Chapter 3

Doublecortin-like is implicated in adult-hippocampal neurogenesis and in motivational aspects to escape from an aversive environment

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Abstract

Doublecortin-like (DCL) is a microtubule-associated protein that is highly homologous to doublecortin and is crucially involved in embryonic neurogenesis. Here, we have investigated the in vivo role of DCL in adult hippocampal neurogenesis by generating transgenic mice producing inducible shRNA molecules that specifically target DCL but not other splice-variants produced by the DCLK gene.

DCL knockdown resulted in a significant increase in the number of proliferating BrdU+ cells in the subgranular zone one day after BrdU administration. However, the number of surviving newborn adult NeuN+/BrdU+ neurons are significantly decreased when inspected 4 weeks after BrdU administration suggesting a blockade of neuronal differentiation after DCL-KD. In line with this, we observed an increase in the number of proliferating cells, but a decrease in post mitotic DCX+ cells that are characterized by long dendrites spanning all dentate gyrus layers.

Behavioural analysis showed that DCL-KD strongly reduced the escape latency of mice on the circular hole board but did not affect other aspects of this behavioural task. Together, our results indicate a key role for DCL in neuronal development but not in hippocampus-dependent memory formation.

Introduction

The doublecortin (DCX) gene family members are involved in structural plasticity and a rapid adaption of cellular shape (for review see Reiner et al., 2006). Proteins encoded by this family are generally microtubule-associated proteins (MAPs) characterized by a-typical microtubule (MT) binding domains, called DC domains. The archetypical member of this family is DCX. Phosphorylation and dephosphorylation of DCX controls cytoskeleton dynamics thereby enabling movement of migrating neuroblasts (Schaar et al., 2004). Consequently, missense mutations in human X-linked DCX are associated with impaired neuronal migration of neuroblasts during embryonic development and are associated with lissencephaly in men and with the double cortex syndrome in females (des Portes et al., 1998;Gleeson et al., 1998).

A complete removal of DCX in mice leads to normal cortical development suggesting that other members of the DCX family compensate for the loss of DCX function (Corbo et al., 2002). One likely member in this respect is the doublecortin-like kinase-1 (DCLK1) gene (for review see Dijkmans et al., 2010). Interestingly, like DCX knockout mice, DCLK1 knockout mice also lack a clear phenotype (Deuel et al., 2006) but DCLK/DCX double knockout mice display profound disorganized cortical layering and a disrupted hippocampal structure, suggestive of a compensatory role for the DCLK1 gene in the migration of neuronal progenitor cells during embryogenesis (NPCs; Deuel et al., 2006;Koizumi et al., 2006). In addition, this suggests that DCX as well as DCLK are necessary for proper neuronal development.

The DCLK gene encodes multiple splice-variants encoding proteins containing DC domains and Ser/Thr kinase domains, such as DCLK-long, or Ser/Thr kinase domains only, like DCLK-short (for review see Dijkmans et al., 2010). In addition, the DCLK gene encodes one splice variant called doublecortin-like (DCL), that lacks a kinase domain and is highly homologous to DCX over its entire length (Vreugdenhil et al., 2007). During embryonic development, DCL functions as a microtubule stabilizing protein of mitotic spindles in vitro and in vivo. In addition, DCL knockdown by RNA-interference technology induces spindle collapse in vitro and in vivo while DCL knockdown in vivo by in utero electroporation leads to significantly reduced cell numbers in the inner proliferative zones and dramatically disrupts most radial processes (Vreugdenhil et al., 2007).

Both DCX and DCL are also expressed in the adult brain. Consistent with a function for DCX in the migration of neuronal progenitor cells, profound DCX expression occurs in well-established neurogenic areas in the adult brain and DCX is generally considered a useful neurogenesis marker (Brown et al., 2003b;Couillard-Despres et al., 2005). DCX+ neuronal progenitors cell's (NPC's) and DCX+ migrating neuroblasts can be found in the subventricular

zone (SVZ) and rostral migratory stream (RMS). DCX+ neuroblasts are well-studied in the subgranular zone (SGZ) of the dentate gyrus where approximately 20% of the DCX+ cells are proliferating NPC's, while the remaining 80% are post mitotic NPC's and/or neuroblasts (Plumpe et al., 2006;Walker et al., 2007). However, DCX expression has also been found generally in lower numbers, in other brain regions such as the telencephalon, hypothalamus or amygdala (Gomez-Climent et al., 2008;Nacher et al., 2001;Werner et al., 2012;Zhang et al., 2009). Previously, we have reported DCL expression that overlaps and co localises with DCX, in the SVZ, in the rostral migratory stream and in the SGZ of the dentate gyrus (Saaltink et al., 2012). As DCX, DCL is also expressed at high levels in other brain areas, like the suprachiasmatic nucleus (SCN), the Islands of Calleja and in hypothalamic tanycytes (Saaltink et al., 2012).

Although a role for the DCLK1 gene in embryonic neurogenesis seems evident, the functional role for DCLK-splice variant DCL in adult neurogenesis and its function in NPCs remains elusive. To begin to address this role, we have generated inducible DCL-shRNA mice to knockdown DCL in vivo. As neurogenesis is well-established in the dentate gyrus and DCX and DCL expression is restricted to progenitor cells in the SGZ, we focus on this neurogenic area of the hippocampus. Furthermore, the functional role of adult neurogenesis in the cognitive performance was studied using a hippocampus-dependent spatial memory task known as circular hole board paradigm. We report here that inducible knockdown of DCL leads to a dramatic reduction of post-mitotic DCX-positive cells. In addition, impaired neurogenesis does not affect spatial memory formation. However, DLC knockdown leads to an increase in the time to escape from the circular hole board suggesting a subtle role for DCL in context discrimination.

Methods

Animals and animal experimentation

Transgenic male mice were obtained from TaconicArtemis GmbH (Köln, Germany). These mice contain an inducible and reversible shRNA expression system (Seibler et al., 2007), which we called DCL-KD mice. The following hairpin sequences targeting the 3'-UTR region of the mRNA encoding DCL (see Fig. 2A) were cloned into the Taconic Artemis system as described previously (Seibler et al., 2007):

5'- TCCC GCTGGTCATCCTGCATCTTGT TTCAAGAGA ACAAGATGCAGGATGACCAGC TTTTTA -3'

3'- CGACCAGTAGGACGTAGAACA AAGTTCTCT TGTTCTACGTCCTACTGGTCG AAAAATGCGC -5'

Transgenic males were the founders of our heterozygous outbred colony with B6129S6F1 mice. The shRNA system was induced by doxycycline (dox) via dox containing food pellets (Dox Diet Sterile S3888, 200mg/kg, BioServ, New Jersey, USA). Animals were put for 4 weeks on dox diet (ad libitum) before they were used for any experiment. Non-induced control animals were fed on identical control diet without dox (S4207, BioServ, New Jersey, USA). Tissues are obtained from transgenic DCL-KD mice and wildtype littermates born in our animal facility. After dox induction animals were killed by decapitation and brains were quickly removed for dissection of olfactory bulb and hippocampus. Tissue for qPCR was put into RNAlater® Solution (Applied Biosystems, The Netherlands) and kept at 4°C for a day and stored at -20°C for later use. Tissue for Western Blot analysis was identically dissected, snap-frozen and stored at -80°C for later use.

All experiments were approved by the committee of Animal Health and Care, Leiden University and performed in compliance with the European Union recommendations for the care and use of laboratory animals.

RNA isolation

Total RNA was extracted using Trizol (Invitrogen, The Netherlands) and checked for concentration and purity using a Nanodrop ND-1000 spectrometer (Thermo Scientific, USA). RNA integrity was checked using RNA nano labchips in an Agilent 2100 Bioanalyser (Agilent Technologies, Inc, USA). To remove genomic DNA, 1µg RNA of each sample was treated with DNAse Amplification Grade (Invitrogen, The Netherlands) and diluted with DEPC-MQ to 50ng/µl RNA. From this purified RNA, cDNA was generated using Biorad iScript cDNA synthesis kit (Biorad, The Netherlands).

shRNA detection

shRNA targeting DCL was measured using a custom designed Taqman microRNA assay on a ABI 7900HT fast real time PCR system (Applied Biosystems, The Netherlands). Specific primers were designed to detect anti-DCL shRNA (ACAAGAUGCAGGAUGACCAGC). For mouse tissue, snoRNA-202 was used as reference gene and the data was analyzed using the ΔΔCt method (Livak and Schmittgen, 2001).

Western blot analysis

Tissue was solubilised in lysis buffer (1% Tween-20, 1% DOC, 0,1% SDS, 0,15M NaCl and 50 mM Tris pH 7,5) and centrifuged at max speed (14000rpm) for 10 minutes. The protein concentration of the supernatant was measured using the pierce method (Pierce® BCA Protein

Assay Kit, Thermo Scientific, Etten-Leur, The Netherlands). Equal amounts of protein (2 µg cell lysate) were separated by SDS-PAGE (10% acrylamyde) and transferred to immobilon-P PVDF membranes (Millipore).

Blots were incubated in a blocking buffer (TBST, Tris-buffered saline with 0.2% Tween 20, with 5% low-fat milk powder) for 60 minutes and then incubated in fresh blocking buffer with primary antibodies as described (Saaltink et al., 2012) anti-DCL, 1 : 2000; monoclonal α-tubulin DM1A, 1:10000; Sigma–Aldrich, The Netherlands) for another 60 minutes. After a five minutes wash (3x) with TBST, horseradish peroxidase-conjugated secondary antibodies were added in TBST. After treatment with 10 ml luminol (200ml 0,1M Tris HCL, pH8. 50 mg sodium luminol, 60 µl 30% H2O2), 100 µl Enhancer (11mg para-hydroxy-coumaric acid in 10 ml DMSO) and 3 µl H2O2 protein detection, was performed by ECLTM western blotting analysis system (Amersham Pharmacia Biotech, Freiburg, Germany).

The developed films were scanned at a high resolution (13200 dpi) and gray-values were measured using Image-J. α-tubulin expression was used to correct for the amount of protein for each sample.

Histology

BrdU treatment

To test whether DCL knockdown had an effect on adult neurogenesis, BrdU was used to label proliferating cells. In the first experiment, wildtype and transgenic animals of 6 weeks old were put on a dox or control diet (n=6 per group). After 4 weeks, mice received a single intraperitoneal injection with BrdU (200 mg/kg BrdU dissolved in 0.9% saline, Sigma Aldrich). After 24 hours the animals were sacrificed and prepared for immunohistochemistry as described previously (Saaltink et al., 2012). In a second experiment, animals received a similar diet described above for 4 weeks. Subsequently, intraperitoneal BrdU (100 mg/kg BrdU dissolved in 0.9% saline, Sigma Aldrich) was administrated for 4 consequential days. The animals were kept on the experimental diet for another 4 weeks were after the animals were killed and prepared for immunohistochemistry as described before.

Immunohistochemistry

To measure proliferation, BrdU was visualized with 3,3'-Diaminobenzidine (DAB) as previously described (Heine et al., 2004a). In short, free-floating sections were incubated in 0.5% H2O2 to block endogenous peroxidase. Subsequently, the sections were incubated in mouse α-BrdU primary antibody (clone: BMC9318, Roche Diagnostics, The Netherlands, 1:1000

overnight) and subsequently in sheep α mouse biotinylated secondary antibody (RPN1001, GE Healthcare, Germany, 1:200 for 2 hrs); both antibodies diluted in 0.1% Bovine Serum Albumin (BSA; sc-2323; Santa Cruz Biotechnology), 0.3% TX-100 and 0.1M phosphate buffer. To amplify the signal, a VectaStain Elite avidin-biotin complex (ABC) Kit (Vector Laboratories, Brunschwig Chemie, Amsterdam, The Netherlands, 1:800 for 2 hrs) and tyramide (TSATM Biotin System, Perkin-Elmer, Groningen, The Netherlands, 1:750 for 45 minutes) were used. Thereafter, sections were incubated with DAB (0.5 mg/ml), dissolved in 0.05M tris-buffer (TB) with 0.01% H2O2 for 15 minutes. Sections were air-dried and counterstained with haematoxylin, dehydrated and cover slipped with DPX (MerckMillipore, Darmstadt, Germany).

To analyze cell survival, chicken α-BrdU (ab92837, Abcam, Cambridge, UK, 1:1000) and mouse α-NeuN (MAB3777, Millipore Billerica, MA, 1:200) were visualized with fluorescent secondary antibodies (Alexa Fluor®488, goat α-chicken and Alexa Fluor®594 donkey α-mouse, Invitrogen, Breda, The Netherlands).

To analyze the immature cell population in the dentate gyrus, DCX was visualized with DAB as previously described (Oomen et al., 2007). Briefly, free-floating sections were incubated in 0.5% H2O2 in 0.05 M tris-buffered saline (TBS; pH 7.6) to block endogenous peroxidise. Before primary antibody incubation, the sections were blocked in 2% low-fat milk powder (Elk, Campina, The Netherlands) in TBS for 30 minutes. Sections were incubated in goat α-DCX (sc-8066; Santa Cruz Biotechnology, Santa Cruz, CA, 1:800 overnight) and subsequently in biotinylated donkey α-goat (sc-2042; Santa Cruz Biotechnology, Santa Cruz, CA, 1:500) for 2 hrs. Both antibodies were diluted in TBS with 0.25% gelatine and 0.1% TX-100. To amplify the signal a VectaStain Elite avidin-biotin complex (ABC) Kit and tyramide were used. Incubation of 15 minutes in DAB (0.5 mg/ml), dissolved in 0.05M tris-buffer (TB) with 0.01% H2O2 finished the staining. Sections were air dried and counterstained with haematoxylin, dehydrated and cover slipped with DPX.

Cell counting

Every tenth section of the collected material (1 series out of 10) was stained according the procedures described above. In case of proliferation, all BrdU positive cells in the dentate gyrus were estimated by counting the cells within this series and multiply this with 10. For cell survival, BrdU and NeuN double positive cells were counted. To analyze the immature population of newborn neurons a distinction based on the dendritic morphology was made between three types of DCX positive cells (Plumpe et al., 2006). We categorized DCX positive cells in proliferative stage (type 1, short of no processes), intermediate stage (type 2, medium processes) and post mitotic stage (type 3, strong dendrites with branches). For all three experiments, the total amount of cells in each section was multiplied by 10.

Circular hole board

Apparatus

The circular hole board paradigm (CHB, Fig. 1) was performed as described previously (Dalm et al., 2009). In short, a round Plexiglas plate (diameter: 110 cm) with 12 holes (diameter: 5 cm; Fig. 1C) was situated 1 meter above the floor (Fig.1D). The holes were connected to an s-shaped tube of 15 cm length. Beneath the tube, the home cage was placed such to enable the animal to leave the plate and enter its cage. At 5 cm depth, the holes could be closed by a lith. One week before the experimental procedure, the animals were trained to climb through the tunnel 3 times.

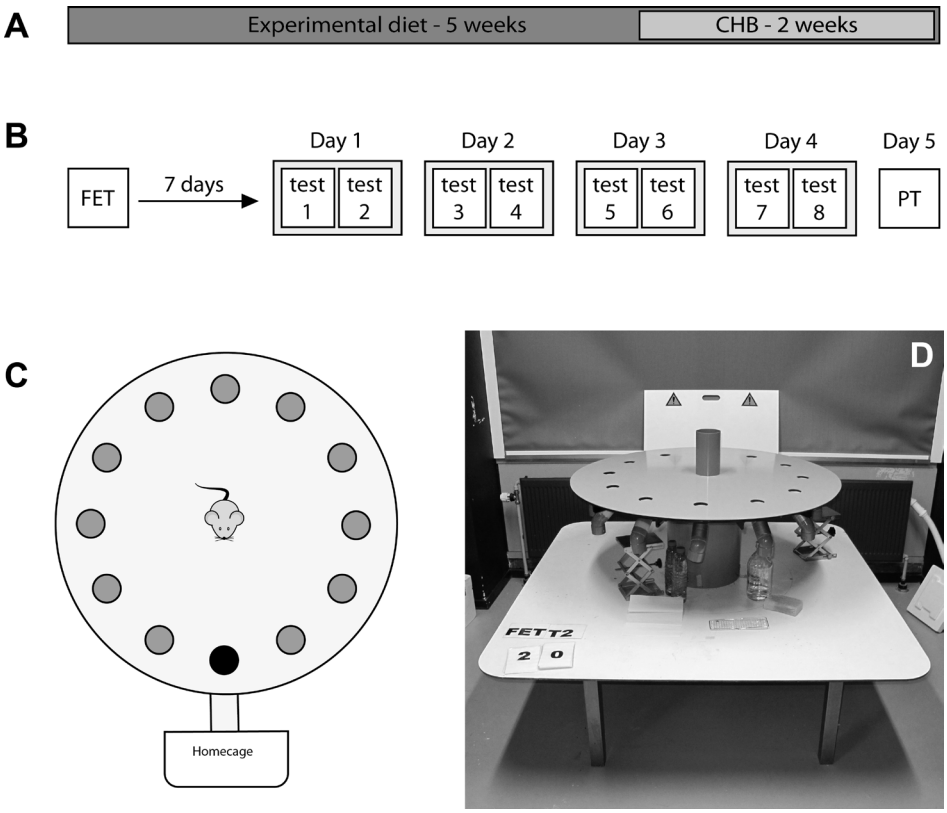

Figure 1: Setup of circular hole board experiment. A: Animals were put on a dox diet for at least 5 weeks before the CHB was started. B: The CHB paradigm started with a free exploration trial (FET). 7 days later the animals followed a training for 4 consecutive days with 2 trials a day. At day 5 the animals were exposed to a probe trial in which the escape hole was closed. C: The hole board was equipped with 12 holes. During the training, 1 hole was open and animals could reach their home cage. D: Picture of the setup in the lab.

Procedure

At day 1, each mouse started with a Free Exploration Trial (FET) of 300 sec. All holes were closed and the mouse was allowed to move freely over the board. Seven days after the FET the animals proceeded with a 4 days training session with two trainings a day (120 sec) in which the mice learned to find the exit to their home cage. One day after the training sessions the animals were once again placed on the board for a FET of 120 sec.

Behavioural assessments

Video recorded behaviour was automatically analyzed (distance moved, velocity) by Ethovision software (Noldus BV, Wageningen, The Netherlands) combined with manually collected data like hole visits, latency to target and the escape latency.

Statistics

Results are expressed as mean ±S.E.M. and unless stated otherwise a Student's t-test was performed using Prism 4.00 (GraphPad Software Inc., San Diego, CA). Behavioural data is tested with a General Linear Model (GLM) for repeated measurements in SPSS statistical software version 20 (IBM, SPSS Inc. Chicago,IL).

Results

Generation of DCL-KD mice.

To create an inducible DCL-specific knockdown mouse, we designed a shRNA molecule that targets the 3'-UTR of the DCL mRNA that is absent in other splice-variants of the DCLK gene (see Fig. 2A) and has no significant homology with other members of the DCX family. This DCL-specific shRNA was used to generate doxycycline-inducible knockdown mice according standard procedures (Seibler et al, 2007). No obvious phenotypic differences were observed with respect to weight, breeding and behaviour in the transgenic DCL-KD mice compared to their littermate WT controls. We checked the expression of DCL-targeting shRNA with or without dox administration by a DCL-specific custom-made qPCR approach.

As expected, no shRNA-DCL expression is detected in WT littermate mice (data not shown). Strong hairpin induction is found in both hippocampus and olfactory bulb of DCL-KD mice (in both cases; student's t-test, n=4, two-tailed, *** $p < 0.0001$). Compared to transgenic littermates on control diet, an 10 (Hi) and 25 (OB) fold higher expression of shRNA was measured in transgenic animals on dox diet (see Fig. 2D). To investigate specificity of the DCL shRNA

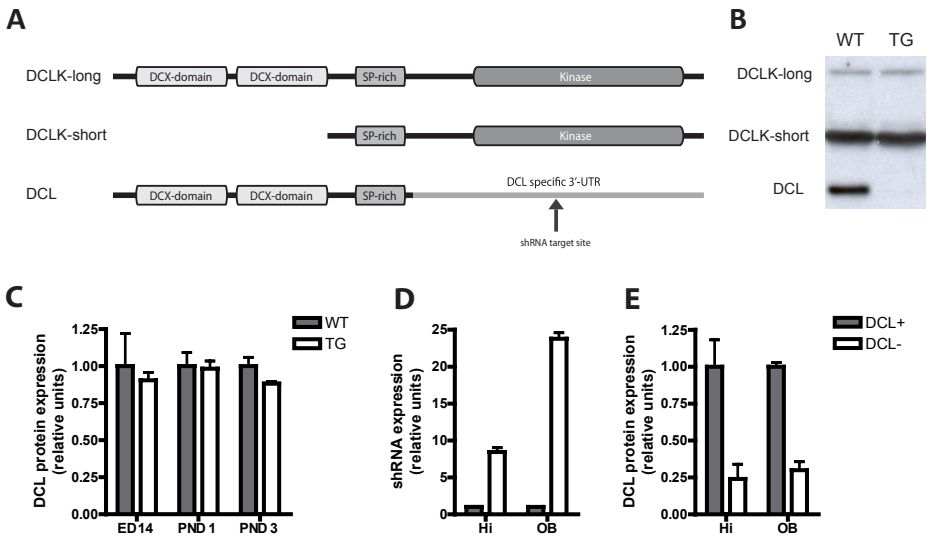

Figure 2: Specific knockdown of DCLK1 splice variant DCL. A) Overview of the three most important DCLK1 splice variants and their functional components. The shRNA target sequence resides in the 3'-UTR of DCL mRNA which is absent in DCLK-long and DCLK-short. B) Western blot analysis reveals splice variant specific knockdown of DCL in dox induced transgenic (TG) animals compared to dox induced wildtype (WT) animals. DCLK-long and DCLK-short expression is not affected. C) Although there is some leakage, this leakage does not affect hippocampal DCL expression during embryonic development. There is no significant difference in DCL expression between non-induced wildtype (WT and transgenic (TG) littermates at embryonic day 14 (ED14) and postnatal day 1 and 3 (PND1 & PND3). D) After dox induction, in the hippocampal tissue (Hi) an almost 10-fold higher shRNA expression measured compared to non induced transgenic littermates.(student's t-test, n=4, two-tailed, *** $p < 0.0001$) In the olfactory bulb (OB) a nearly 25-fold higher shRNA expression is measured (student's t-test, n=4, two-tailed, *** $p < 0.0001$). E) In both hippocampus (Hi, student's t-test, two-tailed, control n=4, dox n=5, ** $p<0.01$) and olfactory bulb (OB, student's t-test, two-tailed, control n=4, dox n=5, *** $p<0.0001$) DCL protein expression is reduced to 25% after dox induction compared to non induced transgenic littermates.

we analyzed the expression of all DCLK1 gene derived proteins by Western blot analysis. DCL protein levels were reduced to 25% after doxycycline administration in both hippocampus and olfactory bulb (Fig. 2E) while the expression levels of other DCLK1 gene-derived proteins are not affected (Fig. 2B). To check for possible fluctuations in DCL expression during neuronal embryogenesis and early postnatal development, a neuronal developmental time-window depending critically on proper expression of DCLK1 gene expression, we inspected DCL expression at embryonic day 14 and postnatal day 1 and 4 by western blot analysis. We found no significant differences in DCL protein levels in DCL-KD animals compared to their littermate WT controls. Together, we concluded that we generated a reliable mouse model with inducible DCL-specific knockdown.

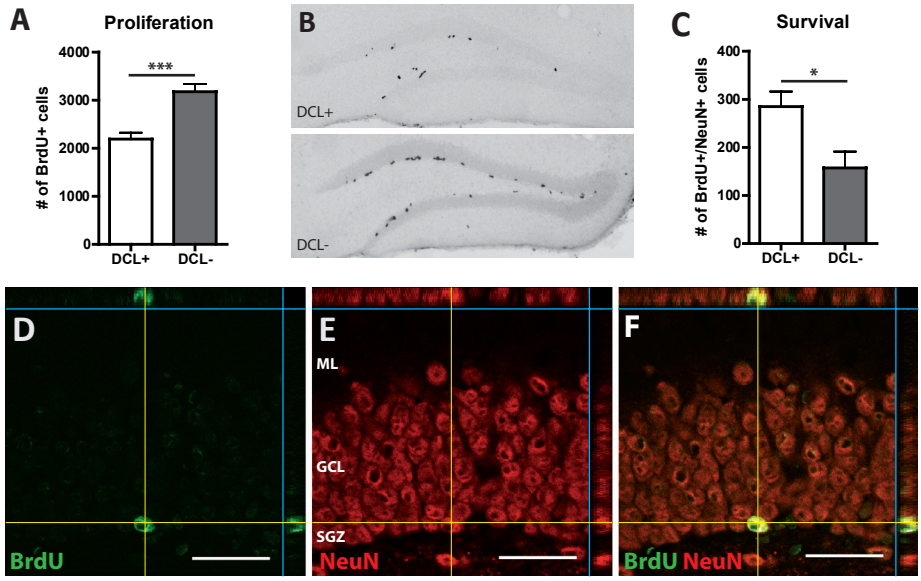

Figure 3: Adult neurogenesis measurement using BrdU labelling. A: 24 hours after a single BrdU injection, a highly significant ($p<0.05$, two-tailed) increase in BrdU positive cells was measured in dox induced transgenic animals (n=6) compared to non-induced transgenic littermates (n=6). B: Examples of hippocampi derived from animals killed 24 hours after BrdU injection. Both sections are stained for BrdU and show mainly BrdU positive cells in the subgranular zone. Tissue is derived from dox induced transgenic animals (DCL-) and non-induced transgenic littermates (DCL+) C: BrdU/NeuN double staining revealed a significant decrease ($p<0.05$, two-tailed) in double positive cells in hippocampal dentate gyrus of dox induced transgenic animals (dox, n=5) compared to non-induced transgenic littermates (control, n=4). D-F: Confocal laser scanning microscopy images showing co localization of BrdU (green in D) and NeuN (red in E). Only cells in the dentate gyrus that are double positive (yellow in F) were counted. Scale bar in D-F measures 25µm.

DCL knockdown stimulate proliferation but reduces survival of NPCs.

During embryonic development and in cell lines, the DCLK1 gene has been implicated in the formation of mitotic spindles and proliferation of NPCs and in survival of neuroblasts. Therefore, to investigate the role of the DCL splice-variant in proliferation and survival of adult hippocampal NPCs in vivo, we administered the proliferation marker BrdU (Fig. 3B,C and E) to DCL-KD mice and sacrifice these animals after 24 hrs (proliferation) and after 4 weeks (survival). DCL-KD mice on dox diet showed 1.51 more BrdU positive cells 24 hours after injection compared to non-induced transgenic littermates (student's t-test, two-tailed, n=6, *** $p<0.001$, see Fig. 3A). We measured the survival of newborn NPC's using BrdU in combination with the adult neuron marker NeuN (see Fig. 3D-F). Dox induced DCL-KD-mice killed 4 weeks after the last BrdU injection showed a significant reduction of almost 50% of BrdU/NeuN double positive cells (student's t-test, two-tailed, control n=4, dox n=5, * $p<0.05$, see

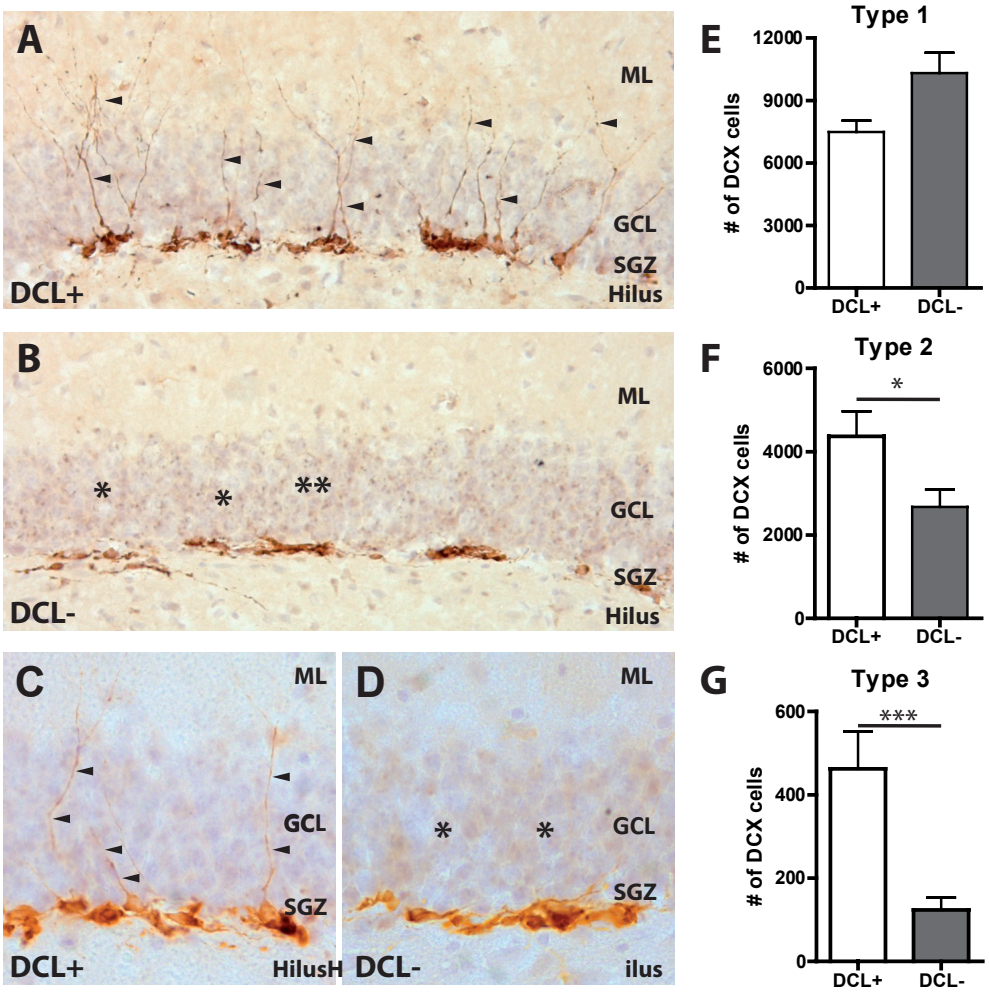

Figure 4: DCX cell morphology. A: DCX expressing cells in the hippocampal dentate gyrus of a transgenic animal on an control diet showing a normal DCX morphology with cell nuclei close to the subgranular zone (SGZ) and dendrites towards the molecular layer (ML). B: Hippocampal dentate gyrus of a dox induced transgenic littermate showing aberrant morphology of DCX positive cells. Hardly any DCX positive cell has dendrites in the granular cell layer (GCL) or ML. C-D: Close-up of DCX expressing cells in the hippocampal dentate gyrus of a transgenic animal on a control diet (C). Several DCX positive cells show dendritic outgrow (arrows) towards the molecular layer which are absent after DCL knockdown (D). E: Number of proliferating type 1 DCX positive cells in transgenic animals on a control or dox diet. There is no significant difference between both groups (t-test, two-tailed, p=0.064). F: Number of intermediate stage type 2 DCX positive cells in transgenic animals on a control or dox diet. There is a significant difference between both groups (t-test, two-tailed, p=0.05). G: Number of post mitotic stage type 3 DCX positive cells in transgenic animals on a control or dox diet. There is a significant difference between both groups (t-test, two-tailed, p=0.001).

Fig. 3C). Proliferation and cell survival in wildtype animals were similar as in non-induced transgenic animals. Together, this dataset suggests that proper DCL expression is necessary for NPC survival in the dentate gyrus of the hippocampus.

To investigate the role of DCL in neurogenesis in more detail, we labelled neuronal progenitor cells with DCX, a well-established marker for neurogenesis (Brown et al., 2003b). The expression of DCX is restricted to two types of proliferating neuronal precursor cells with no or short processes (here called type 1) or medium processes reaching the molecular layer of the dentate gyrus (here called type 2) and post-mitotic neuroblasts characterized by elongated dendrites branching into the granule cell layer and molecular layer (here called type 3; categorized after (Oomen et al., 2010; Plumpe et al., 2006)). Although a trend (p=0.0638) of 1.2 more DCX-positive type 1 cells was found, there was no significant difference in the number of proliferative type 1 DCX cells between induced and non-induced DCL-KD mice (Fig. 4E). However, we found a clear and significant phenotypical difference in the populati-

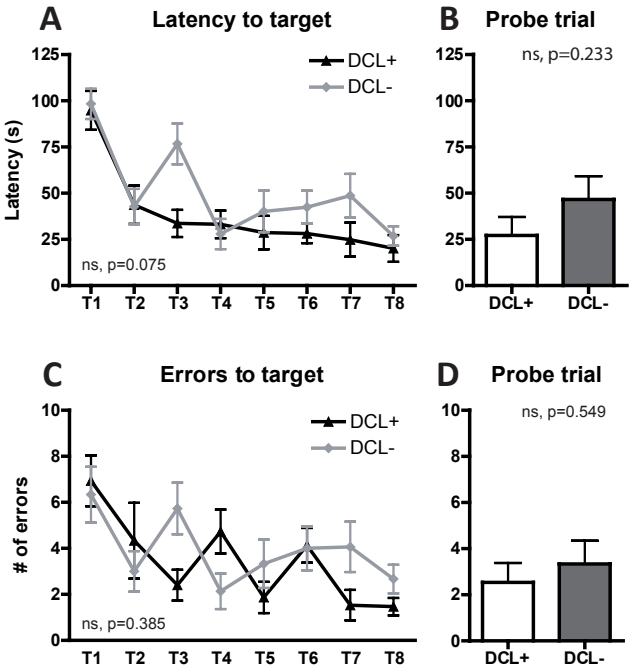

Figure 5: Spatial parameters measured on the circular hole board. DCL knockdown did not affect the latency to target during training sessions (A, GLM, F(1)=3.426, p=0.075) and probe trial (B, t-test, t(28)=1.219, p=0.233). Animals with DCL knockdown did not make more errors before reaching the target during both training (C, GLM, F(1)=0.779, p=0.385) and probe trial (D, t-test, t(28)=0.607, p=0.549).

on of DCX+ type 2 and particularly type 3 cells after DCL knockdown (see Fig. 4A-D). Interestingly, we found significant (p=0.0359) 0.61 less type 2 cells (Fig. 4F) and even 0.27 less type 3 cells (p=0.0008; Fig. 4G) Thus, DCL knockdown clearly leads to a reduction of intermediate proliferating type 2 cells and particularly reduce the number of post mitotic type 3 cells.

DCL-knockdown mice exhibit increased latency to escape from the circular hole board.

Numerous studies indicate that aberrant neurogenesis in the adult hippocampus is associated with disease-associated impaired learning and memory formation (see e.g. Clelland et al., 2009; Fitzsimons et al., 2013; Sahay et al., 2011a; for reviews see Petrik et al., 2012; Samuels and Hen, 2011). To investigate possible functional consequences of DCL-KD induced aberrant neurogenesis, we used the circular hole board paradigm, a behavioural task aiming to study hippocampal memory performance.

Four groups (N=16 each), transgenic mice with and without dox and their wildtype littermate controls were subjected to 8 training sessions during 4 consecutive days followed by a free exploration trial with closed exit hole (probe trial: PT; see Fig. 1). As both wildtype groups were indistinguishable from the transgenic mice without dox, for reasons of clarity we only compare here the with (DCL-KD) and without dox (DCL+) transgenic groups (for all control groups see Fig. 7). DCL knockdown had no effect on the parameters 'latency to target' (t-test, t(28)=1.219, p=0.233, Fig. 5B) and 'errors to target' (t-test, t(28)=0.607, p=0.549, fig. 5D). Both DCL-KD mice and DCL+ mice showed a similar decrease over 4 training days

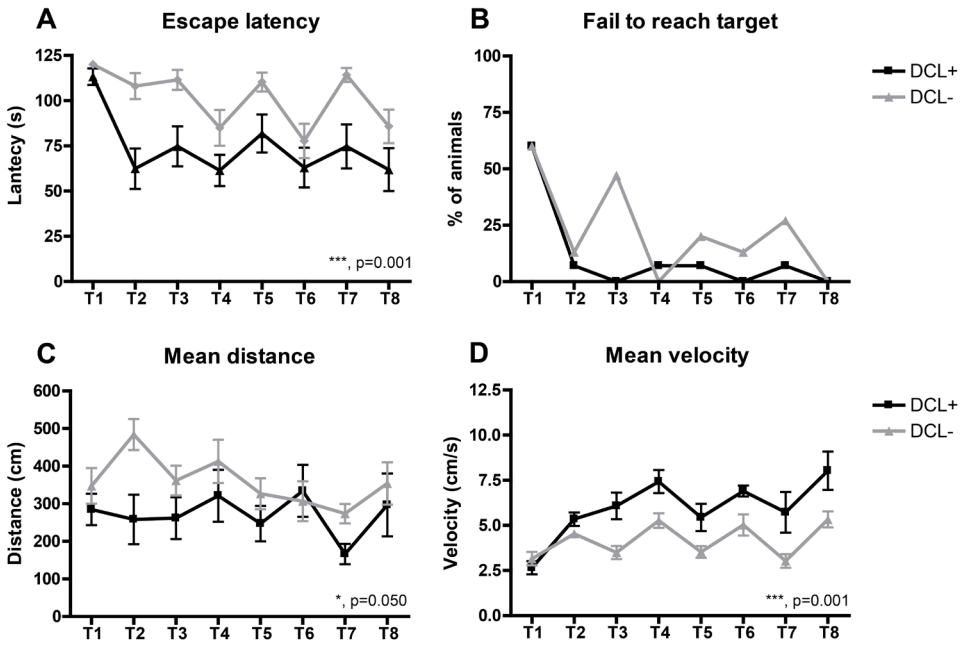

Figure 6: Motivational parameters measured on the circular hole board. A: DCL-KD animals showed a significant longer escape latency compared to DCL+ animals (GLM, F(1)=12.813, p=0.001). B: Percent of animals who did not reach the target within 120 seconds. C: Mean distance moved during each trial. DCL-KD animals move a significant longer distance compare to DCL+ animals (GML, F(1)=4.198, p=0.050). D: Average velocity during each trial. DCL-KD animals are significant slower compared to DCL+ animals (GML, F(1)=15.101, p=0.001).

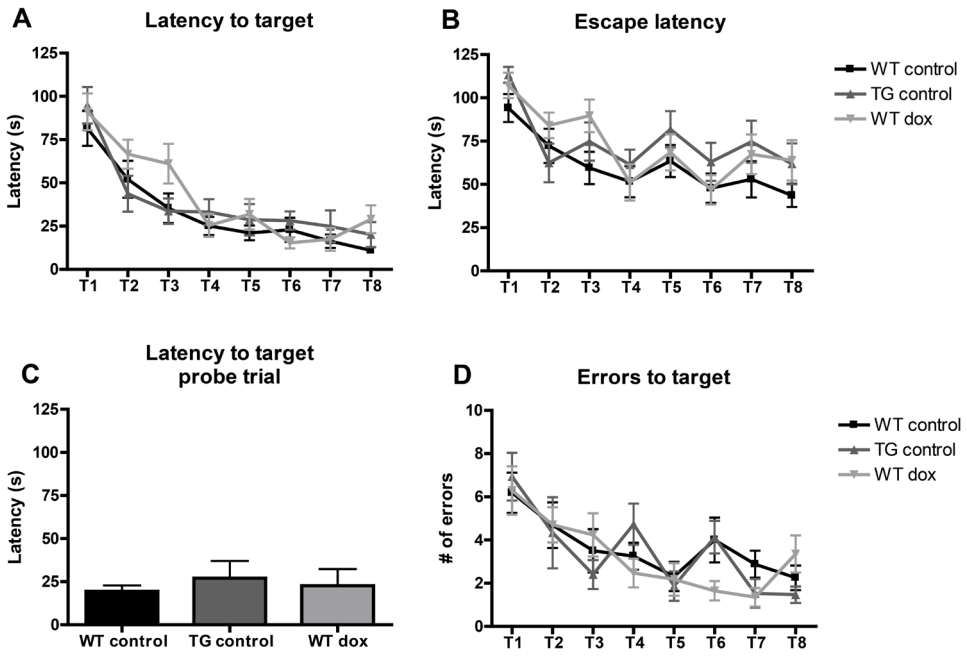

Figure 7: CHB score of all control groups. Wildtype and non-induced transgenic animals did not differ in latency to target (A), escape latency (B) and number of errors to target (D) during the training sessions. There is also no significant difference in latency to target during the probe trial (C).

in latency to target (GLM, $F(3)=18.433$, $p<0.001$) and errors to target (GLM, $F(3)=6.392$. $p=0.001$) and exhibit similar errors to find the target, suggesting that both groups learned the task equally well. Also, we observe no significant differences between the two groups in the probe trial indicating that DCL knockdown does not affect spatial learning parameters in the circular hole board task. However, surprisingly, we observe a highly significant effect on escape latency (GLM, $F(1)=12.813$, $p=0.001$, Fig. 6A) whereby DCL-KD animals exhibit a strong delay in leaving the board after finding the exit hole, to their home cage. This finding is supported by the longer moved distance (GML, $F(1)=4.198$, $p=0.050$, Fig. 6C), lower velocity (GML, $F(1)=15.101$, $p=0.001$, Fig. 6D) and the higher number of animals that failed to reach the target (fig. 6B). This suggests that DCL-KD animals are less motivated to escape from an aversive environment.

Discussion

Here we show that DCL is implicated in adult hippocampal neurogenesis. Surprisingly, DCL knockdown does not affect spatial learning but is significantly associated with reduced escape latency on circular hole board. Knockdown of DCL leads to a significant increase in

the number of proliferating cells in the subgranular zone one day after BrdU administration. However, the number of newborn adult NeuN+ cells are significantly decreased when studied 4 weeks after BrdU administration suggesting a suppression of neuronal development after DCL-KD. In line with this, the number of post-mitotic DCX+ NPC's are dramatically reduced. As other splice-variants of the DCLK1 gene are unaffected and expressed at normal levels, our results demonstrate a role for DCL in the differentiation of newborn neurons that is not compensated for by other DCLK splice variants or other members of the DCX gene family including DCX. Strikingly, DCL-KD strongly reduces the escape latency of mice on the circular hole board but does not affect other aspects of this behavioural task. Together, our analysis indicates a key role for DCL in cell proliferation, migration and maturation. DCL is furthermore involved in motivational aspects to escape from an aversive environment.

DCL-KD leads to a significant decrease in the number of post-mitotic NeuN+/BrdU+ cells while the number proliferating BrdU+ cells are increased. These data suggest involvement of DCL in cell proliferation and subsequent survival of new born neurons. Indeed, the DCLK1 gene has been shown to regulate dendritic development (Shin et al., 2013) and the form of mitotic spindles in embryonic NPC's and neuroblasts in vitro and in vivo (Shu et al., 2006;Vreugdenhil et al., 2007). In C. elegans, the orthologue of the DCLK1 gene, zyg-8, regulate a-symmetric division of fertilized eggs by controlling the length of mitotic spindles (Gonczy et al., 2001). Also in mammals, a correct positioning of mitotic spindles in radial glia cells has been associated with proper differentiation of the resulting neuronal daughter cells (Lancaster and Knoblich, 2012). Initial neuro-epithelial cell division may occur symmetrical and subsequently, neuronal progenitors cells, i.e. radial glia cells, are believed to divide asymmetrically during embryonic neurogenesis. In analogy with such a proliferation and differentiation scheme, type 1 and type 2 DCX+ cells may represent symmetric dividing progenitor cells while type 3 post-mitotic DCX+ cells may be the result of an a-symmetric cell division requiring functional DCL. Additionally, The DCLK gene has been shown to be a pro-survival gene in neuroblastoma cells (Kruidering et al., 2001) and is a target for pro-apoptotic enzymes such as caspases and calpain (Burgess and Reiner, 2001;Kruidering et al., 2001). Moreover, DCLK knockdown by RNA-interference technology leads to the activation of a pro-apoptotic program in neuroblastoma cells (Verissimo et al., 2010a) and to a reduction of neuronal progenitor cells during neocortical development in vivo (Vreugdenhil et al., 2007). As the shRNA molecule targets DCL specifically, leaving other DCLK splice-variants unaltered, our data indicate a role for DCL in the transition and survival of proliferating to post-mitotic DCX+ NPCs.

Knockdown of DCL leads to a phenotypic change of DCX+ cells. This finding suggests that both DCL and DCX are expressed in the same NPC's in the subgranular zone of the dentate gyrus. In line with DCL/DCX co localization are the phenotypic analysis of Dcx/Dclk1 double

knockouts mice showing functional redundancy during hippocampal lamination (Tanaka et al., 2006). Also, gene expression profiling of human primary neuroblasts clearly demonstrate co-expression of DCX and DCL. Moreover, our recent immunohistochemical experiments also showed DCX-DCL co-localization in NPC's in the subgranular of the dentate gyrus and in neuroblasts in the rostral migratory stream (Saaltink et al., 2012). Thus, it seems that co localization of DCX and DCL are required for proper neuronal migration and differentiation. However, at the sub cellular level it seems that DCX and DCL are located at different locations with prominent DCX signals that follows projections forming a dendritic blueprint (see e.g. Fig. 3A) while DCL mainly appeared in speckles at specific dendritic hotspots (Saaltink et al., 2012). Also, detailed immunohistochemical analysis during embryonic development shows spatiotemporal differences in expression of DCX and DCL (Boekhoorn et al., 2008). Thus, it seems that DCL and DCX have different sub cellular functions in within a cell. In this respect, it is interesting to mention the study of Merz & Lie (Merz and Lie, 2013) who did not see altered morphological maturation of adult born dentate granule cells or migration of new neurons in either adult neurogenic niche after siRNA mediated DCX knockdown. This is rather surprising since it is thought that both DCX and DCL play a crucial role in this process.

Our data is not consistent with earlier findings in our lab regarding the role of DCL in intracellular GR transport (Fitzsimons et al., 2008) and the effect of GR knockdown on migration and maturation of new born neurons (Fitzsimons et al., 2013). SiRNA mediated GR knockdown leads to hyperactive neuronal migration and maturation. Since DCL is directly involved in intracellular GR transport, one should expect similar hyperactive neurogenesis after DCL knockdown. Since activated GR's are associated with reduced neurogenesis (Gould et al., 1998), the increased proliferation after DCL knockdown fits into the picture of reduced GR activity although GR knockdown did not affect proliferation (Fitzsimons et al., 2013). More compromising is the strongly reduced migration and maturation of new born neurons which is opposite to GR knockdown mediated hyperactive development. Apparently, DCL serves more functions beside GR transport.

DCL knockdown results in aberrant adult neurogenesis but does not affect spatial learning on the circular hole board. This finding is somewhat unexpected as several studies reported association of reduced neurogenesis and impaired spatial and contextual learning in several behavioural tasks such as contextual fear conditioning (Saxe et al., 2006) and, similar as the circular hole board, the Barnes maze (Imayoshi et al., 2008). However, these findings were not reproduced by numerous other investigators (Martinez-Canabal et al., 2013;Meshi et al., 2006;Shors et al., 2002;Zhang et al., 2008). For example, even complete ablation of neurogenesis in cyclin D2 knockout mice leads to normal spatial learning and contextual memory formation (Jaholkowski et al., 2009;Jedynak et al., 2012;Urbach et al., 2013). Moreover, addition of new neurons is not necessary for hippocampus-dependent learning (Fran-

kland, 2013) but may be involved in forgetting, although this is dependent on the memory task used and its timing in relation to neurogenesis. Recent studies suggest a role for adult neurogenesis in a more subtle cognitive hippocampal function, i.e. pattern separation (Clelland et al., 2009;Sahay et al., 2011a). Thus, the circular hole board paradigm may be too robust to find possible cognitive hippocampus-mediated impairments after DCL knockdown. Alternatively, DCL knockdown leads to approximately 75% reduction of adult-born post-mitotic neurons (Fig. 3G), which may be insufficient to detect neurogenesis-related behavioural differences.

Surprisingly, DCL-KD leads to a highly significant increase in the latency to leave the circular hole board. Possibly, motivation to leave the CHB, might be fear-regulated by the aversive environment created by the board and as such, comparable with context fear conditioning which may be partly regulated by adult neurogenesis (Denny et al., 2012;Drew et al., 2010). Also, this increase in latency is associated with more motor activity with longer moved distances after DCL knockdown, a phenomenon that is also linked to a lessioned hippocampus (Deacon et al., 2002). Alternatively, although DCL has a highly restrictive expression pattern in the hippocampus (Saaltink et al., 2012), we cannot exclude the possibility that other brain areas are involved. In particular, DCL is also highly expressed in the olfactory bulb (OB). Ablation of newly born neurons does not affect olfactory detection levels, however, it might affect downstream processing of odour information (Gheusi et al., 2000;Imayoshi et al., 2008) and as such DCL knockdown might impair olfactory discrimination. Therefore, impaired olfaction might result in impaired recognition of the home cage, which might explain the increased latency to leave the board. However, olfaction is an equally important parameter to learn spatial memory tasks adequately (Machado et al., 2012;van Rijzingen et al., 1995). Moreover, we did not observe any differences, as in the hippocampus, in the form and number of DCX+ cells in the OB (see supplemental Fig. 1) while DCL is also expressed in other brain areas characterized by a high level of neuronal plasticity (Saaltink et al., 2012). Therefore, we favour the hypothesis that the increase in latency is due to impaired structural alterations in the dentate gyrus.

In conclusion

We have successfully generated an transgenic animal model to study the role of a specific splice-variant of the DCLK gene, i.e. DCL, without affecting the expression of the other splice-variants DCLK-long and DCLK-short. Using this model, we found that DCL is involved in the transition of proliferating NPCs into post mitotic neuroblasts. Moreover, behavioural studies show that DCL may be involved in motivational aspects to escape from aversive environments. Our model seems an valuable in vivo tool to study these areas and the role of DCL therein, in a multidisciplinary fashion.

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