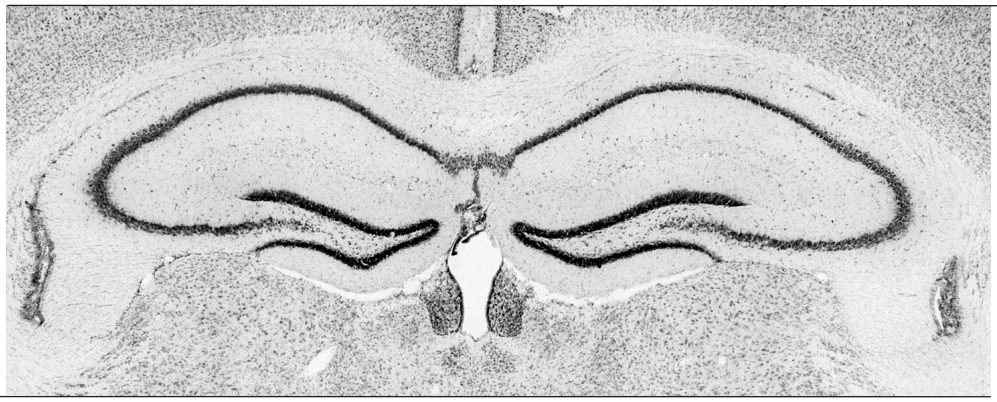
Chapter 2

Doublecortin and Doublecortin-like are expressed in overlapping and non-overlapping neuronal cell population

implications for neurogenesis.



Dirk-Jan Saaltink[1], Bjarte Håvik[2,3], Carla S Verissimo[1], Paul Lucassen[4] and Erno Vreugdenhil[1]

Journal of Comparative Neurology, 2012, 520(13):2805-23.

1 Department of Medical Pharmacology,
Leiden University Medical Center/Leiden Amsterdam Center for Drug research, Leiden, the Netherlands.

2 Department of Clinical Medicine, University of Bergen, Bergen, Norway.

3 Dr. Einar Martens Research Group for Biological Psychiatry,
Center for Medical Genetics and Molecular Medicine, Haukeland University Hospital, Bergen, Norway.

4 Center for Neuroscience, Swammerdam Institute for Life Sciences,
University of Amsterdam, Amsterdam, the Netherlands.

Abstract

We have characterized the expression of doublecortin-like (DCL), a microtubule associated protein involved in embryonic neurogenesis that is highly homologous to doublecortin (DCX), in the adult mouse brain. To this end, we developed a DCL-specific antibody and used this to compare DCL expression with DCX. In the neurogenic regions of the adult brain like the subventricular zone (SVZ), the rostral migratory stream (RMS), the olfactory bulbus (OB) and the hippocampus, DCL co-localizes with DCX in immature neuronal cell populations. In contrast to DCX, we also found high DCL expression in three other brain regions with suspected neurogenesis or neuronal plasticity. Firstly, the radial glia-like, hypothalamic tanycytes show high DCL expression that partly co-localizes with the neural stem cell marker vimentin. Secondly, DCL expression is found in cells of the suprachiasmatic nucleus (SCN), which lacks expression of the adult neuron marker NeuN. Thirdly, a novel region exhibiting DCL expression is part of the olfactory tubercle where DCL is found in the neuropil of the islands of Calleja. Our findings define DCL as a novel marker for specific aspects of adult neurogenesis, which partly overlap with DCX. In addition, we propose unique roles for DCL in adult neurogenesis and we suggest high levels of neuronal plasticity in tanycytes, SCN and islands of Calleja.

Introduction

The doublecortin (DCX) gene family has been associated with several CNS disorders and comprises 11 paralogues in both human and mice (for review see (Dijkmans et al., 2010)). Each member is characterized by the presence of a doublecortin domain enabling the encoded protein to bind to microtubules (Kim et al., 2003) thus defining them as microtubule (MT) associated proteins (MAPs). The archetypical protein of the DCX family is doublecortin or DCX (Francis et al., 1999;Gleeson et al., 1999). Mutations in the x-linked DCX gene has been associated with the doublecortex syndrome in humans (des Portes et al., 1998;Gleeson et al., 1998) and leads to arrest of migrating neuronal progenitor cells (NPCs) during embryonic development (Francis et al., 1999;Gleeson et al., 1999). Because its specific expression in neuronal progenitor cells, DCX is frequently used as a neurogenesis marker (Brown et al., 2003b;Couillard-Despres et al., 2005;Rao and Shetty, 2004;Couillard-Despres et al., 2006). The highly conserved DCX sequences function as MT stabilizers (Horesh et al., 1999) and sub cellular translocators (Fitzsimons et al., 2008;Reiner et al., 2006). However, DCX has also been reported to be expressed in non-neurogenic brain areas where it is thought to play a role in microtubule reorganization and synaptogenesis (Dehmelt and Halpain, 2007;Friocourt et al., 2003;Nacher et al., 2001).

Surprisingly, DCX knockout mice exhibit normal development of the neocortex (Corbo et al., 2002), suggesting a compensatory mechanisms by other DCX gene family members (Tuy et al., 2008). Indeed, mice mutants for both Dcx and doublecortin-like kinase-1 (Dclk1) exhibit disorganized neocortical layering suggesting that (Dclk) gene functions in a partially redundant pathway with Dcx (Corbo et al., 2002;Deuel et al., 2006;Koizumi et al., 2006). Unlike DCX is DCLK1 a complex gene with several alternative splice variants (Vreugdenhil et al., 2001). Interestingly, one splice-variant, called doublecortin-like (DCL) shares 73% amino acid identity with DCX over its entire length of 362 amino acids and is also having two DCX domains. As DCX, DCL is important for corticogenesis. However its spatio-temporal expression pattern during development is remarkably different from DCX, where expression can already be found at ED9 in mitotic spindle structures in ventricular zone cells (Boekhoorn et al., 2008;Vreugdenhil et al., 2007). Also, DCL knockdown at ED13 by in utero electroporation results in disruption of radial processes and DCL-immunoreactive cells display radial glia cell-like morphology and are double-labelled with the radial glia marker vimentin. Thus, during embryogenesis, DCL seems specifically expressed in radial glia cells that are precursor cells generating many, if not all, neurons (Anthony et al., 2004).

In contrast to corticogenesis, little is known about DCL expression in the adult brain. This might be due to the difficulty to produce DCL-specific antibodies that will not recognize other splice-variants of the DCLK-gene (see e.g. Kruidering et al., 2001). Here, we describe

the generation of a DCL-specific antibody that was used to map DCL expression in the adult mouse brain. As expected, we found profound expression in NPCs in the subgranular zone of the dentate gyrus and in progenitor cells of the subventricular zone, the rostral migratory stream and in the bulbus olfactorius where it is co-expressed with DCX. Unexpectedly and in contrast to DCX, we found profound expression of DCL in the islands of Calleja, in the suprachiasmatic nucleus and in tanycytes. Our findings indicate that the roles of DCL and DCX partly overlap, but that they also show differences and reveal other brain areas potentially requiring high levels of neuronal plasticity.

Materials and methods

Animals and tissue preparations

Three month old B6129S6F1 male mice were obtained from our outbred colony (derived from TaconicArtemis, Cologne, Germany). The animals were kept under a 12/12 LD cycle (light on from 7:00 to 19:00h), in a temperature controlled room (23°C). Water and food were available ad libitum. This experiment was approved by the Local Animal Welfare Committee of the University of Leiden, the Netherlands.

Before the procedure the animals were deeply anaesthetized by IP injection of sodium pentobarbital (Euthasol 20%, ASTPharma bv, Oudewater, The Netherlands). Thereafter the mice were transcardially perfused with ice-cold 0.1M phosphate buffered saline (PBS) and subsequently with 4% paraformaldehyde in 0.1M PBS (PFA). After perfusion, the mice were decapitated and the heads kept in 4% PFA overnight at 4°C for post fixation. The next day, brains were removed and put in a 15% sucrose solution (0.1M PBS) overnight at 4°C for dehydration. Subsequently, the brains were put in a 30% sucrose solution for another night at 4°C. At the end of the dehydration procedure the brains were removed from the solution and blotted dry before snap-freezing. The brains were kept at -80°C until used for cryosectioning.

Serial coronal 30µm-thick sections were obtained using a cryostat (Leica CM 1900, Leica Microsystems, Rijswijk, The Netherlands). All brain sections were collected in 2ml eppendorfs containing anti-freeze (50%glycerol, 50% 0.2M PB) and stored at -20°C until further use.

Antibodies

A novel, DCL specific antibody was generated in rabbits by injection of an 18-amino acid-long synthetic peptide (QRDLYRPLSSDDLDSVG-C) corresponding to exon 7 and 8 of DCLK1 which is specific for the splice variants DCL and CARP (Vreugdenhil et al., 2007). Western

blot analysis of liver and brain tissue, cell lysates of transfected and non transfected COS cells (described previously by Vreugdenhil et al., 2007) show that anti-DCL antibody recognizes DCL but not other splice variants like DCLK-long and DCLK-short (Fig. 1B) or the highly homologues DCX protein (Fig. 1C). As expected, no DCL signal was found in the liver.

To show the localization of DCL in specific tissue, several antibodies were used as tissue markers. They are described in detail in table 1. To visualize immature neurons, anti-doublecortin (DCX) was used (sc-8066, Santa Cruz Biotechnology, California, USA), adult neurons were visualized by anti-NeuN (MAB3777, Millipore Billerica, Massachusetts, USA). In our hands, both antibodies showed an expression pattern as expected in neurogenic regions. Tanycytes were visualized using anti-vimentin (AB5733, Millipore, Billerica, Massachusetts, USA) which shows a pattern similar to vimentin positive tanycytes as reported by others (Mullier et al., 2010;Sanchez et al., 2009). To mark the suprachiasmatic nucleus (SCN), anti-AVP (T-5048, Bachem, Bubendorf, Switzerland) was used. The antibody showed a characteristic pattern of AVP expression in the supraoptic nucleus (SON), paraventricular nucleus (PVN) and SCN as described by (Biancardi et al., 2010). In the SCN, anti-AVP showed a specific expression pattern as reported for AVP by (Karatsoreos et al., 2004).

For immunofluorescent staining Alexa Fluor® 488 donkey anti-rabbit IgG or Alexa Fluor® 594 goat anti-rabbit IgG were used to visualize DCL. Alexa Fluor® 594 donkey anti-goat IgG, Alexa Fluor® 594 donkey anti-mouse IgG, Alexa Fluor® 488 goat anti-chicken IgG and Alexa Fluor® 594 goat anti-Guinea pig IgG (Invitrogen, the Netherlands) were used to visualize respectively DCX, NeuN, vimentin and AVP. For the DAB reaction, biotinylated goat-anti-rabbit (sc-2040) was obtained from Santa Cruz to react with the primary DCL antibody.

Immunocytochemistry

DAB staining

Before 3x 10 minutes being washed in 0.05M Tris-buffered saline (TBS), free floating sections were left at room temperature for 15 minutes. To block endogenous peroxidase activity slides were incubated for 15 minutes in 0.5% H2O2 in TBS. After washing in TBS (4x 5 minutes) the slides are incubated in 2% low-fat milk powder (Elk, Campina, the Netherlands) in TBS for 30 minutes. Primary antibodies were applied to the slides in supermix (0.25% gelatine, 0.1% TX-100 in TBS) and left at room temperature for 1 hour followed up by overnight incubation at 4°C.

Subsequently, slides were washed in TBS and incubated in secondary antibody for 2 hours at room temperature. After washing with TBS, the secondary antibody is amplified with avidin-

biotin enzyme complex (ABC kit; Elite Vectastain, Brunschwig Chemie, Amsterdam, 1:800); tyramide (Tyramide Signal Amplification (TSA™), Perkin Elmer, Massachusetts, USA) and developed with di-aminobenzidine (DAB (Sigma-Aldrich; 20 mg/100 ml tris buffer; TB, 0.01% H2O2). After mounting and drying overnight a haematoxylin counterstaining was applied to the slides were after dehydration in an alcohol series and slide covering with DPX was performed.

Immunofluorescent staining

Free floating sections were left at room temperature for 15 minutes before being washed in 0.1M phosphate-buffered saline (PBS) and blocked in 2% bovine serum albumin (BSA, sc-2323, Santa Cruz) in PBS for 2 hours. After three washing steps in PBS the primary antibodies were applied to the slides in PBS with 0.3% TX-100 and left at room temperature for 1 hour followed up by overnight incubation at 4°C. Subsequently the slides were washed in PBS and incubated in secondary antibody for 2 hours at room temperature. After washing with PBS the slides were counterstained with Hoechst (1:10000) for 10 minutes and washed again before they were mounted and covered using Aqua Poly/Mount (Polysciences, Inc.)

Nomenclature

Nomenclature of the brain regions depicted in the figures was based on the atlas of Paxinos and Franklin (Paxinos and Franklin, 2001).

Table 1: Antibodies used.

Antigen	Immunogen/peptide	Species	Catalog nr. and source	Dilution
DCL	QRDLYRPLSSDDLDSVG-C	rabbit polyclonal		1:1000
AVP	H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2	guinea pig polyclonal	T-5048 , lot#A03607, Bachem, Bubendorf, Switserland	1:2000
DCX	DLYLPLSLDDSDSLGDSMC-18 clone	goat polyclonal IgG	Sc-8066, Santa Cruz Biotechnology, California, USA	1:200
NeuN	Purified cell nuclei from mouse brain	Mouse monoclonal IgG1 clone A60	MAB377, Millipore, Billerica, Massachusetts, USA	1:200
Vimentin	Recombinant Syrian gold hamster vimentin	Chicken polyclonal	AB5733, NG1813637, Millipore, Billerica, Massachusetts, USA	1:2000

Photography

Light microscopy for DAB stained slides was performed on a Leica DM6000B microscope (Leica Microsystems, Rijswijk, the Netherlands) and pictures were taken with a Leica DC500 camera on top of the microscope. Fluorescent images were taken on a Nikon TE 2000e in combination with a Nikon C1 confocal scanner.
Images were imported in Adobe (San Jose, CA) Photoshop CS5 for Windows and not manipulated other than slight modifications of the contrast and brightness settings and occasional adjustment of evenness of illumination.

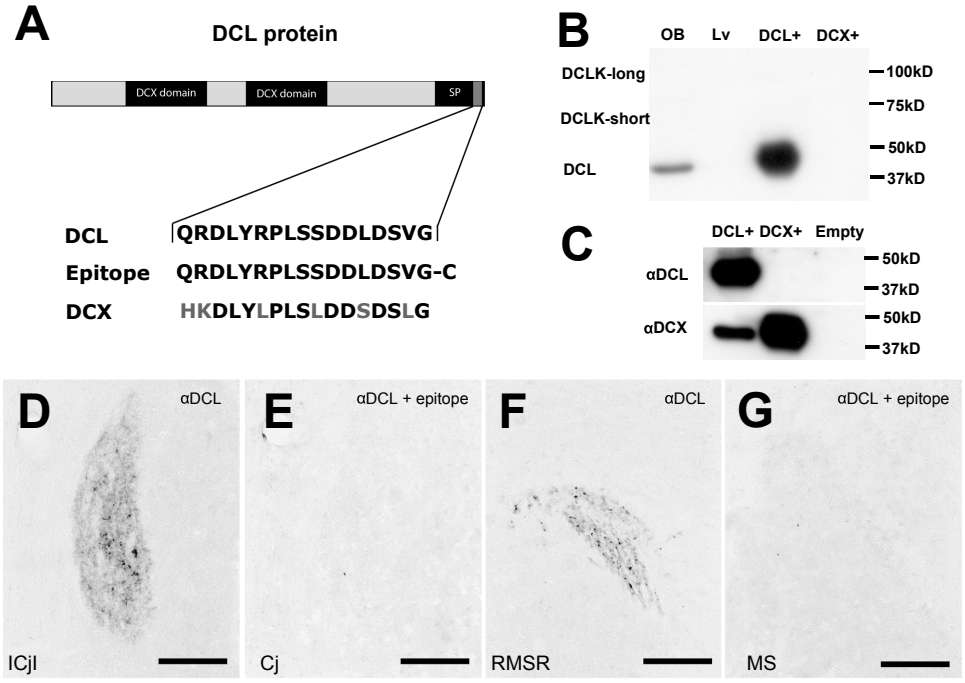


Figure 1: Antibody chatrecteristics of the novel anti-DCL. (A) Location of the antibody target site within the DCL protein and sequence allignment of the epitope, target protein DCL and the nearly homologous DCX. Differences in amino acid sequence between DCL and DCX are indicated in red. (B) Westernblot analysis of anti-DCL on different tissues and cell lysates. A 40kD band is visible in tissue derived from a mouse olfactory bulbus (OB) and COS-cells transfected with DCL protein (DCL+). Mouse liver tissue (Lv) and COS-cells transfected with DCX protein (DCX+) do not shows such a band. No bands are visible around 75 and 100kD which resemble respectively DCLK-short and DCLK-long. (C) Westernblot analysis of anti-DCL and anti-DCX on COS-cell lysates transfected with DCL, DCX or an empty vector. The novel antibody shows only a signal on DCL+ COS-cells, whereas anti-DCX shows a signal at DCX+ COS-cells and a thinner band at DCL+ COS-cells. Cells with an empty vector do not show a band. (D-G) Microphotographs of brain tissue containing ICj (D-E) and rostral migratory stream (F-G) stained with anti-DCL (D&F) and anti-DCL preabsorbtion with the epitope (E&G). Scalebars in D-G meassure 200µm.

Results

Characterization of antibodies

The primary structure of the synthetic peptide used to generate a DCL-specific antibody, corresponds to the C-terminus tail of DCL and CARP (Vreugdenhil et al., 1999;Vreugdenhil et al., 2007) but has no amino acids in common with other splice variants of the DCLK gene. The peptide deviates from DCX for several amino-acid positions (Fig.1A). Western blot analysis revealed a 40kD band, which corresponds to the size of DCL. As expected, it does not stain molecular weight bands that correspond to other DCLK1 splice variants like DCLK-short and DCLK1-long (Fig.1B). Furthermore, the antibody does not recognize to the highly homologues protein DCX. Surprisingly, some cross reactivity of anti-DCX was observed with recombinant DCL although less strong compared to DCX itself (Fig.1C). Incubation of anti-DCL with the epitope strongly reduced immunoreactivity in several brain regions (see Fig. 1D-G). We conclude that we generated a DCL-specific antibody, named anti-DCL, with applications in Western blot analysis and in immunocytochemistry.

Expression in subventricular zone and rostral migratory stream

As DCL is specifically expressed in neuronal progenitor cells (NPCs) during embryogenesis (Vreugdenhil et al., 2007), we first inspected DCL expression in adult subventricular zone (SVZ), a brain area with well-documented ongoing neurogenesis. A strong DCL signal is found in cells of the subventricular zone (SVZ) (Fig.2A) and the rostral migratory stream (RMS; Fig.2B) in the mouse forebrain. DCL positive cells inhabit the SVZ were it co-localizes with DCX (Fig. 2G), a marker for migrating neuroblasts in the SVZ (Brown et al., 2003b). A similar DCL expression pattern is found in cells, which have symmetrical elongated extensions on both sides of the nucleus (Fig.2C) that are reminiscent for migrating type-A cells (Doetsch et al., 1997). Confocal microscopy indicates DCX/DCL sub cellular co-localization in particularly around the nuclei of chains of migrating cells between the corpus collosum (CC) and striatum (STR, Fig 2H-K). However, some DCL+ projections are devoid of DCX signals (see arrows in Fig. 2G). These data indicate overlapping roles for DCX and DCL in migrating neuroblasts in the SVZ while DCL-specific roles may occur in a minority of these cells.

Expression in olfactory bulbus

Migrating and DCX+ neuroblasts are known to reach the olfactory bulbus (OB) (Brown et al., 2003b;Belvindrah et al., 2011). As DCL+ co-localizes with DCX in migrating neuroblasts in the SVZ, we investigated possible DCL expression and DCX co-localization in the OB. We found strong DCL expression in the migrating immature neurons from the RMS (Fig. 3A), in

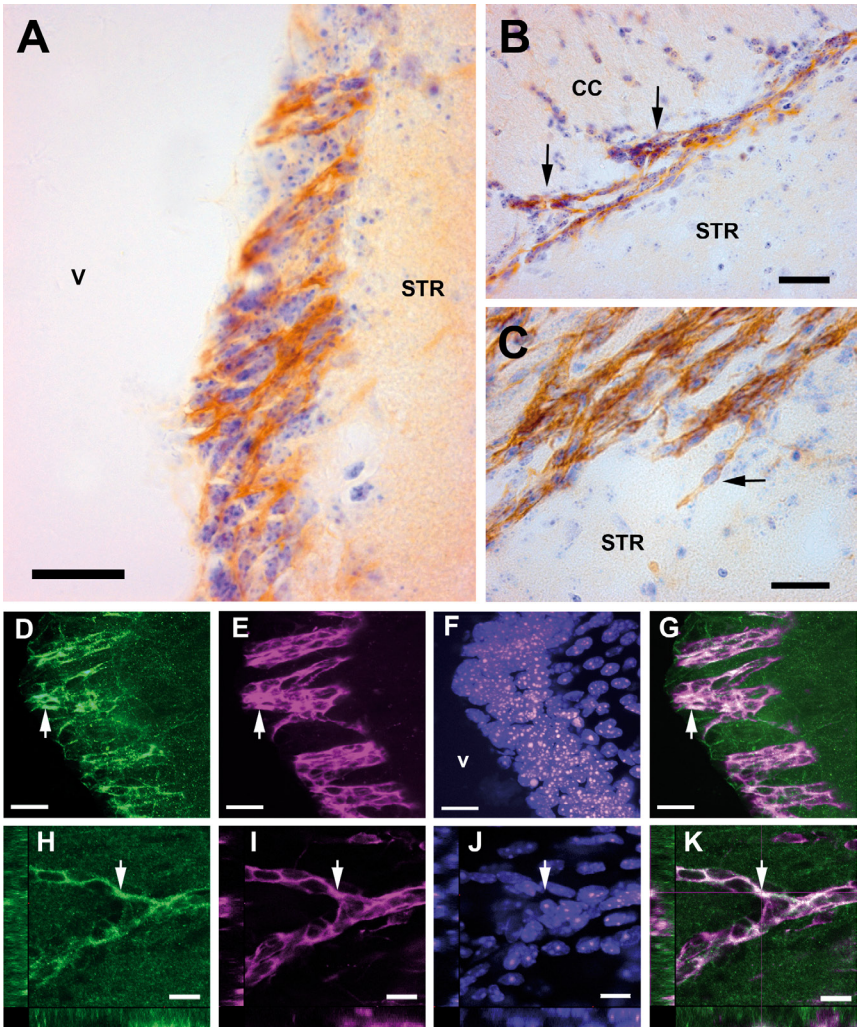


Figure 2: DCL expression in saggital slides showing the (A) the subventricular zone (SVZ) along the lateral ventricles (V) and (B) rostral migratory stream (RMS) between the corpus collosum (CC) and striatum (STR). DCL is expressed in migrating type-A cells which have a typically elongated morphology (C). DCL (D&H) co-localizes with DCX (E&I) in both SVZ and RMS (G&K). D-G represent merged confocal z-stack images, H-K represent single images from confocal z-stacks. Scale bars measure in A and C 20µm, in B 30µm, in D-G 7.5µm and in H-K 10µm.

a number of granule cells (GC's) in the granule cell layer (GCL) (Fig.3B) and in periglomular cells (PGC's) in the glomerula layer (GL; Fig.3C). Beside the DCL expression in these migrating immature neurons, a massive amount of dendrites in the internal (IP) and external (EP) plexiform layers exhibit strong DCL immunoreactivity (Fig.3A). The glomerula are almost completely negative for DCL except some PGC's (Fig.3A).

As in the SVZ and RMS, profound co localization of DCL and DCX is found in the OB (Fig. 4). However, clear differences in DCL and DCX expression patterns are evident. Firstly, inspection of sub cellular DCL immunoreactivity revealed a punctuate pattern with strong DCL+ speckles while DCX immunoreactivity is evenly distributed in the same cells and their projections (e.g. compare Fig. 4A and B). Though less evident, a similar pattern seems present in the SVZ. Secondly, In the GCL, IP and EP, DCL (Fig.3A) is strongly expressed in most fibers whereas DCX is present in cell bodies and a few fibers in the GCL and GL (Fig.4). In general, a strong DCL signal is found (Fig.4A) which overlaps with DCX expression pattern. In the GL, many DCX positive PGCs are found (Fig.4F&J). However, although some of these DCX+ cells are also positive for DCL (Fig.4E), the majority of DCX+ PGCs are DCL negative (Fig4I). The other way around, no DCL+ cell bodies were found that were negative for DCX. Thus, although our data suggest some DCX/DCL co-localization, clear differences in (sub) cellular localization for DCX and DCL is evident in the OB.

Expression in hippocampus

Another brain area with well-documented adult neurogenesis and DCX expression is the subgranular zone (SGZ) of the dentate gyrus. As expected, clear DCL expression is found in cells of the SGZ of the hippocampal dentate gyrus (Fig.5A&B). In line with an immature nature, these DCL+ SVZ cells do not stain with neuronal nuclei (NeuN), a marker for adult neurons that is specifically expressed in nuclei (see Fig.5E). Similarly, DCL co-localizes with DCX in these subgranular cells (Fig.5I), suggesting a DCL role in adult neurogenesis. In line with our previous observations of DCL expression in the nucleus of NPCs in the SVZ during embryogenesis (Boekhoorn et al., 2008), DCL immunoreactivity co-localize with Hoechst staining in cells in the SVZ (Fig.5A and B).

Unexpectedly, a punctuate and speckled staining pattern, similar as was observed in the SVZ and OB, was also observed in different GC layers and in the molecular layer (see Fig. 5C and

Figure 3: DCL expression in coronal slices showing the olfactory bulbus (OB). (A) Overview picture showing the rostral migratory stream (RMS), granule cell layer (GCL), intra plexiformlayer (IP), Mythral cell layer (MCL), extra plexiform layer (EP) and the Glomerula Layer (GL). High DCL signal is found in the RMS and plexiform layers (lower arrow). No strong signal is found in the GL (upper arrow). (B) In the GCL DCL positive granule cells with DCL positive dendrites can be found. (C) In the GL, DCL positive cells can be found (arrow). Scale bars measure in A 250 µm and in B and C 25µm.

Figure 4: DCL and DCX expression in coronal slides containing the olfactory bulbus. (A-D) DCL (A) and DCX (B) co-localization (D) in granule cell and dendrite. (E-H) Co-localization (H) of DCL (E) and DCX (F) in periglomerular cell (PGC). (I-L) A DCL negative (asterix in I) PGC which is DCX positive (J). All slides are counterstained with Hoechst. Cell of interest is indicated with an arrow (C, G & K). Scale bars measure in A-L 10µm.

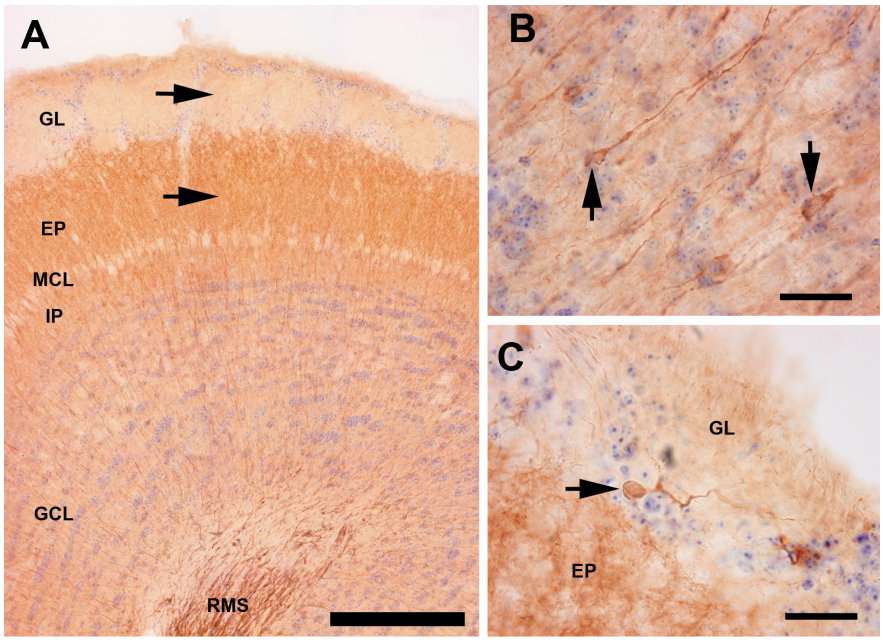


Figure 3

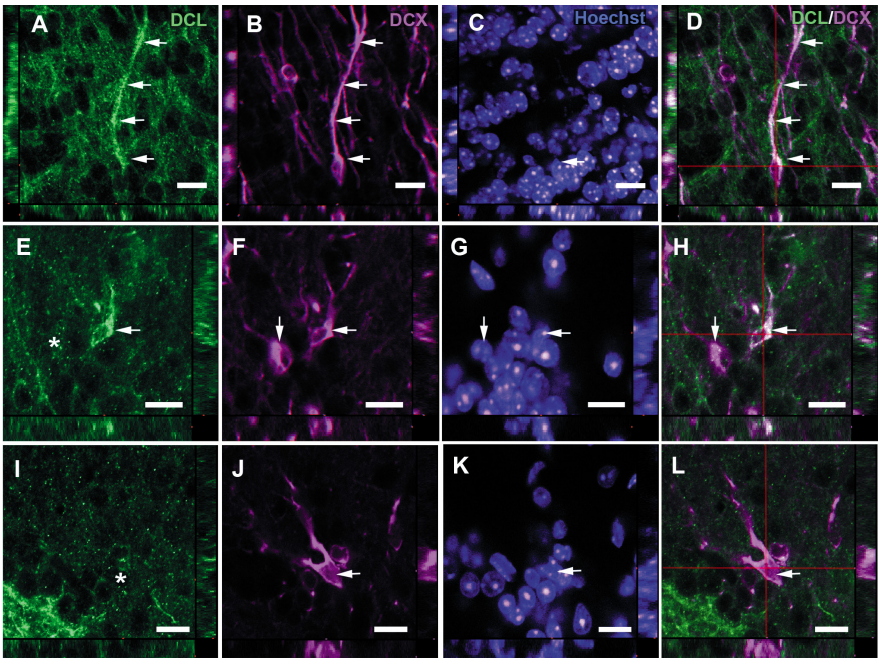


Figure 4

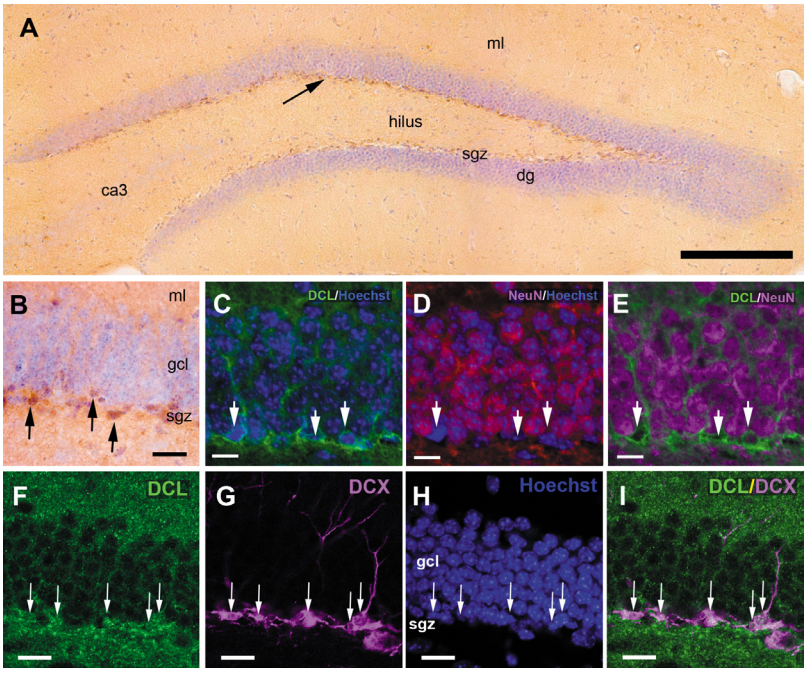

A
ml
hilus
sgz
dg
ca3
B
ml
gcl
sgz
C
DCL/Hoechst
D
NeuN/Hoechst
E
DCL/NeuN
F
DCL
G
DCX
H
Hoechst
gcl
sgz
I
DCL/DCX

Fig. 5

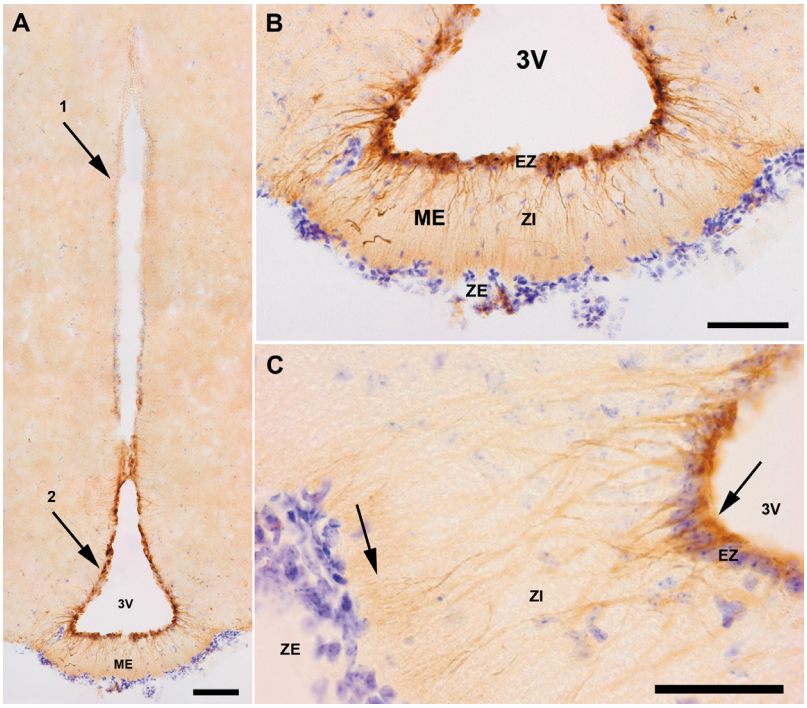

A
1
2
3V
ME
B
3V
EZ
ME
ZI
ZE
C
3V
EZ
ZI
ZE

Fig. 6

5F). However, in GCLs, no DCL+ cell bodies or clear dendritic structures were observed. We conclude that, as DCX, DCL is expressed in immature neurons in the SVZ and, unlike DCX, can also be found in other DG layers.

DCL expression in tanycytes

Tanycytes, non-ciliated ependymal cells that line the third ventricle, exhibit several features of embryonic radial glia cells (RGCs), that have been established as neuronal progenitor cells in the developing and adult CNS (Anthony et al., 2004;Malatesta et al., 2003). Tanycytes exhibit growth factor-induced mitotic activity (Xu et al., 2005), they lack NeuN expression, a marker for mature neurons but they do express vimentin, a marker for neurogenic RGCs. Previously, we have shown specific expression of DCL in RGCs and radial processes located in the ventricle zone in the neuroepithelium of mouse embryos where it co-localize with vimentin, a marker for neurogenic RGCs. Therefore, we have investigated possible DCL expression in the hypothalamus and in particularly in tanycytes. In line with their RG-like characteristics, we found specific DCL expression in tanycytes around the third ventricle (3V, Fig.6A). In the ependymal zone (EZ) of the median eminence (ME) reside DCL positive cells with projections through the internal zone (ZI) towards the external zone (ZE) of the ME (Fig.6B). The projections are thick at the beginning of the nucleus, but branch when they are closer to the ZE (Fig.6C). As in embryonic RGCs we found clear co-localization of DCL with vimentin. However, in contrast to vimentin (Mullier et al., 2010) DCL is only expressed in tanycytes but not in the ependymal cells higher up in the ventricle wall (Fig.6A & 7A-D). Like vimentin, DCL shows high expression in the cytoplasm and fibers of tanycytes. No signal seems to be present in the nucleus (Fig.7F). In contrast to DCL, DCX protein was below de-

Figure 5: DCL expression in coronal slices containing the hippocampal dentate gyrus. (A) Overview picture showing the hilus, molecular layer (ml), ca3 and dentate gyrus (DG) with DCL positive cells (arrow) in the subgranular zone (SGZ). (B) Higher magnification image of the DG showing DCL positive cells in the SGZ (arrows). (C-E) Merged confocal images of DCL and Hoechst (C), NeuN and Hoechst (D) and DCL and NeuN (E). Arrows indicate DCL positive cells. (F-I) Confocal images of the DG stained for DCL (F), DCX (G) and Hoechst (H). A merged image (I) shows DCL expression in positive DCX positive cells. Arrows indicate DCL/DCX positive cells. Scale bars measure in A 200µm, in B 20µm, in C-D 15µm and in F-I 7.5µm.

Figure 6: A DAB staining of coronal slides show DCL expression in hypothalamic tanycytes. (A) Tanycytes located close to the median eminence (ME) in the ventral part (arrow 2) of the wall of the third ventricle (3V) are DCL positive. No such expression was found in ependymal cells higher up along the ventricle wall (arrow 1). (B) Tanycyte cell bodies are located in the ependymal zone (EZ) of the third ventricle (3V) and fibers from these nuclei protrude trough the zona interna (ZI) towards the zona externa (ZE) from the median eminence (ME). (C) The tanycyte fibers branch into thin fibers before they reach the ZE. Scale bars measure in A 1mm, in B 75µm and in C 40µm.

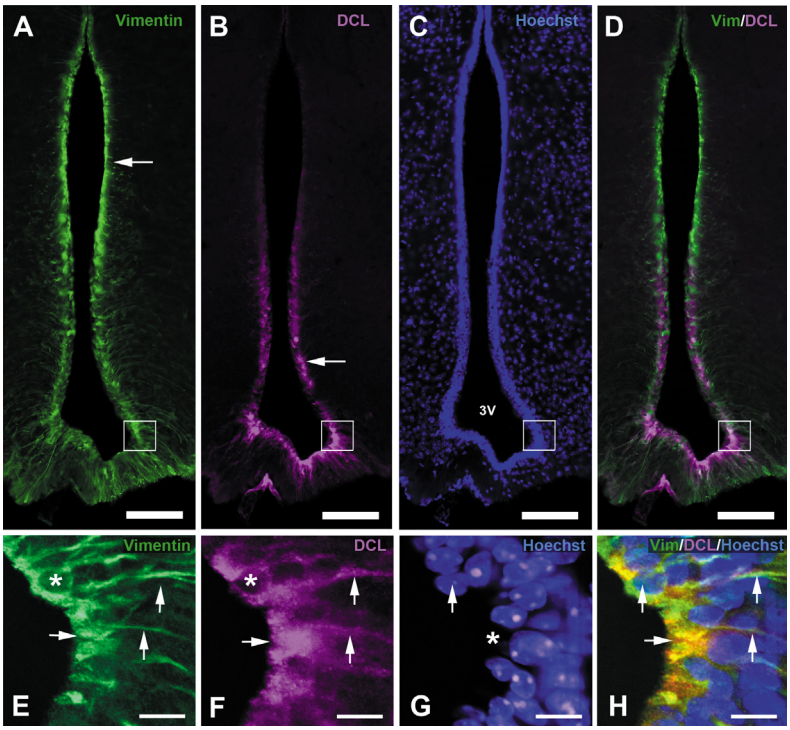


Figure 7: DCL and vimentin expression in the hypothalamus. (A-D) stitched fluorescent overview images of vimentin (A), DCL (B) and Hoechst (C) expression around the third ventricle (3V) in the hypothalamus. Both tanycytes and ependymal cells are vimentin positive (A), DCL is only expressed in the tanycytes (B). Both vimentin and DCL images are merged in (D) which shows the DCL expression in the lower half of the ventricle wall. (E-H) confocal images taken with a higher magnification derived from A-D (square box). Vimentin (E) and DCL (F) not expressed in the nucleus, but in the cytoplasm and dendritic fibers (vertical arrows in E & F). Horizontal arrows show strong vimentin and DCL co-localization (E, F &H) in the ventricle wall. No nucleus is present there (asterix in G). Scale bars in A-D measure 100µm, in E-H 10µm.

Figure 8: DCL and vasopressin (AVP) expression in the hypothalamic suprachiasmatic nucleus (SCN). (A-D) Fluorescent overview pictures showing DCL expression (A) in the SCN above the optic chiasm (OC) but not in the paraventricular nucleus (PVN). AVP is expressed in both SCN and PVN (B). A hoechst staining shows anatomy of the hypothalamic area arround the third ventricle (3V) and SCN (C). (D) A composed image of A & B showing the partial overlap between DCL and AVP. (E-H) The overlapping expression patterns of DCL (E) and AVP (F) are consistent with the subdivision of the SCN into a core and a shell area (G) since AVP is known to be expressed in the SCN shell. (H) A dual laser confocal image shows co-localization between DCL and AVP. (I-L) In this shell area AVP and DCL are not always co-localized in the same cell population. Some DCL positive cells (arrows in I) are AVP positive (arrow in J), some others are AVP negative (asterix in J). (L) Doublelaser confocal image show co-localizetion. Scalebars measure in A 250µm, in B 50µm and in C 10µm.

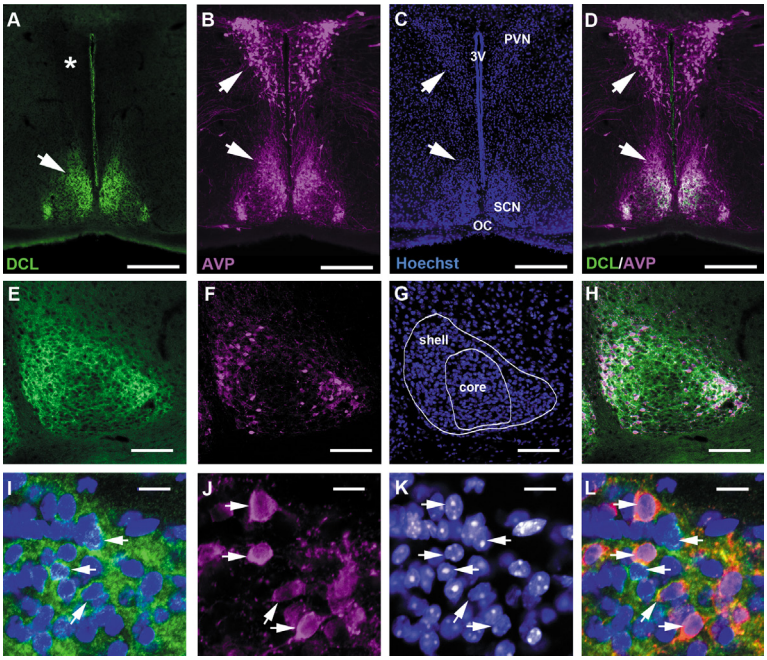


Fig. 8

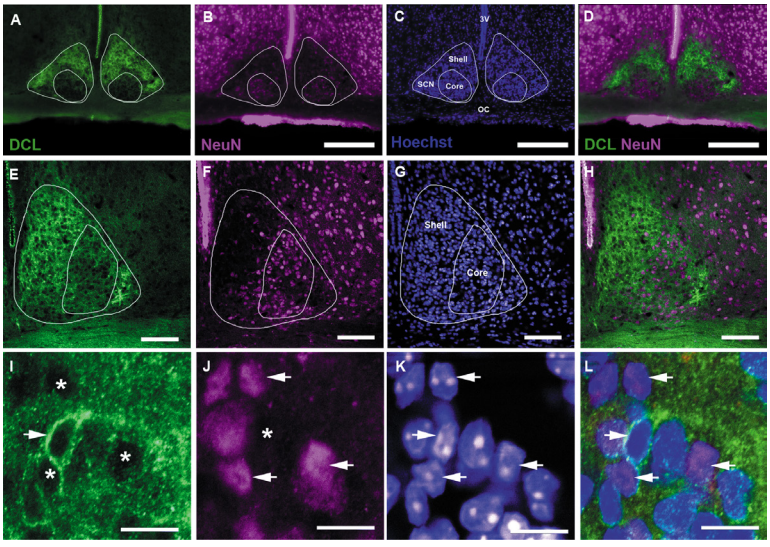


Figure 9: DCL and NeuN expression in the SCN. DCL is not expressed in the NeuN positive (red) subregion of the SCN. DCL is expressed in the shell region of the SCN (A & E) whereas NeuN is expressed in the SCN core region (B & F). Merged fluorescent overview image D and double laser confocal image H do not show co-localization. Close-up images show DCL (arrow in I) and NeuN (arrows in J) are expressed in a different cell population. Scalebars measure in A 200µ, in B 75µm and in C 10µm.

tection level (data not shown) in tanycytes and other cells around the third ventricle. Thus, our findings suggest a unique, but yet unknown role of DCL in the hypothalamus and are in line with the hypothesis that tanycytes have neurogenic properties.

DCL expression in SCN

Recent evidence suggests high neuronal plasticity in the suprachiasmatic nucleus (SCN), which is evidenced by low NeuN expression and the presence of neuroblast-like cells are suggested by DCX expression (Geoghegan and Carter, 2008). To investigate possible DCL-DCX co-localization in the SCN we inspect DCL/DCX expression by confocal microscopy. Surprisingly, we did not detect any DCX staining in the SCN (data not shown). However, clear DCL expression is observed in outer parts of the SCN. DCL immunoreactivity is likely located in the shell area (Leak and Moore, 2001) because it overlaps with the expression vasopressin (AVP), a marker of the shell area (Fig.8 A&B). Detailed inspection of AVP-DCL co localization shows a complex picture of AVP–specific expression in cell bodies, DCL-specific expression in mainly projections and the cytosol of cell bodies but not in nuclei and AVP-DCL co-localization in the cytosol of cell bodies and in projections, suggesting cellular heterogeneity in the shell area of the SCN (Fig. 8C). DCL expression is highly specific for the SCN and is below detectable levels in related nuclei like the paraventricular nucleus (PVN, see Fig 8A) or the supraoptic nucleus (SON; data not shown).

As reported by Geoghegan & Carter (2008), we found NeuN expression in the core region of the SCN. However, DCL expressing cells in the shell region are devoted from NeuN expression while NeuN expressing cells in the core do not express any detectable DCL. Together, we observe a number of DCL+/NeuN- cells in the shell area of the SCN indicating their immature character suggesting a high degree of neuronal plasticity in this brain area.

Expression in islands of Calleja

We noticed persistent and unexpected DCL expression in the islands of Calleja (ICj; Fig.10). The ICj are composed of several small groups of granule cells in the polymorph layer of the olfactory tubercle and one large group, insula magna, which lies along the border between septum and the nucleus accumbens shell (Fallon et al., 1983;Fallon et al., 1978). The islands contain small granule cells (10-20 μm) which appear as rather undifferentiated neurons with poorly developed dendrites and axons. The granule cells surround a core of neuropil or hilus in which some medium sized neurons (20-35 μm) reside (Fallon, 1983;Meyer et al., 1989;Millhouse, 1987). DCL expression is found in all locations like the insula magna or major islands (Fig.10A&J), the ventral group of the islands along the pial border of the basal forebrain (Fig.10A&F) and the rostral group of islands below the semilunar nucleus (Fig.10B).

DCL expression is mainly found in the hilar neuropil (Fig.10C-N). Unlike our findings in the other brain areas, small granule cells in the ICj are NeuN positive (Fig.10K-N). These granule cells are characterized by their relative small size compared to neuronal nuclei outside the islands or inside the hilus (Fig.10F). No DCL is expressed in cell nuclei of ICj granule cells.

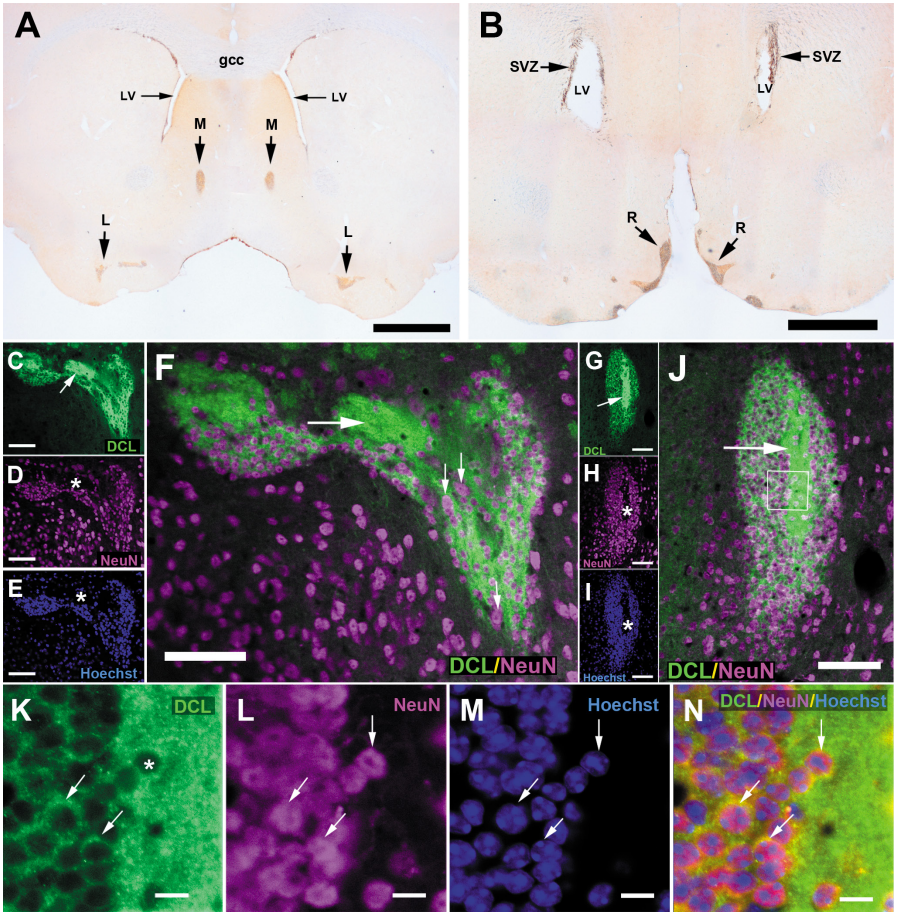


Figure 10: DCL expression in the islands of Calleja (ICj).(A) The major islands (arrows M) are located between the Nucleus accumbens shell and lateral septal nucleus. The lateral islands (arrows L) are located in the ventral pallidum. (B) Also the rostral islands (arrows R) are DCL positive. (C-F) High DCL expression is found in the neuropil or hilus (C & E). The small granule cells of the ICj are NeuN positive (D). Also some characteristic larger NeuN positive neurons are found (arrows in F). (G-J) The highest DCL expression in the major islands is found in the neuropil or hilus of the islands (G & I). Also in the major islands, the granule cells are NeuN positive (H). (K-N) Close up from in J (rectangle). DCL expression is found outside cell nuclei (asterix in L) but overlaps partly on the border of these nuclei (arrows in L, M & N). Scale bars measure in A, 1mm, in B 0.8mm, in C-J 100µm and in K-N 10µm.

Discussion

We have generated a DCL-specific antibody and applied it to characterize the expression of DCL, a neurogenesis-related gene, in the adult mouse brain. As expected we found strong DCL expression in two well-established neurogenic cell niches; i.e. in the SVZ, RMS and olfactory bulbus and in the dentate gyrus of the hippocampus. In these areas, DCL shows co-localization with the DCX expression patterns, suggesting a role for DCL in neuronal migration as reported in the embryonic brain (Deuel et al., 2006;Koizumi et al., 2006;Vreugdenhil et al., 2007). Strikingly, unlike DCX, we found unique DCL expression in specific brain nuclei, i.e. in tanycytes near the third ventricle wall, in the SCN and in the ICj suggesting the presence of immature and/or potential mitotic cells in these brain areas. Finally, unlike DCX, we observed a punctuate and speckled staining pattern in all inspected brain areas suggesting that, besides neurogenesis, DCL may regulate other processes in neuronal plasticity.

Strong DCL expression is found in the neurogenic cells of the SVZ, RMS and olfactory bulbus. In these neurogenic regions strong DCX expression has been reported in rats and mice (Brown et al., 2003b;Couillard-Despres et al., 2005;Nacher et al., 2001;Rao and Shetty, 2004). DCL expression shows strong overlap with the DCX expression patterns, which is in line with our expectation about a role for DCL in neurogenesis. In the subventricular zone, DCL is co-expressed with DCX in cells referred to as type-A cells (Doetsch et al., 1997). Along the RMS this co-expression is continued which suggests a role in neuronal migration as reported before on DCL in the embryonic brain (Deuel et al., 2006;Koizumi et al., 2006;Vreugdenhil et al., 2007). In the granule cell layer (GCL) of the olfactory bulbus, DCX positive immature neurons show DCL co-expression too. In the glomerula layer co-expression is less common, several DCX positive PGC's show co-expression with DCL, but the majority of these cells are DCX positive only. No DCL positive/DCX negative cells were found. This might indicate that DCL expression in neuron maturation ends earlier compared to DCX.

In addition to DCL positive immature granule cells, a clear DCL signal is found in the GCL and plexiform layers of the OB. The nature of this signal is difficult to pin point, since this area primarily consists of dense neuropil formed by fibers from the GCL and the mitral cell layer (MCL) (Ennis et al., 2007). The signal is low compared to immature DCL positive granule cells. The function of DCL in these fibers remains unknown, however, continuous replacement of newborn neurons in the OB (Imayoshi et al., 2008;Murata et al., 2011) might need some dendritic rearrangement of the existing network (see review by Wilson et al., 2004).

DCL positive cells are mainly found in the subgranular zone of the dentate gyrus and the majority of these cells co-express DCX but not NeuN. DCX is a reliable frequently-used marker for migrating neuroblasts (Brown et al., 2003b;Rao and Shetty, 2004;Couillard-Despres et

al., 2005;Garcia et al., 2004). Given their high homology and their co-expression, our data indicate similar functions for DCL with DCX in migrating neuroblasts. In line with this notion are studies on the role of DCX and the DCLK gene in the development of the neocortex. Both DCX and DCLK mouse mutants show normal development of the neocortex (Corbo et al., 2002;Shu et al., 2006). However, DCX/DCLK double mouse mutants exhibit severe malformations of the neocortex and other brain areas (Koizumi et al., 2006;Deuel et al., 2006;Dehmelt and Halpain, 2007) suggesting that DCX/DCL(K) proteins interactions are necessary for proper neurogenesis. Our data indicate that DCX/DCL interaction continues in the adult brain. We observe DCL expression in nuclei in the SGZ that are negative for NeuN suggesting a DCL function in proliferating NPCs. In line with this, in C. elegans, the analogue of the DCLK gene, zyg-8, is involved in a-symmetric cell divisions and controls mitotic spindle positioning by promoting microtubule assembly during the anaphase (Gonczy et al., 2001). In mouse embryos, in vivo DCL knockdown by in utero electroporation in embryonic ventricle zone cells reduces the number of NPCs (Vreugdenhil et al., 2007). In vitro, DCL co localizes with mitotic spindles in neuroblastoma cells and DCL knockdown leads to apoptotic cell death (Verissimo et al., 2010b;Vreugdenhil et al., 2007). Together with our present results, all these data points to a role for DCL in proliferation of NPCs in the adult dentate gyrus. However, such a role seems unique for the hippocampus as we did not observe nuclear DCL localization in other brain areas.

A novel population of DCL-expressing cells are tanycytes. These cells are derived from embryonic radial glia cells and express many markers which are found in radial glia and neuronal precursor cells (for an overview see Rodriguez et al., 2005;2010). For example, hypothalamic tanycytes express GFAP, vimentin and nestin, all markers for neuronal stem cells, but do not express NeuN, a marker for adult neurons. A low frequency of ongoing adult neurogenesis in hypothalamic tanycytes has been reported (Rodriguez et al., 2005;Xu et al., 2005;Kokoeva et al., 2007). Since DCL is linked to adult neurogenesis, DCL might be involved in hypothalamic neurogenesis. However, only a subpopulation, e.g. α-tanycytes, is thought to have regenerative capacity (Rodriguez et al., 2005). DCL is expressed in all types of tanycytes around the third ventricle and therefore might serve a broader function than neurogenesis alone. The functions of tanycytes are complex and are for a large degree unknown. Tanycytes are involved in the release of gonadotropin releasing hormone (GnRH) (Rodriguez et al., 2005;Prevot, 2002) and also thyroid hormone signalling is regulated by thyroxine deiodinase enzymes which are expressed in tanycytes (Tu et al., 1997). Interestingly, tanycytes may be involved in circadian or circa-annual cycles as they may regulate seasonal production and release of thyroid hormone and GnRH (Kameda et al., 2003; for review see Hazlerigg and Loudon, 2008). Since tanycytes are in contact with the cerebrospinal fluid (CSF) they are also thought to orchestrate sensing of glucose levels and therefore may play a role in glucose metabolism (Millan et al., 2010). Thus, although the precise functional significance in tanycytes is presently unknown DCL expression might be related to cell movement due

to seasonal changes and/or to microtubule-guided transport of signalling proteins as was shown for the glucocorticoid receptor (Fitzsimons et al., 2008).

We found high DCL expression in the SCN, a brain area crucially involved in the regulation of circadian cycles and, as tanycytes, has been associated with seasonal timing. The SCN is dynamic in temporal and spatial expression of genes (Welsh et al., 2010;Morin, 2007). The SCN has a plastic nature which facilitates daily structural rearrangements within the SCN (Meijer et al., 2010;Girardet et al., 2010a). In the structural organization of the SCN, a clear distinction can be made between cells expressing vasopressin (AVP) defining the SCN shell and cells expressing vasoactive intestinal polypeptide (VIP) defining the core. Although both core and shell show structural rearrangements between light and dark phases (Becquet et al., 2008), the VIP containing core is believed to have a more dynamic character compared to the AVP expressing shell (Girardet et al., 2010b). However, our finding of DCL+/ AVP+ cells in the shell of the SCN that, in contrast to cells in the core, are negative for NeuN (see below), suggest an immature character and suggests strong cytoskeleton rearrangements in this part of the SCN. DCL might be involved in SCN plasticity similarly as has been reported for polysialic acid (PSA) and neural cell adhesion molecule (NCAM) (Bonfanti et al., 1992;Glass et al., 2003;Shen et al., 1997;Shen et al., 1999;Prosser et al., 2003). PSA-NCAM is thought to play a key role in daily structural rearrangement of the SCN (Girardet et al., 2010a;Glass et al., 2003;Prosser et al., 2003) and is thereby crucial for the circadian organization of behaviour (Fedorkova et al., 2002;Shen et al., 1997;Prosser et al., 2003). In line with DCL expression in the SCN, PSA-NCAM is also a well-known marker for neurogenesis in the adult brain and often is co-expressed with DCX (Varea et al., 2009; reviewed by Bonfanti, 2006). Besides neurogenesis, DCL is involved in fast microtubule-guided retrograde transport of signalling molecules in neuronal progenitor cells (Fitzsimons et al, 2008) and thus might play such a role in the shell of the SCN too. Further experiments aiming to manipulate DCL expression in the SCN, are required to address this point.
Interestingly, DCX expression in the core of the rat SCN has been reported (Geoghegan and Carter, 2008). However, we were unable to reproduce this finding, which might be due to technical, antibody-related issues or to the differences between species used in our study (mice) and used by Geoghegan and Carter (rat). We find specific DCL expression in the shell of the SCN but not in the core. This points towards different roles of DCL and DCX in the SCN.

Unexpectedly, we observed high DCL expression in the ICj. Although the ICj are not well known for their neurogenic capacity, their granule cells are derived from the SVZ (De Marchis et al., 2004). In postnatal and adolescent mice, an alternative migratory route named ventral migratory mass, ends as granule cells in the ICj. In addition, NeuN/BrdU double positive cells in the ICj are found 11 days after BrdU injection (Shapiro et al., 2009). The possible role of DCL in this area remains speculative. We did not find cells which look like migratory type-A

cells as found in the RMS. The fact that DCL is mainly expressed in the hilus or neuropil, which mainly consists of unmyelinated axons and dendrites, points towards a similar expression pattern of DCL as seen in the granular and plexiform layer of the olfactory bulbus. Tracing studies revealed input from the substantia nigra-ventral tegmental area (SN-VTA), olfactory bulbus and cortical input from the piriform and periamygdaloid cortex (Fallon, 1983). ICj projections are found towards the surrounding olfactory tubercle, ventral striatum and ventral pallidum (Fallon, 1983;Ubeda-Banon et al., 2008). The ICj might play a role in encoding the proper reward component to olfactory and emotional information (Shapiro et al., 2009).

Compared with DCX, we observe a distinct staining pattern for DCL. Whereas DCX staining is homogeneous and nicely highlights complete dendrites of NPCs, DCL has a speckled and rather punctuate appearance. Moreover, this DCL pattern can sometimes be found outside neurogenic areas such as the GCLs of the dentate gyrus suggesting additional roles for DCL in neuronal plasticity. Interestingly, several neurogenesis-related proteins such as calbindin and PSA-NCAM (for review see (Duan et al., 2008) exhibit a similar speckled and punctuate staining pattern (Bonfanti, 2006). Interestingly, both Calbindin and PSA-NCAM have also been associated with the novo formation with synapses (Dityatev et al., 2004;Rami et al., 1987) indicating a possible DCL role in synaptic plasticity. However, the functional significance of this similarity in staining patterns, if any, is at present unknown and requires further detailed structural and morphological analysis.

In conclusion, DCL is expressed in the adult neurogenic niches, where it exhibits strong co-expression with DCX, suggesting that dcx/dcl interaction is required for proper adult neurogenesis. Moreover, we identified hypothalamic tanycytes, the SCN and the ICj as novel sites with specific DCL expression, but not DCX. As DCL is characteristic for dynamic processes such as cellular migration that requires drastic reorganization of the microtubule cytoskeleton, our data suggests the existence of high levels of neuronal plasticity in these brain areas.

Acknowledgements

The authors thank Jasper Laboyrie and Sander Griepsma for technical assistance and Joke Meijer for reviewing the manuscript. This work was supported by Top Institute Pharma (project T5-210), The Netherlands.