protein kinases I/IV. *J Biol Chem.* 281(29):20427-39.

Papadopoulos T, Korte M, Eulenburg V, Kubota H, Retiounskaia M, Harvey RJ, Harvey K, O'Sullivan GA, Laube B, Hülsmann S, Geiger JR, Betz H. 2007. Impaired GABAergic transmission and altered hippocampal synaptic plasticity in collybistin-deficient mice. *EMBO J.* 26(17):3888-99.

Papaleo F, Crawley JN, Song J, Lipska BK, Pickel J, Weinberger DR, Chen J. 2008. Genetic dissection of the role of catechol-O-methyltransferase in cognition and stress reactivity in mice. *J Neurosci.* 28(35):8709-23.

Pedotti P, 't Hoen PA, Vreugdenhil E, Schenk GJ, Vossen RH, Ariyurek Y, de Hollander M, Kuiper R, van Ommen GJ, den Dunnen JT, Boer JM, de Menezes RX. 2008. Can subtle changes in gene expression be consistently detected with different microarray platforms? *BMC Genomics.* 9:124.

Rodgers RJ, Cao BJ, Dalvi A, Holmes A. 1997. Animal models of anxiety: an ethological perspective. *Braz J Med Biol Res.* (3):289-304.

Rodgers RJ, Dalvi A. Anxiety, defence and the elevated plus-maze. 1997. *Neurosci Biobehav Rev.* 21(6):801-10.

Rudolph U, Crestani F, Benke D, Brünig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Möhler H. 1999. Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. *Nature.* 401(6755):796-800.

Rudolph U, Möhler H. 2006. GABA-based therapeutic approaches: GABAA receptor subtype functions. *Curr Opin Pharmacol.* 6(1):18-23.

Sapir T, Horesh D, Caspi M, Atlas R, Burgess HA, Wolf SG, Francis F, Chelly J, Elbaum M, Pietrokovski

S, Reiner O. 2000. Doublecortin mutations cluster in evolutionarily conserved functional domains. *Hum Mol Genet.* 9(5):703-12.

Schenk G.J., Engels B., Zhang Y.P., Fitzsimons C.P., Schouten T., Kruidering M., de Kloet E.R., Vreugdenhil E. 2007. A potential role for calcium / calmodulin-dependent protein kinase-related peptide in neuronal apoptosis: in vivo and in vitro evidence. *Eur J Neurosci*. 26(12):3411-20.

Shang L, Kwon YG, Nandy S, Lawrence DS, Edelman AM. 2003. Catalytic and regulatory domains of doublecortin kinase-1. *Biochemistry.* 42(7):2185-94.

Silverman M.A., Benard O., Jaaro H., Rattner A., Citri Y., Seger R. 1999. CPG16, a novel protein serine/threonine kinase downstream of cAMP-dependent protein kinase. *J Biol Chem*. 29;274(5):2631-6.

Sommer W, Möller C, Wiklund L, Thorsell A, Rimondini R, Nissbrandt H, Heilig M. 2001. Local 5,7-dihydroxytryptamine lesions of rat amygdala: release of punished drinking, unaffected plus-maze behavior and ethanol consumption. *Neuropsychopharmacology.* (4):430-40.

Tanaka T., Koizumi H., Gleeson J.G. 2006. The doublecortin and doublecortin-like Kinase 1 genes cooperate in murine hippocampal development. *Cereb Cortex.* 16 Suppl 1:i69-73.

Tozuka Y, Fukuda S, Namba T, Seki T, Hisatsune T. 2005. GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. *Neuron.* 47(6):803-15.

Treit D, Pesold C, Rotzinger S. 1992. Noninteractive effects of diazepam and amygdaloid lesions in two animal models of anxiety. *Behav Neurosci.* 107(6):1099-105.

Treit D, Menard J. 1997. Dissociations among the anxiolytic effects of septal, hippocampal, and amygdaloid lesions. *Behav Neurosci.* 111(3):653-8.

Trivedi MA, Coover GD. 2004. Lesions of the ventral hippocampus, but not the dorsal hippocampus, impair conditioned fear expression and inhibitory avoidance on the elevated T-maze. *Neurobiol Learn Mem.* 81(3):172-84.

Tuy F.P., Saillour Y., Kappeler C., Chelly J., Francis F. 2008. Alternative transcripts of Dclk1 and Dclk2 and their expression in doublecortin knockout mice. *Dev Neurosci*. 30(1-3):171-86.

Vidal M., Morris R., Grosveld F., Spanopoulou E. 1990. Tissue-specific control Elements of the Thy-1 gene. *EMBO J*. 9(3):833-40.

Vreugdenhil, E., N. Datson, B. Engels, J. de Jong, S. van Koningsbruggen, M. Schaaf, and E.R. de Kloet. 1999. Kainate-elicited seizures induce mRNA encoding a CaMK-related peptide: a putative modulator of kinase activity in rat hippocampus. *J Neurobiol*. 39:41-50.

Vreugdenhil, E., B. Engels, R. Middelburg, S. van Koningsbruggen, J. Knol, B. Veldhuisen, and E.R. de Kloet. 2001. Multiple transcripts generated by the DCAMKL gene are expressed in the rat hippocampus. *Brain Res Mol Brain Res*. 94:67-74.

Vreugdenhil, E., Kolk, S.M., Boekhoorn, K., Fitzsimons, C.P., Schaaf, M., Schouten, T., Sarabdjitsingh, A., Sibug, R., Lucassen, P.J. 2007. Doublecortin-like, a microtubule-associated protein expressed in radial glia, is crucial for neuronal precursor division and radial process stability. *Eur J Neurosci*. 25(3):635-48.

Whiting PJ. 2006. GABA-A receptors: a viable target for novel anxiolytics? *Curr Opin Pharmacol.* 6(1):24-9.