Doublecortin-like knockdown in the adult mouse brain

implications for neurogenesis, neuroplasticity and behaviour

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Voor Isabel, Olivia & Liselot

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List of abbreviations

ADX	adrenalectomy
ARC	Arcuate nucleus
AVP	arginine vasopressin
BrdU	Bromodeoxyuridine
CARP	CaMK-related peptide
CFC	Contextual Fear Conditioning
CHB	Circular Hole Board
D2	Deiodinase 2
D3	Deiodinase 3
DCL	Doublecortin-like
DCLK	Doublecortin-like kinase
DCX	Doublecortin
DG	Dentate gyrus
dox	Doxycycline
EC	Enthorhinal cortex
GC	Granule cell
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid Receptor
HPA-axis	Hypothalamic pituitary adrenal axis
HPT-axis	Hypothalamic pituitary thyroid axis
ICj	Islands of Calleja
KD	Knockdown
MAP	Microtubules associated protein
ME	Median Eminence
ML	Molecular layer
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
NPC	Neuronal progenitor cell
NPY	Neuropeptide Y
NSC	Neuronal stem cell
OB	Olfactory bulb
PE	Periventricular area
PGC	Periglomerular cell
PVN	Paraventricular nucleus
RG	Radial glia
RMS	Rostral migratory stream

List of abbreviations

SCN	Suprachiasmatic nucleus
SGZ	Subgranular zone
shRNA	Short hairpin RNA
siRNA	Short interference RNA
SVZ	Subventricular zone
TRH	Thyroid Releasing Hormone
TSH	Thyroid Stimulating Hormone

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

Chapter 1

General introduction

Part of this chapter is published as review

Saaltink & Vreugdenhil 2014

Stress, glucocorticoid receptors and adult neurogenesis: a balance between excitation and inhibition?

Cellular & Molecular Life Sciences.

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1 Embryonic neurogenesis

In the mouse, brain development starts around embryonic day (E)8 when the neuroepithelium lines the neural tube. A few days later massive proliferation of neuronal progenitor cells (NPC's) marks the start of a explosive biological phenomenon called neurogenesis in which many NPC's are born that further differentiate and migrate towards their final destination. This process is well defined and many genes play an important role in this complex event (Gupta et al., 2002; Kriegstein and Alvarez-Buylla, 2009). Furthermore, migration patterns seem to be complex with different types of migration routes and sites of origin (Kriegstein and Alvarez-Buylla, 2009). Especially in the neocortex many neurons are born that migrate over long distances towards their destination. By a tightly timed schedule, the neocortex is built layer by layer until 6 layers are created together forming the cortical plate (CP) between the ventricular zone (VZ) and the pial surface (PS; Gupta et al., 2002; Marin and Rubenstein, 2003). The magnitude of this process decreases after birth when the formation of the brain is nearly completed. However, on a few locations in the brain NPC's have not ceased proliferation. At the adult subventricular zone (SVZ) and the hippocampal dentate gyrus (DG) NPC's continue to proliferate and thereby creating new neurons. Although still under debate, other brain regions are suspected of neurogenic capabilities (Gould, 2007). In this chapter, I will describe the field of developmental and adult neurogenesis to present a complete overview of the neurogenesis process.

1.1 Stem cell lineages

For long it was thought that neurons and glial cells derived from two different progenitor pools. Neurons were generated by neuroblasts whereas spongioblasts, now called radial glia cells (RG), where thought to be precursors for astroglial cells, because RG's finally become astrocytes when they finish functioning as proliferating unit. However, recent research showed that RG's give also rise to differentiated neurons (for an extensive review see Kriegstein and Alvarez-Buylla, 2009).

The primary progenitor cells are called neuronal progenitor cells (NPC's). At several developmental stages, these cells give rise to cell lineages that differentiate become neurons or glia cells. Neurons and glia cells are not directly generated from NPC's but develop trough several intermediate stages. Another cell type during development are transit amplifying cells, also called intermediate progenitor cells (IPC's) that have a more restricted potential. Three types of IPC's exists; 1) neuron-generating IPC's (nIPC); 2) oligodendrocytes-generating IPC's (oIPC) and; 3) astrocytes-generating IPC's (aIPC) (Kriegstein and Alvarez-Buylla, 2009). The name glia cell is confusing since it is used for both NPC glia cells as well as differentiated glia cells. Further below I will elaborate on the function of RG's as NPC's.

1.2 Proliferation

After neurulation, a pseudo stratified epithelium forms the central nervous system (CNS) in which radially arranged bipolar cells form the neural tube. At the apical side (close to the ventricle) proliferation takes place. Marked by changes in gene expression early progenitor cells transform into neuroepithelial progenitors (NEP's). NEP's divide initially symmetrical at the apical side of the neural tube from where they are also connected to the pial surface via long radial processes. In time they start to divide also asymmetrically giving rise to a NEP and alternatively to a neuron or a basal progenitor that will undergo mitosis at a significant distance from the VZ generating the subventricular zone (SVZ). Already at this stage cells seem to be committed to specific fates although this is not yet linked to the expression of specific markers (Malatesta et al., 2008).

After the appearance of the first neurons, NEP's transform a second time. They start to acquire molecular and cytological features typical of the astroglial lineage and become RG's. Well known features typical for mature or reactive astrocytes are for example calcium binding protein (S100β) and intermediate filament vimentin but there are several more (see Malatesta et al., 2008) for an overview). In contrast to primates, glial fibrillary acidic protein (GFAP) is not such a marker in rodents.

1.3 Radial glia cells

Radial glia cells serve a double function in the developmental process. They form a scaffold from VZ to PS were along post mitotic neurons migrate. In the mean time they serve as an NPC from which new neurons and basal progenitor cells (nIPC's) are born. They form actually the main source of newborn neurons in the neocortex (Kriegstein and Alvarez-Buylla, 2009;Malatesta et al., 2008;Marin and Rubenstein, 2003).
Time-lapse video analysis revealed the proliferation process of RG's (Noctor et al., 2001;Noctor et al., 2008). Asymmetric divisions result in one daughter cell retaining the basal process and maintain the radial glia like phenotype, while the other cell will migrate along her sister's process towards the PS and differentiate into a neuron. After neuronal production ceases, RG's will differentiate into astrocytes (Kriegstein and Alvarez-Buylla, 2009;Malatesta et al., 2008;Marin and Rubenstein, 2003).
It is thought that the RG population can be divided in subpopulations committed to different fates. The commitment of cells seems to be present from the beginning of the development, which suggests a role of neuroepithelium in fate determination. The occurrence of early commitments to late cell fates suggests that already committed progenitors can remain quiescent even for long periods (Kriegstein and Alvarez-Buylla, 2009;Malatesta et al., 2008).

1.4 Migration

There are two modes of migration i.e. radial migration and tangential migration (for reviews see Ayala et al., 2007). The majority of neurons (80%-90%) arises from proliferative zones in the dorsal telencephalon and migrates radially along RG processes. These processes reach from VZ to PS and form the guiding scaffolding for migratory NPC's. Time-lapse video analysis revealed that NPC's can migrate in two different ways, by somal translocation or cellular locomotion (Nadarajah et al., 2001). Cells, which migrate by somal translocation form long processes towards the PS where after the processes shorten again and the cell soma migrates upwards to the cortical layers. Somal translocation happens in mice around E12-E13 when the PP is split in two. Cells migrating by locomotion have short unbranched processes, which remain stable in length. They migrate together with the cell soma along the RG processes towards the cortical layers (Nadarajah et al., 2001;Gupta et al., 2002). This process happens slightly later (E15-E16) and can probably not proceed in absence of RG's (Gupta et al., 2002).

Cells born elsewhere in the brain (e.g. ganglionic eminences) migrate tangentially through the IZ and SVZ towards the cortical layers. Two major tangential routes are identified (Ayala et al., 2007); cells born in the medial ganglionic eminence (MGE) migrate towards neocortex and hippocampus and cells born in the lateral ganglionic eminence (LGE) migrate towards the olfactory bulb. The latter migratory route is still active in the adult brain where it is called the rostral migratory stream (RMS). In the cortical layers, tangentially migrating neurons start to migrate radially to reach their final destination (Ayala et al., 2007;Kriegstein and Noctor, 2004).

Migration is a complex phenomenon, which is guided by many factors. Extracellular signalling cues are received by intracellular signalling pathways linked to the cytoskeleton, which plays a crucial role in cell movement. The cytoskeleton network consists of several components of which the actin and microtubules are the main components (Ayala et al., 2007). Actin filaments are involved in the axonal wrist of a migrating neuron where microtubules give stability to the growing neuritis (Dehmelt and Halpain, 2004). Regulators called microtubules associated proteins (MAP's) serve several functions like stabilizing the microtubules or regulate its direction or length. One of such regulators is doublecortin (DCX), which I will discuss in further detail in paragraph 5. Actins and microtubules interact together via shared regulators like DCX or Lis1. However, there is little known about this interaction and how it involves migration (Ayala et al., 2007).

1.5 Neocortical development

To summarize the process of neocortical development, I will go up one level and focus on cell populations instead of individual cells. A first wave of post mitotic neurons migrates from the VZ towards the PS creating a neuronal layer known as the pre-plate (PP). A second wave of neurons splits the PP into a superficial marginal zone (MZ) and the subplate (SP). Both MZ and SP form the boundary of what will develop into the CP. Consecutive migration waves build-up the CP from inside-out which means that early born neurons make up the inner layers of the brain and the newer cells end up in the more superficial layers. Finally, a cortical plate consisting of 6 distinguishable layers forms the neocortex of a postnatal animal (Gupta et al., 2002).

2 Adult neurogenesis

Altman and Das published their findings of postnatal neurogenesis in the rat hippocampus already in 1965 (Altman and Das, 1965). However, theories about adult neurogenesis did not proliferate before Goldman & Nottebohm published their findings on neurogenesis in adult canaries in 1983 (Goldman and Nottebohm, 1983). Nowadays the concept of adult neurogenesis is widely accepted (Abrous et al., 2005;Alvarez-Buylla and Lim, 2004;Kempermann et al., 2008;Lim et al., 2008;Lledo et al., 2006;Ming and Song, 2005;Ming and Song, 2011) although there is some debate about additional brain areas, which might be regenerative too (Gould, 2007).

The main focus in the field of adult neurogenesis is on the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) along the walls of the lateral ventricles. Newborn neurons in the SVZ migrate via the rostral migratory stream (RMS) towards the olfactory bulb (OB) where they replace local interneurons (Imayoshi et al., 2008). In the DG a similar process is seen, however the distance of migration is much shorter. The primary progenitor cells in the adult brain have similar glial features as RG's. Because the neurogenic process in both DG and SVZ differ in many aspects, I will discuss both processes separately.

2.1 Adult Subventricular zone and olfactory bulb neurogenesis

After developmental brain formation, several cell layers disappear like the subplate and the RG rich VZ loses its regenerative character. However, the subventricular zone (SVZ) along the walls of the lateral ventricles still harbours neural stem cells (NSC's). The local SVZ environment is pro-neurogenic as transplantation experiments of SVZ cells grafted homotopically into the SVZ of a recipient animal gives rise to OB interneurons (Alvarez-Buylla et al., 1994). In contrast, when grafted into a non-proliferative brain region like the striatum, no neuro-

nal differentiation occurs. Instead, grafted NSC's will develop into glia cells (Herrera et al., 1999). A variety of different terminology is used, which may be explained by individual preferences of many scientists that worked on this (Abrous et al., 2005;Alvarez-Buylla and Lim, 2004;Lim et al., 2008;Lledo et al., 2006;Ming and Song, 2005;Ming and Song, 2011;Nissant and Pallotto, 2011). In this introduction, I will use the terminology of Limand colleagues (Lim et al., 2008) which is mainly based on the work of Doetsch et al., (1997).

Table 1: Immunocytochemical characteristics of different cell type dwelling in the SVZ (derived from Doetsch et al., 1997).

Cell type	PSA-NCAM	TuJ1	Nestin	GFAP	Vimentin	DCX
Type-A, migrating neuroblast	+++	+++	+	-	-	+++
Type-B, astrocyte	-	-	++	++	+	n.d.
Type-C, precursor	-	-	+	-	-	n.d.
Type-D, tanycytes	-	-	n.d.	+++	n.d.	n.d.
Type-E, ependymal	-	-	+++	+	++	n.d.

Subventricular zone

The SVZ harbours 5 main cell types (A, B, C, D and ependymal (E) cells), which differ in morphological, immunocytochemical and ultra structural characteristics (Doetsch et al., 1997). Type-B cells are astrocytes, which descended from embryonic RG's. They function as multipotent NSC's and give rise to type-C cells, which function as IPC's to and give rise to type-A cells, which are migratory neuroblasts. This type-A cells form a chain of migrating cells from the SVZ to the core of the OB. This chain is called the rostral migratory stream (RMS, reviewed in Lim et al., 2008;Ming and Song, 2005). Type D cells are tanycytes occasionally residing between ependymal cells (type-E) at the ventricle wall (Doetsch et al., 1997).

Migrating type-A cells are isolated from the ependymal layer and striatum by type-B cells, which surround the tangentially orientated neuroblasts. On several 'hotspots' along the migratory stream, type-C cells function as proliferating precursors. At these hotspots, the sheath of type-B cells is open, which suggests interaction via these 'ports' with the local environment (Doetsch et al., 1997). At several locations along the ventricle wall type-B cells protrude trough the ependymal layer and make a direct connection to the ventricle. It might be possible that ependymal cells function here as niche cells and give rise to type-B cells (Lim et al., 2008).

Different cells of the SVZ can be characterized by cell-specific markers (see Table 1). Type-A cells express polysialylated neural adhesion cell molecule- (PSA–NCAM), TuJ1- (α-tubulin),

and nestin but not GFAP- and vimentin. Type-B cells are also nestin positive but lack the expression of PSA-NCAM and TuJ1. They share GFAP and vimentin expression with ependymal cells. Tanycytes are positive only for GFAP expression (Doetsch et al., 1997). Doublecortin (DCX) is a popular marker for immature and migrating neurons in the hippocampus (Couillard-Despres et al., 2005;Plumpe et al., 2006;Rao and Shetty, 2004) but is not well documented in relation to SVZ neurogenesis. Although not explicitly named as a marker for SVZ neurogenesis, DCX is depicted in one of the figures in the extensive review of Ming and Song, (2005). Originally, DCX has been characterized as a MAP that is specific and crucial for immature and migrating neurons (Francis et al., 1999;Gleeson et al., 1999). Therefore it is assumed that only type-A cells are DCX positive (Lim et al., 2008).

Rostral migratory stream

When the chain leaves the SVZ, no type-C cells are found around the chain anymore. The migrating neuroblasts are only sheathed by type-B cells. In vitro, these neuroblasts form automatically migratory chains in which type-A cells migrate with a relative high speed of 122µm/hr (Wichterle et al., 1997). They migrate with a combination of nuclear translocation and locomotion. First leading processes are extended with a growth cone where after the cell soma is translocated towards the growth cone tip. This process is repeated until the cell reaches the OB (Wichterle et al., 1997).

Type-A cells are highly positive for DCX, which is also highly expressed during neuronal migration in the developing embryonic brain (Francis et al., 1999). Together with collapsing response mediator protein-4 (CRMP-4), DCX seems to play a role in the migration process although the exact function is not known yet (Gleeson et al., 1999;Nacher et al., 2001). In paragraph 1.x, I will elaborate on DCX further more. Several other molecules in the cells and extra cellular matrix (ECM) are thought to play a role in type-A migration like PSA-NCAM, 9-O-acetyl GD3 and Tenascin-C (reviewed by Lim et al., 2008).

Olfactorius bulb

Reaching the core of the OB, tangential migration comes to an end and radial migration towards the periglomerula starts. Reelin seems to play an important role in the detachment of type-A cells from the RMS and the start of radial migration into the OB. Tenascin-R (Saghatelyan et al., 2004) and Prokineticin-2 (Ng et al., 2005) play a comparable role in this process.

After RMS detachment, type-A cells migrate to several layers of which the OB consists. The OB core is filled with newborn neurons, which mainly become GABAergic interneurons.

Around this core of immature neurons the granule cell layer (GCL) makes out the majority of cells. Around the GCL the internal- (IP) and external plexiform (EP) layers surround the mitral cell layer (MCL). The "shell" of the OB is formed by the glomerula layer (GL), which is a heterozygous layer with many different cell types (Kosaka et al., 1995;Ng et al., 2005;Parrish-Aungst et al., 2007). Upon arrival in the core of the OB, type-A cells can develop into two kinds of interneurons, granule cells (GC's) or periglomerular cells (PGC's; Lim et al., 2008). GC's are in general GABAergic and calretinin positive whereas 40% of PGC's are GABAergic of which 60-70% is also dopaminergic. PGC's can be further divided into calretinin, calbindin or glutamic acid decarboxylase (GAD) positive cells (Kosaka et al., 1995).

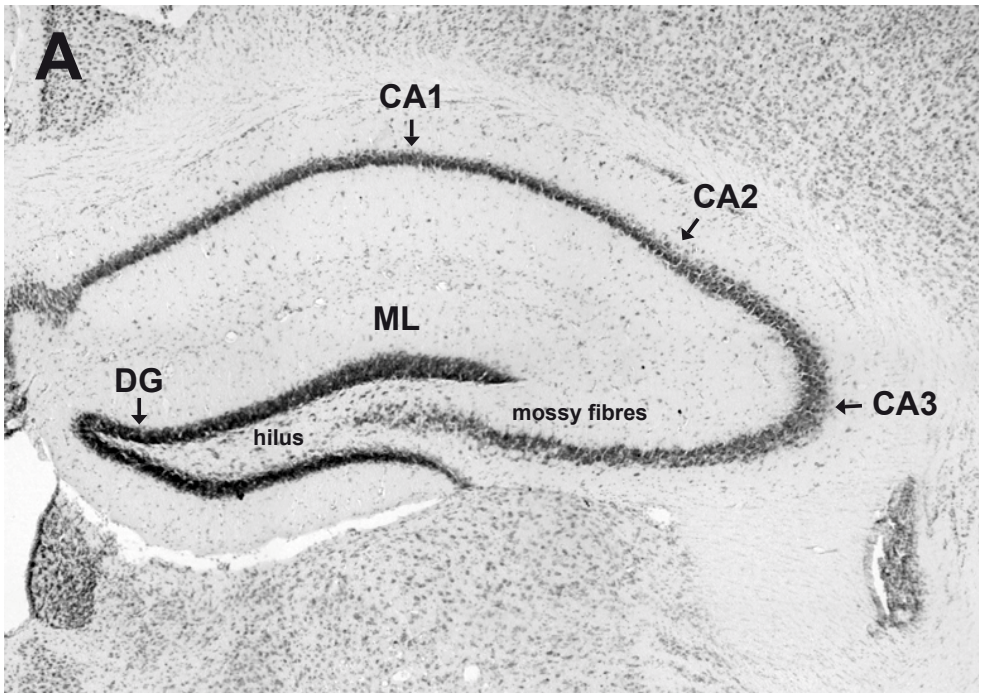
How the specification into several different interneurons is defined, has been intensively studied. Some crucial factors in the development of different PGC types are Paired box 6 (Pax6), a homeobox transcription factor, Olig2 (Hack et al., 2005;Kohwi et al., 2005), and the transcription factor Sp8 (Waclaw et al., 2006), Emx1 and Dlx5/6 (Kohwi et al., 2007). These factors seem to form a combinatorial "code" to define the final end product of the neurogenesis process providing information from where the cells came from and what they have to become (Lim et al., 2008). The anatomical origin of the cells seems determine the final destiny of the newborn neuron. So cells which differentiate into GABAergic and dopaminergic PGC's come from a different part of the SVZ than progenitors, which are only GABAergic. When type-A cells finally develop into OB interneurons, their long and complex journey has come to an end.

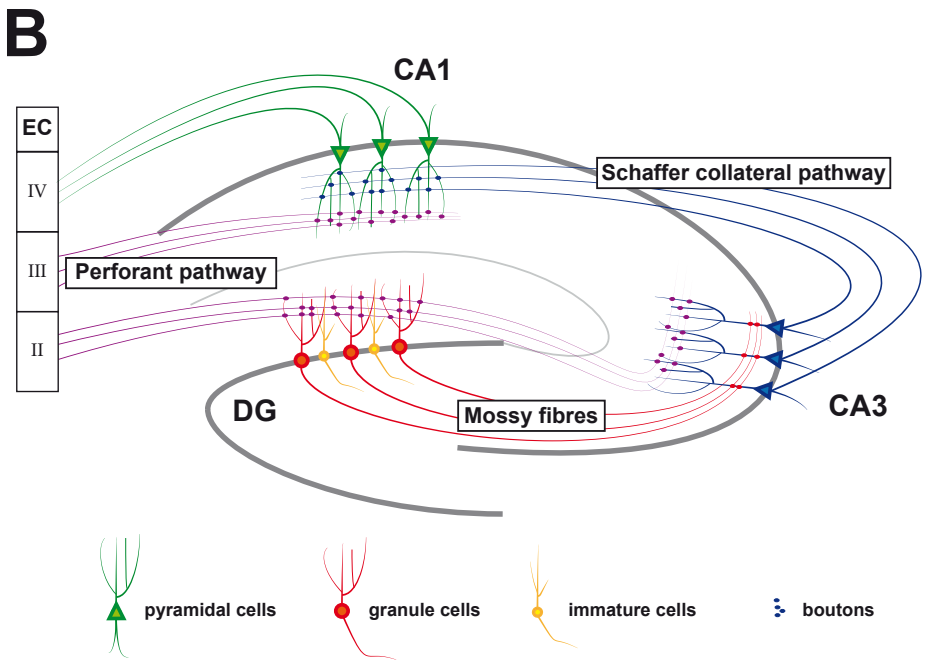
2.2 Hippocampal neurogenesis

Beside the SVZ another adult brain region continues to generate new neurons. In the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus, new granule cells are born and integrated into the network, (Abrous et al., 2005;Alvarez-Buylla and Lim, 2004;Kempermann et al., 2008;Lledo et al., 2006;Ming and Song, 2005;Ming and Song, 2011). This process differs from the SVZ neurogenesis since the DG is not close to ventricles and the migratory distance is much shorter. Also the final adult cell type is more homogeneous then the interneuron population in the OB. However, the hippocampus is an important crossroad in the brain. Unresolved questions to answer are the molecular mechanisms underlying hippocampal neurogenesis, the functional integration of newborn cells in hippocampal networks and the functional implication on hippocampus-depending cognition.

Hippocampal anatomy

The hippocampus can be divided into several sub regions like CA1, CA3 and DG (Fig.1). Together they form the trisynaptic pathway and get input from the enthorhinal cortex (EC) and


A
CA1
CA2
ML
DG
hilus
mossy fibres
CA3


B
CA1
EC
IV
III
II
Perforant pathway
Schaffer collateral pathway
DG
Mossy fibres
CA3
pyramidal cells
granule cells
immature cells
boutons

anterior commissure (AC) to which the hippocampus also projects its output. The DG forms a side loop on the projection from EC to CA3.

Ongoing neurogenesis occurs only in the DG and there is no evidence that other hippocampal regions like CA1 or CA3 generate new neurons (Kempermann et al., 2008). The DG is inhabited by several types of neurons and can be further divided into sub regions (Amaral et al., 2007). The DG consists of three layers with each their own characteristics. The upper layer is the molecular layer (ML), which is a relative cell free layer and is mainly occupied by the dendrites of granule cells from the granule cell layer (GCL) where they meet fibers from the perforant pathway that originate in the entorhinal cortex. The principal layer is the GCL, which is densely packed with granule cells. At the border between GCL and polymorphic layer (PL) reside some pyramidal basket cells. This border zone is also called subgranular zone (SGZ) and inhabits neural stem cells (NSC's), which are the starting point of ongoing adult neurogenesis. In the PL several cell types reside from which the mossy cell is the most prominent type (reviewed by Amaral et al., 2007). Because only granule cells are generated during adult life, I will discuss this cell type in further detail.

DG granule cell and cell layer

Granule cells in the DG have an elliptical cell body of about 10x18 µm. They are tightly packed and are not sheathed by glia cells. Granule cells have a cone shaped dendritic tree, which projects into the ML until the hippocampal fissure. On the other end of the cell body, granule cells give rise to unmyelinated axons called mossy fibers. These fibers have large boutons, which connect to mossy cells in the PL and pyramidal cells of the CA3. Principal mossy fibers give rise to several collaterals, which innervate cells in the PL before entering the CA3 (Amaral et al., 2007).

Figure 1: Overview of the hippocampal formation and its trisynaptic pathway. A: Nissl-stained coronal section through the hippocampal formation of the mouse. The major fields are indicated. Dentate gyrus (DG) encloses the hilus from which the mossy fibres project towards the CA3. Further along the pyramidal cell layer the CA2 and CA1 are situated. The DG is surrounded by the molecular layer (ML). B: Trisynaptic pathway which is formed by projections between CA1, CA3 and DG. Input from the entorhinal cortex (EC) layer II projects via the perforant pathway (purple) towards the dendrites of the DG granule cells (red). This perforant pathway projects further towards the CA3 were they end in boutons which enclose dendrites of pyramidal cells (blue). Mossy fibres (red) originating in the DG granule cells project to the same pyramidal cells in the CA3. Via the Schaffer collateral pathway (blue) the CA3 projects towards pyramidal neurons (green) in the CA1 which also receive input from the EC layer III. CA1 pyramidal neurons project (green) towards layer IV of the same EC. Only in the DG new granule cells (yellow) integrate into this network.

The SGZ contains a microenvironment that favours neuronal development. This is called a neurogenic "niche" and comprises precursor cells, their immediate progeny, immature neurons and some other supportive non-neuronal cell types (Kempermann et al., 2008). The process of adult neurogenesis is well studied and a detailed description of neuronal development is known nowadays. In the next paragraph I will elaborate into more detail about the distinct stages of neuronal development in the DG.

Neuronal development

SGZ proliferation differs slightly from SVZ proliferation, although general principles are the same. Terminology used in the extensive cell phenotyping study of (Doetsch et al., 1997) is the standard for SVZ neurogenesis. However, for hippocampal neurogenesis there is more variety in the use of terminology, see Alvarez-Buylla and Lim, (2004) versus Kempermann et al., (2008). Because Kempermann describes the full process in more detail, I will stick to their terminology in this chapter.

The neuronal development in the adult DG can be divided into several phases. The first distinction can be made between the expansion phase in which precursor cells proliferate, and the survival and elimination phase in which cells maturate and survive by integration into hippocampal networks or die. A subdivision of these two phases can be made; a precursor cell phase, an early survival phase, post mitotic maturation phase and a late survival phase. Furthermore, based on morphology and marker proteins six milestones can be separated from each other (reviewed by Kempermann et al., 2008).

Cell proliferation

During the precursor cell phase numerous cell types can be distinguished morphologically (Seri et al., 2001;Seri et al., 2004). They do not so much represent different cell populations as well reflect milestones of a developing process (Kempermann et al., 2008). As mentioned above, the primary NSC in the DG is a radial glia-like astrocyte and according to the nomenclature of (Kempermann et al., 2008) called a type-1 cell. Like RG's, these NSC's have long processes which project into the ML. There are also horizontal astrocytes, however, it is unclear whether they generate neurons or oligodendrocytes (Seri et al., 2004). NSC's are like normal glia cells and are not electrically excitable because they lack voltage gated sodium channels. Instead, they have voltage-independent potassium channels resulting in a low electrical input resistance of about 70 MΩ (Fukuda et al., 2003).

Progeny of these type-1 cells are type-2 cells, which function as nIPC. They are horizontally orientated with two small processes. Type-2 cells go through a developmental process in which several markers are expressed at different time points. Therefore two type-2 subtypes

can be distinguished; type-2a and type-2b (Steiner et al., 2006). Major difference between these two subtypes is the expression of GFAP and DCX. Type-2a cells have still some GFAP expression which type-2b cells do not have. However, type-2b cells do express DCX, which is a marker for immature and migrating neurons. Other markers for early granule cell development are Prox-1 and NeuroD1 (Steiner et al., 2006). Type-2 cells are thought to generate the bulk of NPC's because these cells in particular proliferate by neurogenesis-boostering factors such as exercise or antidepressant drug administration (Encinas et al., 2006;Kronenberg et al., 2003). Interestingly, before DCX expression, the glucocorticoid receptor (GR) is expressed in type-1 and type-2a cells. GR expression disappears when DCX comes to expression. GR expression revives when DCX expression fades away. At this time, the mineralocorticoid receptor (MR) is also expressed in these new mature neurons (Garcia et al., 2004).

With the development into type-2 cells, voltage-independent potassium channels disappear and the input resistance increases to 500 MΩ (Fukuda et al., 2003). In the mean time, γ-amino-n-butyric acid A (GABAA) receptors start to be expressed and the NPC's can be activated by tonic GABA (Wang et al., 2005). This ambient GABA input drives the differentiation of NPC's towards a neuronal phenotype (Ge et al., 2006).

The proliferative stage comes to an end when type-2 cell differentiate into type-3 cell. Although these cells can proliferate in certain conditions like epilepsy (Jessberger et al., 2005) they lose nestin expression and DCX becomes the primary marker to visualize immature neurons (Couillard-Despres et al., 2005;Plumpe et al., 2006;Rao and Shetty, 2004).
Post mitotic, immature neurons

When NPC's exit the cell cycle, a critical period for newborn cells begins. Firstly, under influence of GABA they start to develop dendrites and axons towards respectively the ML and CA3 regions. In the beginning, these dendrites lack any spines but receive functional GABAergic input (Esposito et al., 2005). At this stage, GABA has an excitatory effect, which induces dendritic growth. When this excitatory signalling is blocked, immature neurons develop abnormally (Ge et al., 2006).

Around two weeks after cell birth, GABAergic excitation changes towards GABAergic inhibition due to a change in Cl- transporter expression (Ge et al., 2006). At the same time, dendrites start to develop spines and receive their first glutamatergic input and the newborn cells become highly excitable (Esposito et al., 2005;Ge et al., 2006;Zhao et al., 2006). On the other end of the neuron, axons start to reach the CA3 region via the mossy fiber pathway. The first small boutons make contact with interneurons and CA3 pyramidal cell dendrites were after dendritic spines invade into the molecular layer where they make contact with in size increasing boutons (Faulkner et al., 2008). In the mean time, glutamatergic output

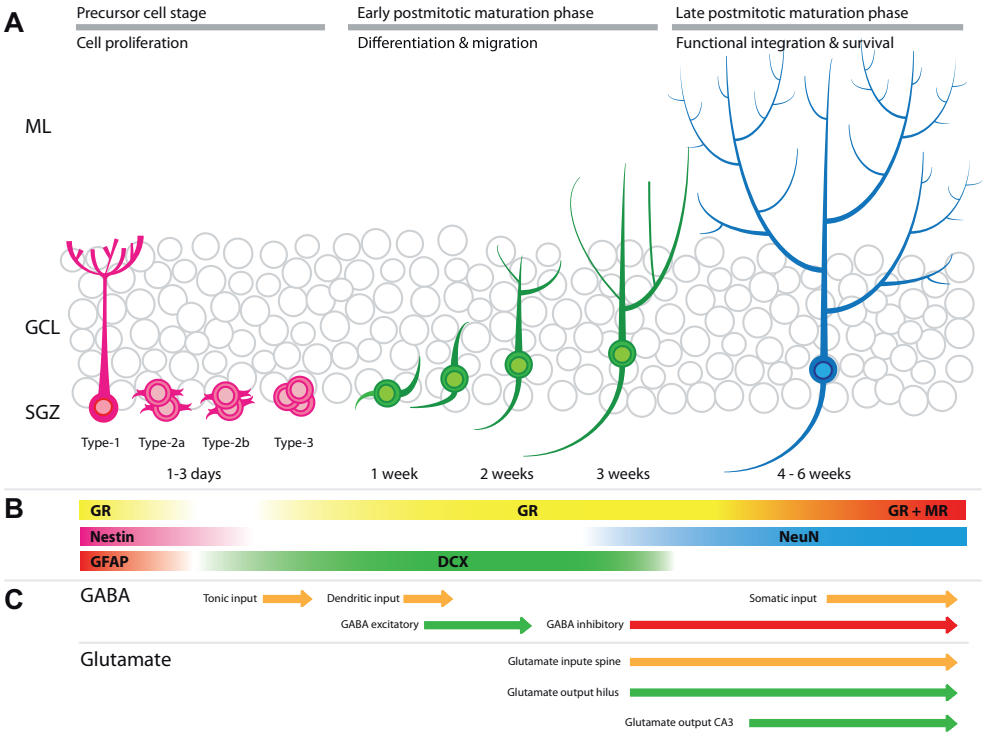


Figure 2: Overview of the adult neurogenesis process in the hippocampal dentate gyrus. A: The process can be divided into 3 different program sections. In the precursor cell stage, stem cells (Type-1) residing in the subgranular zone (SGZ) give birth to neuronal precursor cells (NPCs). Three different subtypes of NPCs (Type-2a, Type-2b and Type-3) can be distinguished depending on phenotype and marker expression. In the early post mitotic maturation phase, NPC's develop into immature neurons by outgrow of their neurites and migration into the granule cell layer (GCL). This process takes about 1 to 3 weeks. In the late mitotic maturation phase (4 to 6 weeks), the neurites develop further into mature dendrites by growing into the molecular layer (ML) and developing electrophysiological properties. B: The neurogenesis process is characterized over time by several markers. Stem cells and early NPC's are characterized by nestin and glial fibrillary acidic protein (GFAP) expression. Also glucocorticoid receptor (GR) is expressed in these stem cells and early NPCs. From NSC Type-2b onwards, doublecortin (DCX) is expressed during the early post mitotic maturation phase in these immature neurons. GR expression is absent during the time of DCX expression. In the late post mitotic phase, DCX expression fades away and is superseded by neuronal nuclear antigen NeuN. In these mature neurons, GR expression revives together with the expression of the Mineralocorticoid receptor (MR). C: Post mitotic cells start to develop electrophysiological properties. First GABA starts to become active. First in an excitatory role, but later, when glutamate becomes active, in an inhibitory role. At the end of the immature stage, when glutamate becomes active, the neurons show mature electrophysiological properties.

towards the CA3 region starts to appear (Faulkner et al., 2008;Toni et al., 2008). They will first make contact with interneurons in both hilus and ML at the moment when they are highly excitable, thereby creating temporal clusters of hippocampal connections which might reflect long-term episodic memories (Aimone et al., 2006;Toni and Sultan, 2011).

Not all newborn cells will survive and a number of cells die by an apoptotic process (Biebl et al., 2000;Kuhn et al., 2005). Already 3 days after BrdU injection (labelling of dividing cells), the number of BrdU positive cells decreases. The number of labelled cells keeps on decreasing until 4 weeks after labelling where after the number of BrdU positive cells remains stable for a long period of time (Kempermann et al., 2003). How much neurogenesis contributes to the total population of granule cells is not exactly known (Kempermann et al., 2008). Several factors contribute to cell survival or cell death. For an animal, enriched environments or learning tasks do induce cell survival (Dobrossy et al., 2003;Gould et al., 1999b;Kempermann et al., 1998b). Cell death of newborn neurons seems also necessary for proper learning, blocking apoptosis impairs learning, learning-induced cell survival and proliferation (Dupret et al., 2007).

The immature neurons do not stay in the SGZ but migrate radially into the DG. However, they do not migrate further than the inner third layer (Esposito et al., 2005;Kempermann et al., 2003). The majority of GC's, which inhabit the middle and outer third layers are born during early postnatal development (Muramatsu et al., 2007). Several factors regulate this migration like disrupted in schizophrenia 1 (DISC1) and glucocorticoid receptors (GR's) and when manipulated, migration is affected and adult born GC's end up in middle or outer third layer. Both factors seem to temper migration speed and distance (Duan et al., 2007;Fitzsimons et al., 2013). In addition, epileptic seizures can 'push' newborn neurons towards the other direction into the hilus (Jessberger et al., 2007).

Late, post mitotic maturation phase

Cells, which survived, integrate into the local DG network and are at the end functional similar to other granule cells (Esposito et al., 2005;Laplagne et al., 2006;van Praag et al., 2002;Zhao et al., 2006). Although newborn cells lose immature markers around 4 to 5 weeks after cell birth they are not fully differentiated. Full maturity of the excitatory input is only reached around 60 days after cell birth (Laplagne et al., 2006). After the first weeks they continue to extend their dendritic and axonal processes and many new connections are formed, also with glutamatergic synapses from entorhinal cortex and out-put to pyramidal cells in the CA3. Not only newborn GC's modulate their connectivity, also mature GC's change connectivity.

Mature GC's lose some of their connections with entorhinal cortex derived axons and are replaced by newborn GC's dendrites. It seems that synapse connections from newborns GC's compete at entorhinal boutons with old synapses from mature GC's. Dendritic spines start as small filopodia searching for synaptic clusters at presynaptic axonal boutons also called multiple synapse boutons (MSB). Tony and colleagues (Toni et al., 2007;Toni and Sultan, 2011) suggest the filopodia are attracted to these MSB's by synaptic activity mainly induced by glutamate spill over. The filopodia develop into synapses connected to these MSB's, which later in time replace the old synapses. Only the synapse from newborn neurons survives this temporarily double connected stage. As mentioned earlier, this temporal double connected situation might be functional in generating temporal clusters of long-term episodic memories (Aimone et al., 2006;Toni and Sultan, 2011).

This pattern of competitive synaptic plasticity in the molecular layer seems also to be present in the hilus and CA3 region at the axonal end of newborn neurons. Although boutons actively connect to synapses already 17 days after cell division, the connections are fully mature after 2 months post cell division (Faulkner et al., 2008;Toni et al., 2008).

2.3 Adult neurogenesis function

About two decades ago, the field of adult neurogenesis was dominated by the discussion about the existence of the process but at present an incredible amount of work convinced the majority of scientist to accept adult neurogenesis. The main focus nowadays is the function of the neurogenesis process. Why does it happen and why does it happen only in the SVZ and DG? Evidence of adult neurogenesis in humans is emerging (Curtis et al., 2003;Curtis et al., 2007;Eriksson et al., 1998;Lucassen et al., 2010b;Sanai et al., 2007;Wang et al., 2011) but is still difficult to proof as experiments cannot easily be performed. Presently, the presence of adult neurogenesis in the human brain is still under debate.

Neurogenesis in the mammals

Of all higher animals, mammals share a unique feature, which is the proliferation of neuronal stem cells (NSC's) in the hippocampal dentate gyrus. Where the proliferation of NSC's in vertebrates is limited to the walls of the lateral ventricles, mammals do show a second area of NSC proliferation (Altman and Das, 1965;Gould, 2007;Kaplan and Hinds, 1977;Taupin and Gage, 2002). At several other sites evidence for newborn neurons is found like the olfactory tubercle and hypothalamus (see Table 2 for a full overview). However, the existence of adult neurogenesis in these regions is still under debate (Bonfanti and Peretto, 2011;Gould, 2007;Rakic, 2002;Rakic, 2006). The rate of adult neurogenesis is very low and might represent technical artefacts or new neurons are only found after physical or chemical induction

of damage. To conclude, the mammalian brain harbours two sites with ongoing neurogenesis with a relative high turnover of newborn cells and the existence of silent areas with inducible neurogenesis cannot be excluded.

Although the exact function of neurogenesis is not yet known, research in the lab focuses mainly on the mechanism of neurogenesis and how it is influenced by different kinds of factors especially stress. In the past few years, several transgenic animal models appeared in which neurogenesis can be blocked on demand. Since smell is not a primary sense of human beings, functional research on hippocampal neurogenesis got far more attention compared to the question about the function of adult neurogenesis in the olfactory bulb. Nonetheless for these two sites, functional questions are asked and studies performed.

The majority of work in the lab is performed with rats and mice. Laboratory strains are often for decades in captivity devoid of any natural/environmental selection criteria. There are many mouse strains and differences between these strains in regard to adult neurogenesis are present, which suggests genetic involvement (Kempermann et al., 1997a). Also external cues can manipulate adult neurogenesis: exercise and cage enrichment can boost proliferation and survival of newborn cells in laboratory rodents (Kempermann et al., 1997b;van Praag et al., 1999b;van Praag et al., 1999a). Since laboratory mice live in rather small and dull environments compared to wild conspecifics, their baseline neurogenesis might not reflect a natural situation. However, comparative studies have been performed with several rodent species but not with wild and laboratory living Mus musculus (Amrein et al., 2004a). However, a comparative study on several laboratory and wild derived rats revealed not a

Table 2: Overview of all brain areas in which adult neurogenesis is reported.

Brain region	Literature
Neocortex	Altman, 1963; Bernier et al., 2002; Bloch et al., 2011; Cai et al., 2009; Dayer et al., 2005; Gould et al., 1999c; Huang et al., 1998; Kaplan, 1981; Zhang et al., 2009
Striatum	Bedard et al., 2002; Bedard et al., 2006; Dayer et al., 2005; Luzzati et al., 2006
Amygdala	Bernier et al., 2002; Fowler et al., 2002; Fowler et al., 2005; Zhang et al., 2009
Piriform cortex	Pekcec et al., 2006
Olfactory tubercle	Bedard et al., 2002; De et al., 2004
Hypothalamus	Cifuentes et al., 2011; Dietrich and Horvath, 2012; Fowler et al., 2002; Fowler et al., 2005; Huang et al., 1998; Kokoeva et al., 2005; Kokoeva et al., 2007; Lee et al., 2012; Matsuzaki et al., 2009; Pencea et al., 2001; Perez-Martin et al., 2010; Rodriguez et al., 2005; Sousa-Ferreira et al., 2011; Xu et al., 2005
Substantia nigra	Zhao et al., 2003; Zhao and Janson Lang, 2009 but see Frielingsdorf et al., 2004
Brain stem	Bauer et al., 2005

great difference in neurogenesis parameters (Epp et al., 2009). Only Sprague-Dawly rats seem to have reduced proliferation compared to wild and captive Brown Norway and Long Evens rats. Such a difference in neurogenesis, however, is absent using DCX as marker. This is a surprising finding considering the difference in environments like stressors, exercise and other environmental enrichment.

Neurogenesis theories

Most of the theories explaining the function of adult neurogenesis are from a human, medical perspective. Since most work is performed on rodents and studies on human tissue are rare, the importance of adult neurogenesis in the human brain is under debate. Compared to rodents, the olfactory bulb seems to be of a rudimentary magnitude in the human brain so the most theories just focus on hippocampal neurogenesis (Abrous and Wojtowicz, 2008;Aimone et al., 2006;Aimone et al., 2010;Aimone et al., 2011;Deng et al., 2010;Gould et al., 1999b;Kempermann, 2008;Sahay et al., 2011b;Wiskott et al., 2006) although specific literature on the olfactory bulb can be found (Lazarini and Lledo, 2010;Lledo, 2008;Lledo and Saghatelyan, 2005;Lledo et al., 2006). Recent work suggests a role for neurogenesis in reproduction and kin recognition by scent (Lau et al., 2011;Mak et al., 2007;Mak and Weiss, 2010).

The first theories appeared at the end of the 20th century and pointed towards a role in memory formation (Gould et al., 1999b). In 2006, Aimone and colleagues (Aimone et al., 2006) suggested a role for adult neurogenesis in the encoding of time in new memories. They called it the 'temporal association memory hypothesis'. New neurons are thought to be important to incorporate a time label into memories. In this hypothesis, the neurogenesis process in the olfactory bulb is neglected. In the same year, Wiskott and colleagues (Wiskott et al., 2006) suggested that neurogenesis helps to prevent catastrophic interference in the hippocampus when adapting to new environments. New, plastic neurons help to prevent the hippocampus against negative side effects due to rearranging networks, induced by new, changing environments. Also Wiskott and colleagues (Wiskott et al., 2006), leave the role of the olfactory bulb nearly unexplained. Kempermann (2008) introduced another theory, 'the neurogenic reserve hypothesis'. Based on older theories concerning neurodegenerative diseases, this hypothesis suggests neurogenesis keeps the brain flexible and plastic for moments when an animal encounters novel and complex situations. This theory is comparable with the one posted (Lledo and Saghatelyan, 2005) which concerns the olfactory bulb.

The latest theories suggest a role for adult neurogenesis in pattern separation. In 2011, two review articles appeared in Neuron discussing the role of neurogenesis in the adult brain.

Sahay and colleagues (Sahay et al., 2011b) suggest newborn neurons are involved in pattern separation in the hippocampus and olfactory bulb. Pattern separation is a process in which overlapping or similar representations are transformed in to less similar outputs (Sahay et al., 2011b). The opposite of pattern separation is pattern completion. In this process a reconstruction of complete stored representations is based on partial inputs that match with parts of stored representations. It is thought that pattern completion takes place in the CA3 and pattern separation in the DG. According to Sahay and colleagues (Sahay et al., 2011b), there is a balance between pattern completion and separation, which is regulated by neurogenesis. An increase in neurogenesis pushes the balance towards pattern separation whereas a decrease in neurogenesis pushes the balance towards pattern completion. Pattern separation leads to increased discrimination and cognitive flexibility whereas pattern completion leads to generalization and inflexibility. Problem with this theory is the fact that pattern separation and completion might not be specific for the hippocampus and OB. In the same issue of Neuron, Aimone and colleagues (Aimone et al., 2011) argue that an underlying mechanism (memory resolution) affects these processes specifically in the neurogenic regions. According to Aimone and colleagues (Aimone et al., 2011) the amount of adult neurogenesis affects memory resolution instead of pattern separation. Subsequently, this memory resolution affects the pattern separation process. A key feature of this process is the developmental cascade of a newborn neuron. Newborn neurons of 4 to 8 weeks old have different electrophysiological properties compared to mature neurons in the DG or OB. Immature neurons provide complete but low-informative representations of experiences whereas mature neurons provide incomplete but high-informative representations. The combination includes both representations with the capacity to include novel situations via immature neurons. However, solid evidence has to be found for both theories.

Although theories appear frequently, many of them are hardly tested. They raise new questions and fuel discussions about neurogenesis. However, recently new techniques with sophisticated animal models appear (see paragraphe 3). More specific ablation of newborn neurons in healthy tissue might bring better tools to study the function of adult neurogenesis. Together with a broader view than rodents and a human medical perspective, the function of adult neurogenesis may be revealed.

Neurogenesis and mental disorders

Several studies showed the presence of adult neurogenesis in the human brain (Curtis et al., 2007;Eriksson et al., 1998;Lucassen et al., 2010b;Wang et al., 2011) (reviewed by Curtis et al., 2011). However, the evidence is scarce and just shows the fact that new neurons might be present in the adult human brain. Despite the fragile evidence, for several psychiatric diseases the involvement of adult neurogenesis is suggested.

Depression and adult neurogenesis

Adult neurogenesis is an ongoing process in the adult brain although it decreases over time in the ageing animal (Kuhn et al., 1996;Amrein et al., 2004a;Amrein et al., 2004b). However, many internal and external factors can affect the rate of neurogenesis like environmental enrichment (Kempermann et al., 1997b;Kempermann et al., 1998b;Gould et al., 1999a;Brown et al., 2003a;Fowler et al., 2002;Sandeman and Sandeman, 2000;Scotto et al., 2000), physical activity (van Praag et al., 1999b;van Praag et al., 1999a;Rhodes et al., 2003;Farmer et al., 2004) and stress (Mirescu and Gould, 2006). Since the hippocampus is an important chain in the HPA-axis (Jacobson and Sapolsky, 1991) and adult neurogenesis contributes to the function of the hippocampus (Ming and Song, 2011;Aimone et al., 2011;Sahay et al., 2011b), adult neurogenesis might play a role in stress (Snyder et al., 2011), and stress related diseases like major depressive disorder (MDD) (Warner-Schmidt and Duman, 2006;Sahay and Hen, 2007;Jacobs et al., 2000) and schizophrenia (Reif et al., 2006;Pickard, 2011).

Hippocampal volume

There are several indications suggesting a role of adult neurogenesis in depression. An often used argument is that patients suffering from MDD show a decrease in hippocampal volume (Sheline et al., 1996;Sheline et al., 1999;Bremner et al., 2000;Steffens et al., 2000;MacQueen et al., 2003). Since adult neurogenesis is also affected by dysregulated glucocorticoid signalling, adult neurogenesis is often mentioned in relation to hippocampal volume loss (Duman, 2004;Warner-Schmidt and Duman, 2006;Jacobs et al., 2000). However, the underlying cellular alterations are far from clear. Evidence points to atrophy of neuropil instead of major loss of cell nuclei (Sapolsky, 2000;Watanabe et al., 1992;Stockmeier et al., 2004). A recent study found a correlation between the onset of depression age and hippocampal volume, but not between hippocampal volume and HPA-axis activity (Gerritsen et al., 2011). It is therefore unlikely that the rate of adult neurogenesis contributes significantly to the loss of hippocampal volume (Sapolsky, 2001;Sapolsky, 2004;Czeh and Lucassen, 2007).

Antidepressants and adult neurogenesis

Another factor suggesting involvement of adult neurogenesis in the regulation of stress-related diseases are antidepressant drugs. Several anti-depressant drugs booster adult neurogenesis (Malberg et al., 2000;Czeh et al., 2001;Li et al., 2004;Encinas et al., 2006;Xu et al., 2006) although not all mouse strains are susceptible (Holick et al., 2008;Huang et al., 2008). However, in humans such a strong effect is not found. No differences were found between patients suffering from MDD and healthy controls (Reif et al., 2006). Also patients treated with antidepressants did not show increased proliferation although another study did find

a relation between the use of antidepressant drugs and increased proliferation in humans (Reif et al., 2006;Boldrini et al., 2009). Interestingly, MDD patients treated with selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) showed increased immunoreactivity for proliferation markers nestin and Ki-67 compared to untreated MDD patients and healthy controls. This study suggests that depressed patients do not suffer from reduced neurogenesis, but treatment with antidepressants induces neurogenesis. However, these studies only included markers for proliferation and later neurogenic stages are not studied.

The other way around: can altered adult neurogenesis affect the functioning of antidepressant drugs? Several methods are used to reduce adult neurogenesis like X-ray or gamma-ray irradiation (Meshi et al., 2006;Santarelli et al., 2003;Holick et al., 2008;Wang et al., 2008;Surget et al., 2008;Airan et al., 2007;David et al., 2009), antimitotic drugs like methylazoxymethanol acetate (MAM) (Bessa et al., 2009;Jayatissa et al., 2009) and transgenic mouse models (Saxe et al., 2006;Li et al., 2008) providing interesting tools to unravel the relationship between neurogenesis, stress and antidepressant treatments. In the mean time these studies show differences between mouse strains in the response to adult neurogenesis reduction on antidepressant treatment and anxiety/depression related behaviours (reviewed by David et al., 2010). For example, 129SvEv mice show a response to AD treatment on proliferation, survival and maturation stages of neurogenesis (Meshi et al., 2006;Santarelli et al., 2003;Wang et al., 2008) whereas BalBcJ mice a resilient to such treatments at all neurogenic stages (Holick et al., 2008;Huang et al., 2008). On top of that, some effects of AD treatment like novelty suppressed feeding and coat state seem to be neurogenesis dependent whereas other effects like open field and forced swim test are independent from adult neurogenesis (David et al., 2010). These data implicate that future experiments have to be designed carefully considering the proper behavioural test and genetic background. On the other hand, differences between genetic backgrounds should be evaluated carefully whether these differences are functional relevant and not artificially induced due to inbreeding.

Glucocorticoids and adult neurogenesis

As mentioned before, the hippocampus is an important chain in the HPA-axis (Jacobson and Sapolsky, 1991). The hippocampus is involved in the negative feedback regulation of glucocorticoid release via the presence of GR and MR (Reul and deKloet, 1985;de Kloet et al., 1998). Chronic or heavy acute stress can unbalance the HPA-axis homeostasis and subsequently result in depression (de Kloet et al., 2005;Mcewen, 2007;Holsboer and Ising, 2010). Fifty percent of patients with MDD show dysregulation of the HPA-axis and thereby altered levels of glucocorticoids (Young et al., 1991;Gold and Chrousos, 2002).

The process of adult neurogenesis is very vulnerable to artificial altered glucocorticoid levels (Gould et al., 1992;Cameron and Gould, 1994;Cameron et al., 1998;Wong and Herbert, 2004) and also various ways of stress affect adult neurogenesis like subordination stress (Gould et al., 1997), resident-intruder stress (Gould et al., 1998), isolation (Dong et al., 2004;Ibi et al., 2007), predator odour (Falconer and Galea, 2003;Tanapat et al., 2001), early life stress (Mirescu et al., 2004), restraint stress (Pham et al., 2003;Bain et al., 2004), inescapable shock (Malberg and Duman, 2003;Vollmayr et al., 2003), cold immobilization or swimming (Lee et al., 2002;Heine et al., 2004b) and sleep deprivation (Mirescu et al., 2006).

However, a direct link between the levels of glucocorticoids and the rate of adult neurogenesis is far from clear. Reduction of glucocorticoid circulation by adrenalectomy (ADX) boosts proliferation levels (Gould et al., 1992;Cameron and Gould, 1994) and chronic treatment with glucocorticoids reduces neurogenesis (Cameron et al., 1998;Wong and Herbert, 2004) suggesting that the amount of glucocorticoids directly regulates the amount of adult neurogenesis. However, high levels of glucocorticoids are not always negative for the hippocampal neurogenesis (de Kloet et al., 1999;Lehmann et al., 2013).

Although glucocorticoids are released into the plasma in a pulsatile manner during the sleep/wake cycle, glucocorticoids still peak in a circadian fashion around the onset of activity and are nearly gone at the onset of the rest period (Lightman et al., 2008;Dickmeis and Foulkes, 2011). Although opposite findings have been reported (Guzman-Marin et al., 2007), studies on proliferation with BrdU and Ki-67 expression in the DG revealed no differences in the amount of proliferation between activity and rest periods during baseline conditions.

However, compared to sedentary animals, running wheel activity induces a significant increase of proliferation during the activity period but not during the rest period In rats, exercise initially increases glucocorticoid levels, but animals adapt to the new conditions and the increased levels of glucocorticoids drop back to baseline levels after several weeks of exercise (Fediuc et al., 2006). The return to normal levels of proliferation after chronic exercise is shown in mice that are exposed to 32 days of exercise.
Interestingly, Van Praag and colleagues (van Praag et al., 1999b) did not find differences in corticosterone levels between runners and sedentary mice. Also Kannangara and colleagues (Kannangara et al., 2009) did not find differences in levels of corticosterone between running and sedentary mice, while running animals do show increased levels of proliferation. However, when these animals were restrained for 15 minutes, no increase of proliferation was found in running animals, which were group housed. Single housed animals did not show such a response (Kannangara et al., 2009). Stress might undo exercise induced increase in proliferation, but single housed mice seem to be non-responsive. These findings are in contrast with studies on rats, which seem to be more susceptible to isolation. Although

they show also an increase in corticosterone levels, their proliferation rates decrease when a running wheel is provided (Stranahan et al., 2006).

Although there are a number of contradictions in the findings with exercised-induced neurogenesis and the level of glucocorticoids, the relative short peak of exercise (several weeks) associated with increased glucocorticoid levels seems to match the short peak of increased proliferation. Since also lifelong reduction of glucocorticoid levels does not alter the rate of adult neurogenesis at later age (Brunson et al 2005) the body seems to adapt after chronic changed glucocorticoid levels towards a new homeostasis, a process known as allostasis (Mcewen, 1998;Mcewen and Gianaros, 2011).

In these cases, increased neurogenesis comes along with increased levels of glucocorticoids. Interestingly, this exercise induced increase of glucocorticoids and neurogenesis seems to correlate with anxiety-like behaviours (Fuss et al., 2010), again a paradox with a contradictive role for adult neurogenesis. Maybe the context in which the levels of glucocorticoids are perceived might induce or inhibit neurogenesis. On the other hand, the presence of adult neurogenesis also influences the glucocorticoid response. When adult neurogenesis is ablated, the negative feedback on glucocorticoid levels in the blood take longer to reach basal levels compared to animals which do have adult neurogenesis (Snyder et al., 2011). Without the small hippocampal subpopulation of newborn cells, the negative feedback is partly disrupted and thereby increasing the weight of perceived stressors. Together with many other factors, adult neurogenesis takes part in a system that incorporates many and often paradoxical signals. Whether adult neurogenesis is an important target to treat depression or just a powerful biomarker remains to be elucidated.

Summary

Neurogenesis is wide spread through the animal kingdom. Even simple life forms like hydra seem to have generation of neurons in later life. In vertebrates, neurogenesis exists mainly along the ventricle walls of the brain. However, mammals share the existence of neurogenesis in the ventricular zone and hippocampus. Less is known about the function of adult neurogenesis from an evolutionary perspective. Just a few wild living species are studied and comparisons between wild living animals and their well studied laboratory conspecifics are scarce. Most theories are formulated from a human, medical perspective and several human psychiatric diseases are linked to adult neurogenesis malfunctions although the evidence for neurogenesis in humans is scarce. Glucocorticoids play an important role in the regulation of the HPA-axis. Glucocorticoids regulate neurogenesis and play a major role in stress-related diseases like depression and mood-disorders. Cause and effect in these processes need to elucidated.

3 Animal models

In the first studies on adult neurogenesis in the sixties, Altman and Das used [H3]-thymidine-incorporation to mark newborn neurons (Altman, 1963;Altman and Das, 1965;Altman, 1969). Later, the incorporation of bromodeoxyuridine (BrdU) was developed which is nowadays a useful method to label newborn cells (Taupin, 2007;Wojtowicz and Kee, 2006). Because BrdU incorporation has some limits and transgenic mice became popular, a wide variety of constitutive reporter mice were developed (for an overview see Dhaliwal and Lagace, 2011). Reporter mice have constructs inserted in which a regulatory element (promoter) of a specific gene of interest is combined with a construct transcribing fluorescent protein (for example green fluorescent protein, GFP). Regulatory elements of several endogenous markers used for cell typing newborn neurons like GFAP, Nestin or DCX are used to transcribe GFP. Especially in combination with BrdU, such reporter mice proved their usefulness within the field of adult neurogenesis (Dhaliwal and Lagace, 2011).

Beside the visualization of the neurogenesis process, it became necessary to manipulate the neurogenic process to study its function. Several more or less specific methods were developed like the use of antimitotic drugs (Bessa et al., 2008;Jayatissa et al., 2009), X-ray or gamma-ray irradiation (Airan et al., 2007;David et al., 2009;Holick et al., 2008;Meshi et al., 2006;Sahay et al., 2011a;Santarelli et al., 2003;Saxe et al., 2006;Surget et al., 2008;Wang et al., 2008), retro- and lentivirus-mediated gene transfer and transgenic mice (Bai et al., 2003;Duan et al., 2007;Fitzsimons et al., 2013). Since the construction of the first transgenic mouse in 1974 (Jaenisch and Mintz, 1974), a wide variety of possibilities has emerged. Beside classical knockout mice (Corbo et al., 2002;Kappeler et al., 2006), also conditional and inducible transgenic (Imayoshi et al., 2006;Imayoshi et al., 2008;Zhang et al., 2010) mice are developed.

3.1 Retro- and lentivirus-mediated gene transfer

One of the first successful specific manipulations of adult neurogenesis was the study of Duan et al. (Duan et al., 2007) were they injected retroviral vectors containing short hairpin (sh) RNA expressing backbones targeting the Disrupted In Schizophrenia 1 protein (DISC-1). These retroviral vectors are injected into the neurogenic niche of the hippocampus were it transfects proliferating cells only that subsequently start to transcribe shRNA. This shRNA targets DISC-1 mRNA and thereby down regulating DISC-1 protein levels. Strikingly, newborn neurons with DISC1 knockdown showed an aberrant morphology and migrated much further into the granule cell layer compared to cells transfected with scrambled shRNA.

A similar phenotype was found by Fitzsimons and colleagues (Fitzsimons et al., 2013) after shRNA-mediated knockdown of the glucocorticoid receptor (GR). Also in this study, newborn cells with GR knockdown migrated further into the DG. Moreover, dendritic arborisation and dendritic spines of these newborn neurons showed abnormalities, showing that the GR plays an important role in the adult neurogenesis process.

The technique of virus-mediated gene transfer is quite effective although there are negative aspects as well. Practically, stereotactic delivery is difficult and needs training and experience. Furthermore, injections may induce inevitable scars and other damage into the brain, thus requiring appropriate controls such as scrambled shRNA. However, the advantage of this system is the possibility of easy switching between shRNA vectors and avoids the time-consuming generation of transgenic mice.

3.2 Classical knockout mice

An interesting animal model to study adult neurogenesis might be the doublecortin knockout mouse (Corbo et al., 2002;Kappeler et al., 2006). Since the doublecortin protein is key for migration of immature neurons and is abundantly expressed in neuronal progenitor cells but not in adult neurons or other brain cells, it might be possible that DCX knockout will have deleterious consequences for the neurogenic process without affecting mature neurons and other cell types. However, whereas subtle mutations causing a single amino acid substitution in the DCX protein in humans have dramatic consequences for brain development, complete knockout of the DCX gene in mice exhibit no clear phenotype. Since local and acute DCX knockdown in the developing cortex by RNA-interference technology results in a clear phenotype, it is postulated that genetic ablation is compensated by other proteins with an comparable function like the homologous DCLK-gene (Deuel et al., 2006;Koizumi et al., 2006;Weimer and Anton, 2006) (more about the doublecortin protein family see paragraph 4). Therefore, classical knockout animals might not be the ideal method for studying adult neurogenesis, especially when developmental neurogenesis should be unaffected.

3.3 Conditional transgenic mice

Site-specific recombinases (SSRs) were developed to study functional consequences of gene ablation in specific neuronal populations. In mammalian tissue, the most widely used SSR is Cre, a P1 bacteriophage derived λ integrase. Cre recognizes loxP sites in genomic DNA. These LoxP sites can be introduced by genetic manipulations flanking a gene of interest. Subsequently, activated CRE excises the DNA, and thus the gene of interest, between the two loxP sites. The expression of Cre can be cell type specific when the expression of Cre is controlled by a specific promoter of interest(Branda and Dymecki, 2004). Recombination of

Cre with a mutated ligand binding domain (LBD) of the estrogen receptor (ER) resulted in the possibility of temporal control of Cre-mediated recombination. Administration of tamoxifen, a synthetic estrogen antagonist, will activate Cre in specific tissues at a specific time of interest. The CreER site is not susceptible for natural ligands of LBD. In the field of adult neurogenesis, the nestin promoter is widely used in combination with this CreER system. Also several other promoters of well known neurogenesis markers are used like GFAP or DCX (for an overview see Imayoshi et al., (2011) and Dhaliwal and Lagace, (2011). Although these systems work well to study adult neurogenesis, real temporal control is not available since Cre activity cannot be reversed.

3.4 Inducible and reversible transgenic mice

Transgenic models with reversible gene knockout technologies are the reverse tetracycline-controlled transactivator (rtTA) regulated models (also known as Tet-On or Off systems). A tet-on system is activated when doxycycline (dox) is administered in food or drinking water whereas a tet-off system blocks the transcription of targeted genes when dox is present (Dhaliwal and Lagace, 2011;Imayoshi et al., 2011). Within the field of adult neurogenesis just one such model is well-studied (Dupret et al., 2008). In this model, the bax protein is transcribed when dox binds to the rtTA protein. The rtTA protein is transcribed under control of the nestin promoter. When bax transcription is activated, the protein induces cell death in cells were nestin is activated (Dupret et al., 2008). The studies of Dupret and

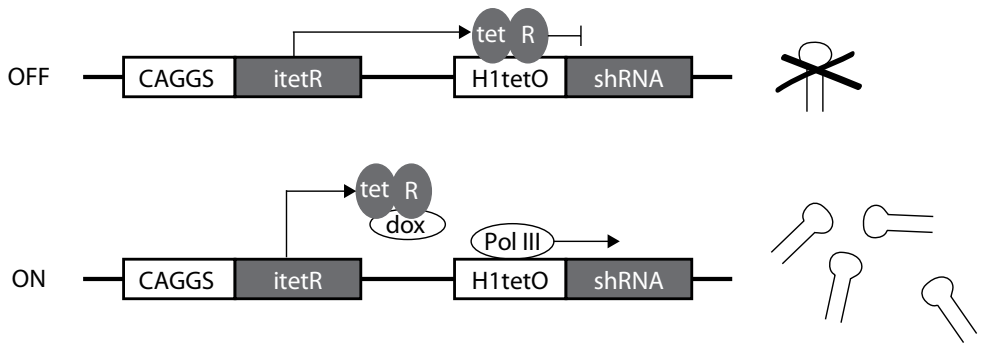


Figure 3: Inducible and reversible shRNA transcribing construct. In a normal condition (OFF), the CAGGS promoter transcribes the tet repressor (tetR) which blocks the H1tetO promoter. No shRNA is transcribed. When dox is applied (ON), dox binds to the tetR which can no longer bind to the H1tetO promoter. Polymerase III (Pol III) can transcribe shRNA. This shRNA binds to their target mRNA which disintegrates preventing protein expression.

colleagues showed that ablation of hippocampal neurogenesis results in impaired spatial relational memory and that adult neurogenesis is necessary for complex forms of hippocampus-mediated learning (Dupret et al., 2008). This Tet-system can also be used to study gene function by manipulating the transcription of genes of interest using short interference RNA (siRNA) (Seibler et al., 2007). In such a tet-on system, the transcription of a short hairpin RNA (shRNA) is induced with doxycycline (Fig. 3). shRNA targets mRNA of a specific gene of interest and prevents protein synthesis by breaking down mRNA. Theoretically, this system allows the knockdown of unique splice-variants of any given gene without affecting other splice-variants.

Summary

To unravel the biological significance, major efforts have been undertaken in the last decade to manipulate adult neurogenesis. Whereas first attempts were not very specific and with numerous side effects (e.g. as is the case with the use of antimitotic drugs and radiation), the subsequent development of viral vector mediated gene transfer and specific transgenic mice provide excellent opportunities to study the function of newborn neurons in rather heterogeneous cell populations in the hippocampal DG and olfactory system.

However, although these models revealed a great part of the neurogenesis process, the function of adult neurogenesis is hardly known. There is a huge amount of evidence that points towards hippocampal memory and cognition but the majority of the work is done in artificial animal models. Evolution history can provide the answer to the question about neurogenesis function. Therefore, new hypothesis based on ecology and evolution of wild living species are needed together with a more systematically description of adult neurogenesis in a wide variety of animal species. This question is beyond the scope of this thesis.

4 Doublecortin & Doublecortin-like

Doublecortin (DCX) is a well known marker for newborn neurons since it seems to be mainly expressed in migrating neuroblasts (Brown et al., 2003b;Couillard-Despres et al., 2005;Nacher et al., 2001). DCX gives also its name to a whole family of proteins (Coquelle et al., 2006;Dijkmans et al., 2010;Reiner et al., 2006) of which doublecortin-like (DCL) is the family member which is most homologous to DCX (Vreugdenhil et al., 2007). However, DCL is an alternative splice variant of a much more complex gene named doublecortin-like kinase 1 (DCLK1). Since the role of DCL is neurogenesis is central in this thesis, I would like to discuss in more detail our knowledge on DCX and in particular the DCLK1 gene.

4.1 Doublecortin gene family

The archetypical gene of the DCX family is doublecortin (DCX), which was first discovered in humans in 1998 as a gene associated with the double cortex syndrome, a disease which is characterized by a malformation of the neocortex (des Portes et al., 1998;Gleeson et al., 1998). Nowadays, DCX is widely established as a marker of immature neurons in the adult brain(Brown et al., 2003b;Couillard-Despres et al., 2005;Nacher et al., 2001). The doublecortin gene family comprises 11 members in humans and 11 in mice (Coquelle et al., 2006;Dijkmans et al., 2010;Reiner et al., 2006). Humans and mice have 10 proteins in common and each have 1 unique protein, which has no human or mouse orthologue. All family members contain one or two DCX domains. These DCX domains are characterized by their microtubule binding properties and are thought to function as stabilizers of microtubules (Cierpicki et al., 2006;Kim et al., 2003). Therefore they belong to the group of microtubule-associated proteins (MAPs) (Cierpicki et al., 2006;Gleeson et al., 1999;Horesh et al., 1999). Beside these DCX domains, three other conserved domains are found within the DCX family. Firstly, the FLJ46154 gene comprises a ricin-type beta-trefoil lectin (RLD) domain, which may be involved in binding carbohydrates (Liu et al., 2000). Secondly, three complex genes named doublecortin-like kinases (DCLK) comprise a calcium/calmodulin-dependent protein kinase (CaMK) domain and thirdly, two of these DCLK genes encode a serine/proline (SP) rich domain, which they share with DCX (for an overview see (Dijkmans et al., 2010). Spatiotemporal expression of the DCX family shows also similarities between genes and species. Both human and mouse orthologues of RP1, RP1L1, DCDC1, DCX, DCLK1 and DCLK2 are expressed in the eye. Beside the eye, DCX and DCLK family members are also uniquely expressed in the brain (Coquelle et al., 2006;Dijkmans et al., 2010;Reiner et al., 2006). Since our focus is on adult neurogenesis, I will discuss in more details the DCX and DCLK1 genes, which are both involved in neurogenesis and neuronal migration.

4.2 Doublecortin (DCX)

Doublecortin is a relative simple gene without multiple splice variants. It comprises two functional microtubule binding domains and an SP-rich region which can interact with other proteins. Doublecortin was discovered in the late 90's as a X-linked gene and a key component in several connatural brain abnormalities like lissencephaly and subcortical band heterotopia also called smooth brain disease or doublecortex syndrome (des Portes et al., 1998;Gleeson et al., 1998;Sossey-Alaoui et al., 1998). Point mutations in DCX are associated with impaired migration of neuronal progenitor cells leading to aberrant positioning cortical layers resulting in lissencephaly in males and subcortical band heterotopia in females (Bai et al., 2003;Francis et al., 1999;Friocourt et al., 2007;Gleeson et al., 1999). Later studies showed a causal link between the DCX microtubule binding domains and cell motility

caused by microtubule rearrangements (Horesh et al., 1999). DCX seems to function as a anti-catastrophe factor without affecting the microtubule growth rate(Moores et al., 2006). Beside stabilizing the microtubule cytoskeleton, DCX may also function as MAP involved in anterograde transport (Fitzsimons et al., 2008;Reiner et al., 2006). Predictions based on sequence analysis indicate a role for the SP-rich domain in this protein-protein interaction (Dijkmans et al., 2010).

During embryogenesis, DCX controls radial migration of neuroblasts (Francis et al., 1999;Gleeson et al., 1999), (Bai et al., 2003;Friocourt et al., 2007). DCX is also expressed in the adult brain (Geoghegan and Carter, 2008;Liu et al., 2008;Nacher et al., 2001) and is frequently used as adult neurogenesis marker (Brown et al., 2003b;Couillard-Despres et al., 2005;Couillard-Despres et al., 2006;Rao and Shetty, 2004). Although DCX is prominent expressed in neurogenic areas in the adult brain like in the subventricular zone of the dentate gyrus in the hippocampus and in the olfactory bulb, also several other regions with less well-established neurogenesis, show DCX expression. For example, DCX-immunoreactive cells are found in the corpus callosum, in piriform cortex layer III and in striatum. In these brain regions the expression of DCX co-localizes with PSA-NCAM which suggests a role of DCX in axonal outgrowth or synaptogenesis (Nacher et al., 2001).

Despite the prominent role of DCX in embryonic neurogenesis and the severe effects of missense mutations in the DCX gene on human brain development, DCX knockout mice showed normal brain development (Corbo et al., 2002); a finding that suggest the existence of compensation by other, functional-related genes (Deuel et al., 2006;Pramparo et al., 2010;Tanaka et al., 2006;Tuy et al., 2008). In particular, the DCLK1 gene which functions in a partially redundant pathway with DCX (Corbo et al., 2002;Deuel et al., 2006;Koizumi et al., 2006), may compensate the functional loss of DCX.

4.3 Doublecortin-like kinase 1 (DCLK1)

Three members of the DCX gene family encode proteins containing kinase domain with high resemblance to CaMK's. These genes are known as the doublecortin-like kinases (DCLK) and numbered DCLK1 to 3 (Dijkmans et al., 2010;Reiner et al., 2006). Unlike DCX and DCLK2 & 3 (Edelman et al., 2005;Tuy et al., 2008), DCLK1 is a complex gene with multiple splice variants (Burgess et al., 1999;Sossey-Alaoui and Srivastava, 1999;Vreugdenhil et al., 2001). Were the DCX gene encodes a single protein, the DCLK1 gene encodes at least 4 different proteins that are generated by means of alternative splicing. These proteins are called DCLK-long, DCLK-short, doublecortin-like (DCL) and CaMK-related peptide (CARP). All 4 proteins contain a common SP-rich region but are different with respect to the kinase domain and DCX domain; DCLK-long and DCLK-short both contain the CaMK domain, DCLK-long and DCL

both include two DCX domains and finally CARP lacks both CaMK and DCX domains (for an overview see Dijkmans et al., 2010).

Like DCX, DCLK1 is expressed during embryonic neurogenesis and bound to microtubules and growth cones (Burgess et al., 1999;Burgess and Reiner, 2000;Lin et al., 2000;Shu et al., 2006). Furthermore, DCLK1 is involved in mitotic spindle formation in neuroblasts (Lin et al., 2000;Shu et al., 2006;Vreugdenhil et al., 2007), apoptosis (Kruidering et al., 2001;Schenk et al., 2007;Verissimo et al., 2010a), neuronal differentiation (Dijkmans et al., 2008), neuronal migration (Deuel et al., 2006;Koizumi et al., 2006) and retrograde transport of glucocorticoid receptors (GR) (Fitzsimons et al., 2008). More specific, both DCLK-long and DCL proteins are associated with embryonic neurogenesis (Boekhoorn et al., 2008;Vreugdenhil et al., 2007), apoptosis (Kruidering et al., 2001;Schenk et al., 2007;Verissimo et al., 2010a) and neuronal migration (Deuel et al., 2006;Koizumi et al., 2006), which is in line with DCX characteristics. DCLK-short is not expressed during embryogenesis but is postnatally expressed in the adult brain (Burgess and Reiner, 2002;Engels et al., 2004;Vreugdenhil et al., 2001) and might be involved in neuronal differentiation (Dijkmans et al., 2008). The smallest splice variant CARP (Berke et al., 1998;Vreugdenhil et al., 1999) is below detection under normal conditions. However, CARP expression can be induced by kainate-induced seizures (Vreugdenhil et al., 1999), D1-receptor agonists or cocaine (Berke et al., 1998;Glavan et al., 2002) and BDNF-induced long term potentiation (LTP) (Wibrand et al., 2006), which suggests a role for CARP in elevated neuronal activity (Schenk et al., 2007).

One of the splice variants generated by the DCLK gene appears to be a DCX look-a-like. Doublecortin-like (DCL) is of similar length as DCX (around 360 amino acids) and overall, DCX and DCL shares 73% sequence identity which is even higher in both DCX domains and in the SP-rich region (Vreugdenhil et al., 2007). As mentioned before, DCL is highly expressed in the embryonic brain were it is involved in mitotic spindle formation and radial migration. Knockdown of DCL by in utero electroporation of plasmids expressing DCL-targeting shRNAs, resulted in reduced cell proliferation and disrupted radial migration. In vitro, DCL knockdown induces spindle collapse in dividing neuroblastoma cells and DCL over expression induces elongated and asymmetrical mitotic spindles (Vreugdenhil et al., 2007). Eventually, DCL knockdown induces cell apoptosis in these neuroblastoma cells (Verissimo et al., 2010a). Although there is a strong overlap in sequence identity and expression pattern, DCX and DCL are differentially expressed especially in early stages of neocortical development (Boekhoorn et al., 2008). DCL expression appears a few days earlier compared to DCX. Unlike DCX, due to the complexity of DCLK1 splice variants, it is not possible to visualize specifically DCL protein expression in the adult brain (Nacher et al., 2001) as epitopes recognized by available anti-bodies are present in different DCLK1 gene-derived proteins (Kruidering et al., 2001). Therefore, it is unknown if and were DCL is expressed in the adult brain. Since DCX

plays an important role in adult neurogenesis and the homologues DCL plays a similar role in the developing brain, it is relevant to study the involvement of DCL in adult neurogenesis.

5 Thesis outline

Rationale

As mentioned above, in the last decades a huge amount of work was dedicated to describe ongoing neurogenesis in the adult brain. In the meantime theories were developed about the function of neurogenesis including its possible role in human brain disorders like depression. However, for both problems (adult neurogenesis and depression) were no good specific animal models available. Models concerning adult neurogenesis were quite unspecific and the manipulations affected more than adult neurogenesis alone. However, the progress in the field of mouse genetic engineering technology has permitted more specific interventions. For example, with such techniques we can study the role of specific genes or even splice variants in the adult neurogenesis process without affecting developmental neurogenesis. Using such an approach, we can study in one single model both gene and neurogenesis function. For this purpose we focus on DCL because several previous studies suggest that DCL might also play a role in adult neurogenesis (Boekhoorn et al., 2008;Vreugdenhil et al., 2007).

Objective

Our main objective is to study the role of the DCLK1 splice variant DCL in adult hippocampal neurogenesis. We have designed experiments to validate a new mouse model based on doxycycline inducible expression of shRNA targeting specifically DCL, which makes novel studies on both DCL and adult neurogenesis possible.

Hypothesis

We postulate that DCL plays an important role in adult neurogenesis in a way similar to its established function in embryonic neurogenesis. To support this hypothesis with evidence, we focus on the following specific aims.

- To examine DCL expression in neurogenic area's like hippocampus and olfactorius bulb.
- To target specifically DCLK1 splice variant DCL in a conditional siRNA expressing mouse line.
- To test whether DCL knockdown can affect the neurogenesis process in the adult brain.
- To investigate the effect of DCL knockdown on hippocampal dependent memory tasks.

Approach

With a novel antibody we will map the distribution of immunoreactive DCL protein in the adult brain of the male mouse. Since gene knockout can induce compensatory mechanisms (with DCX), we will exploit a novel methodology to knockdown DCL in the adult brain by using an 'on demand' inducible knockdown construct, which doesn't affect embryonic brain development. Since we propose that DCL does play a crucial role in adult neurogenesis, we expect that DCL knockdown will affect adult neurogenesis in the dentate gyrus of the hippocampus. Therefore we also will study behavioural performance of mice with reduced DCL in hippocampus dependent memory tasks.

Chapters

In chapter 2 we will report the regional distribution of DCL expression in the adult brain. Using a DCL antibody we will demonstrate that DCL expression occurs in neurogenic regions like hippocampal dentate gyrus and olfactory bulb. At the same time we will show that DCL is expressed in other brain regions as well bringing up a new question on the function of this newly discovered cellular DCL expression.

In chapter 3 we will describe the validation of a mouse model in which DCL is knocked down on demand using a conditional siRNA transgenic technology. DCL protein expression will be measured and the effect of knockdown will be studied using stereological techniques. The findings will address the crucial question on the role of DCL knockdown in adult neurogenesis and subsequently hippocampus-dependent learning tasks.

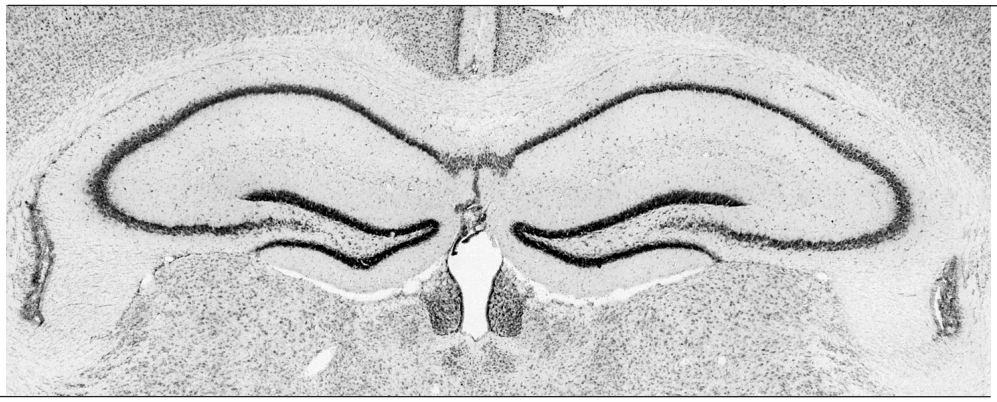
In chapter 4 we describe an additional hippocampus-dependent learning task to seek confirmation in the circular hole board results. By using the contextual fear memory paradigm we can distinguish hippocampus-dependent fear memory formation from amygdala-dependent memory formation.

In chapter 5 we focus on the possible implications of our discovery that DCL is also expressed abundantly in hypothalamic tanycytes and explore the functional consequences of DCL knockdown in this brain area.

Chapter 2

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

implications for neurogenesis.



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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.

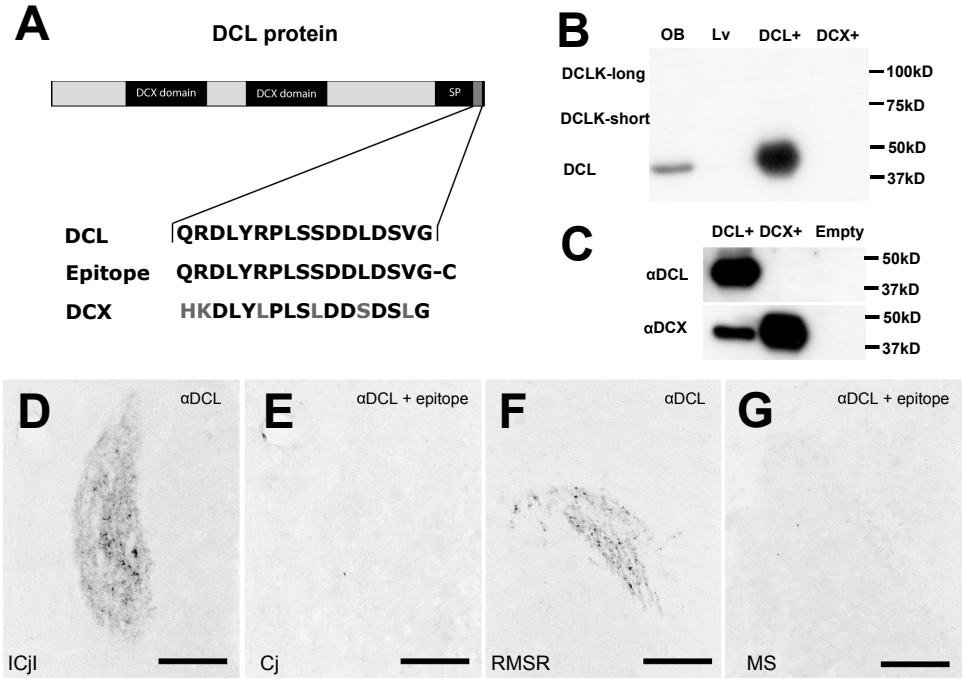

Figure 1: Antibody chatrecteristics of the novel anti-DCL. (A) Location of the antibody target site within the DCL protein and sequence allignment of the epitope, target protein DCL and the nearly homologous DCX. Differences in amino acid sequence between DCL and DCX are indicated in red. (B) Westernblot 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

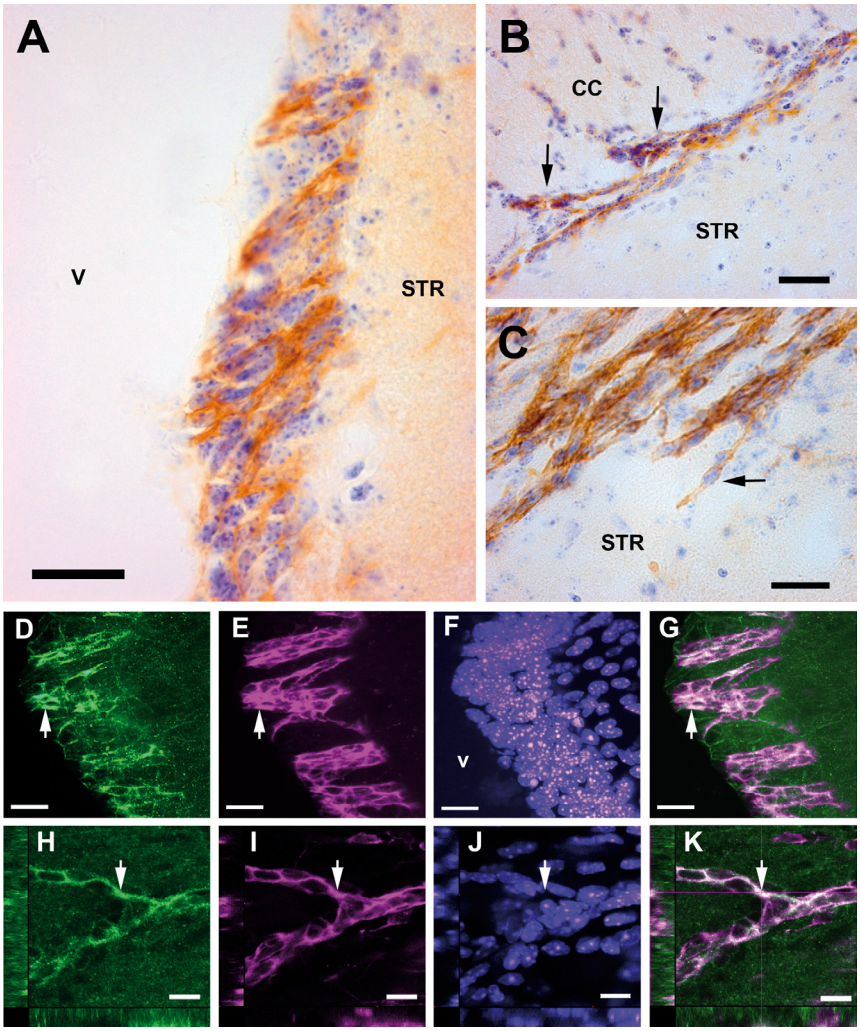

Figure 2: DCL expression in saggital slides showing the (A) the subventricular zone (SVZ) along the lateral ventricles (V) and (B) rostral migratory stream (RMS) between the corpus collosum (CC) and striatum (STR). DCL is expressed in migrating type-A cells which have a typically elongated morphology (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.

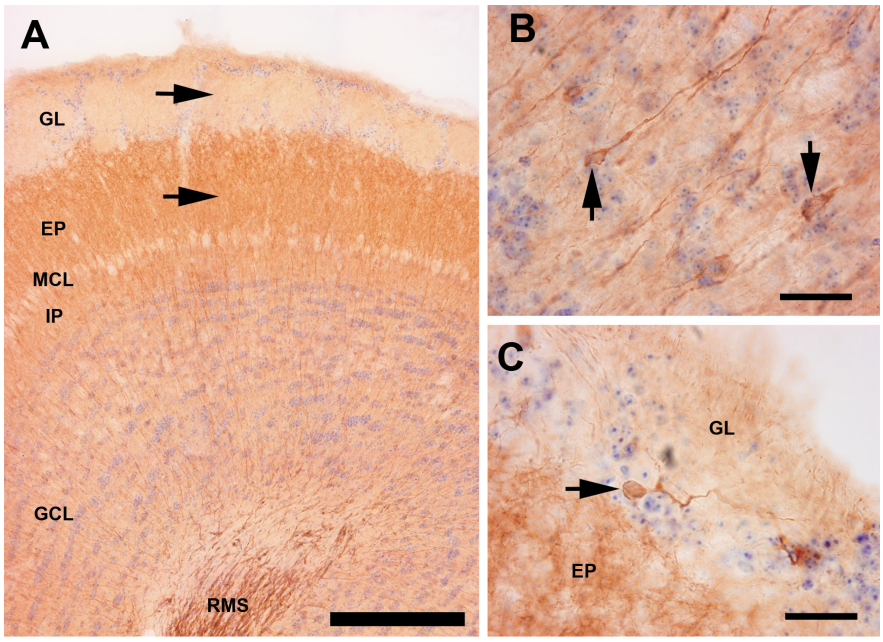

Figure 3

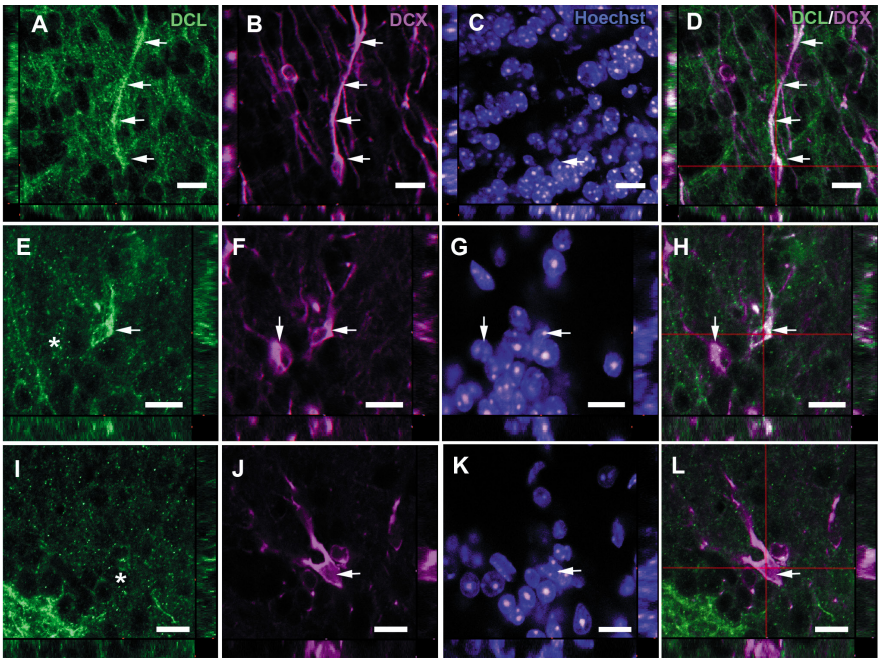

Figure 4

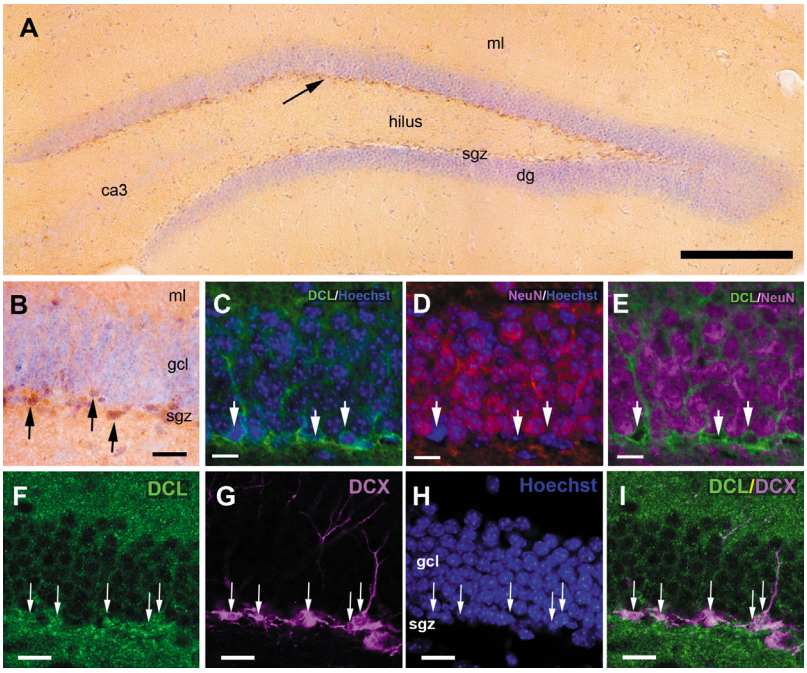
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Fig. 5

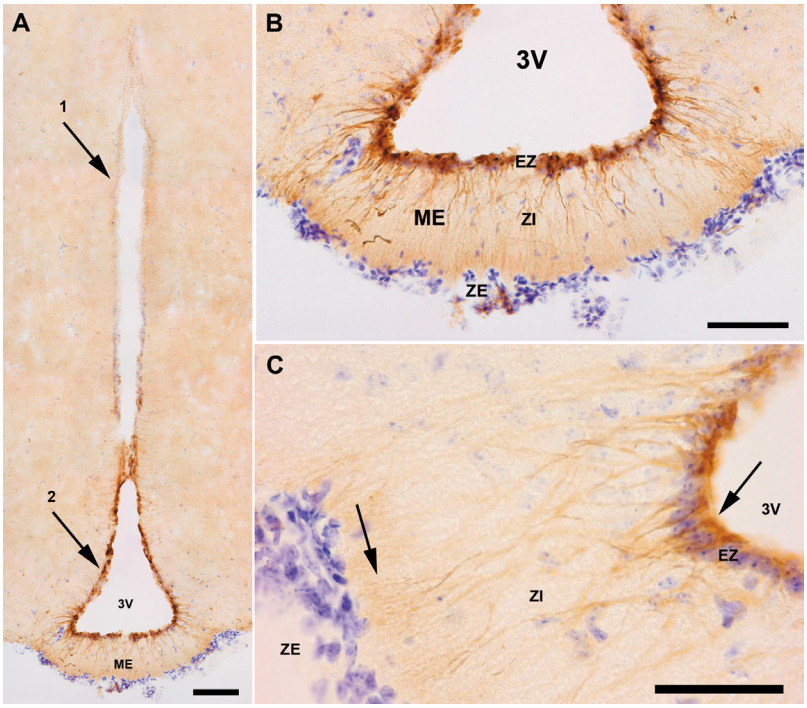
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Fig. 6

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.

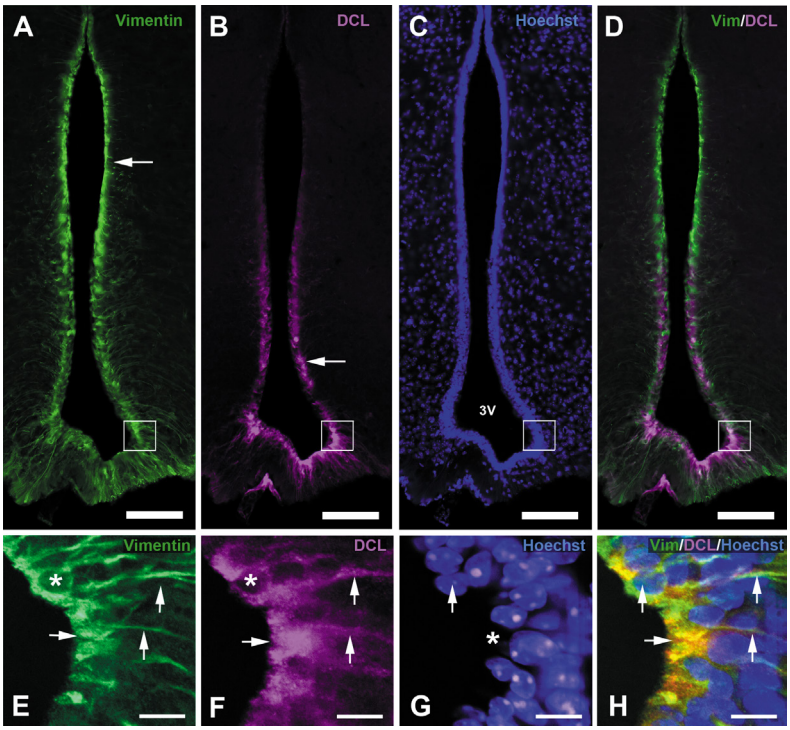

Figure 7: DCL and vimentin expression in the hypothalamus. (A-D) stitched fluorescent overview images of vimentin (A), DCL (B) and Hoechst (C) expression around the third ventricle (3V) in the hypothalamus. Both tanycytes and ependymal cells are vimentin positive (A), DCL is only expressed in the tanycytes (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.

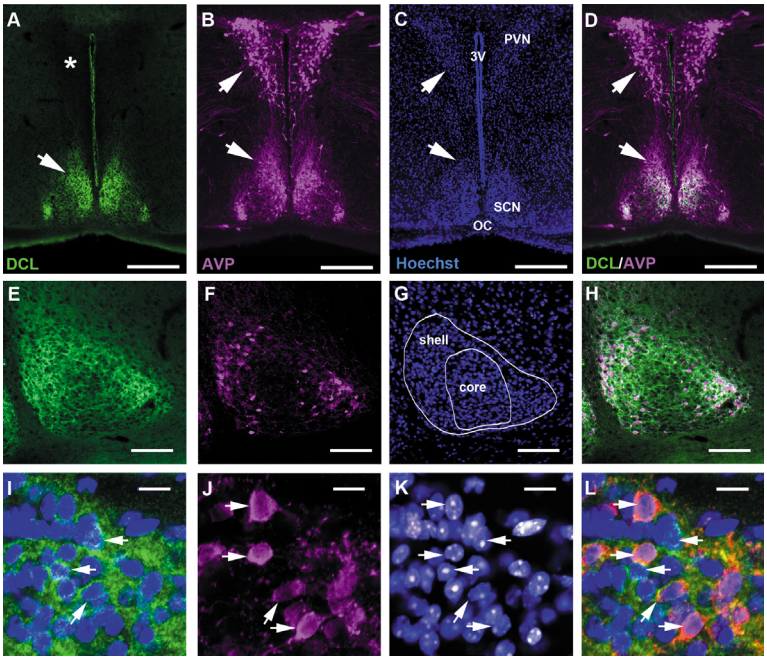

Fig. 8

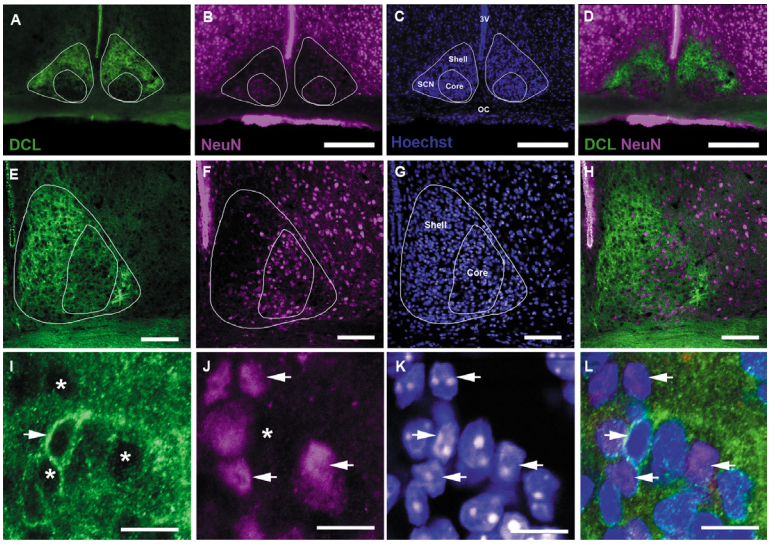

Figure 9: DCL and NeuN expression in the SCN. DCL is not expressed in the NeuN positive (red) subregion of the SCN. DCL is expressed in the shell region of the SCN (A & E) whereas NeuN is expressed in the SCN core region (B & F). Merged fluorescent overview image D and double laser confocal image H do 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.

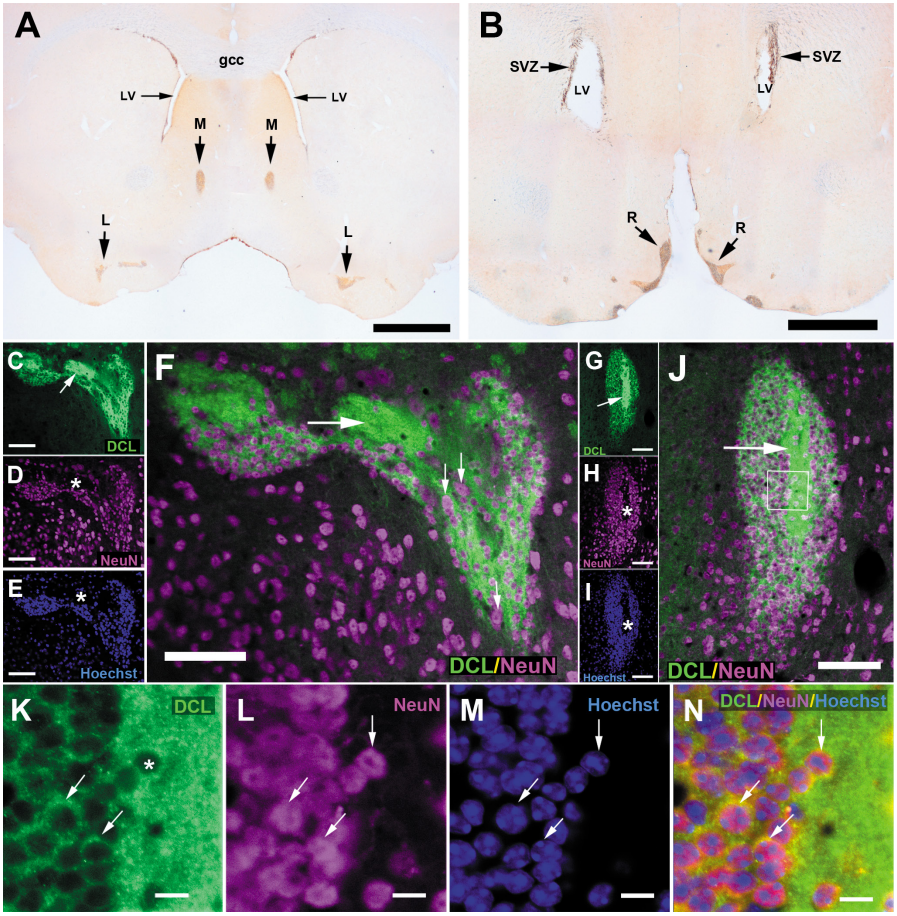

Figure 10: DCL expression in the islands of Calleja (ICj).(A) The major islands (arrows M) are located between the Nucleus accumbens shell and lateral septal nucleus. The lateral islands (arrows L) are located in the ventral pallidum. (B) Also the rostral islands (arrows R) are DCL positive. (C-F) High 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

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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.

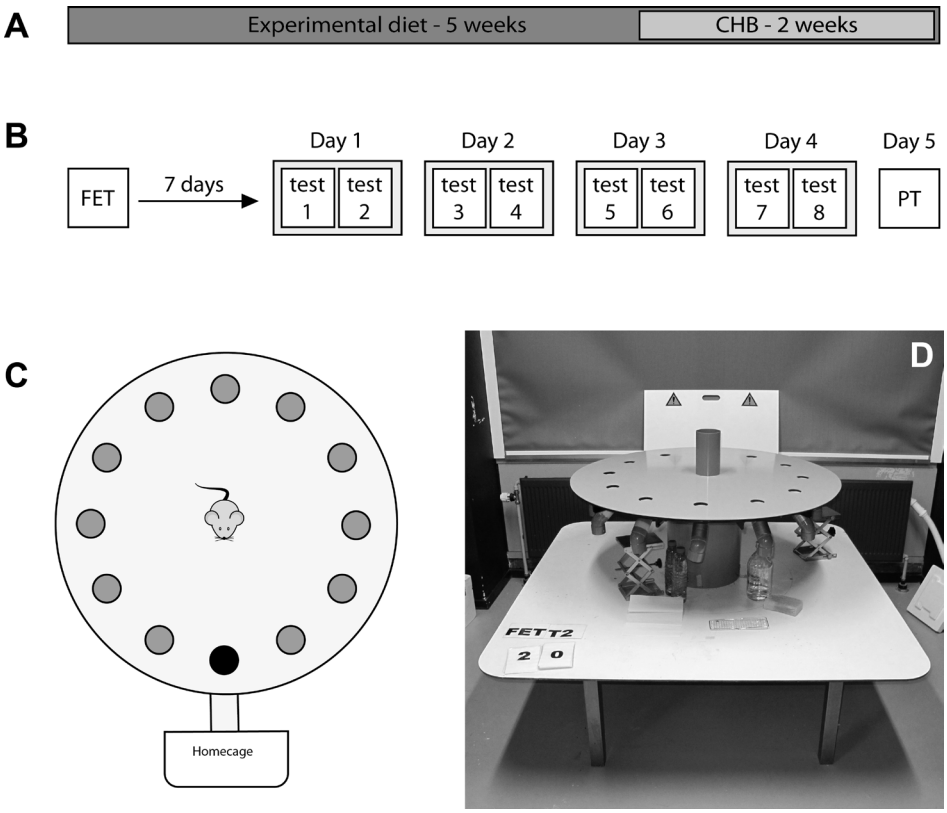

Figure 1: Setup of circular hole board experiment. A: Animals were put on a dox diet for at least 5 weeks before the CHB was started. B: The CHB paradigm started with a free exploration trial (FET). 7 days later the animals followed a training for 4 consecutive days with 2 trials a day. At day 5 the 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

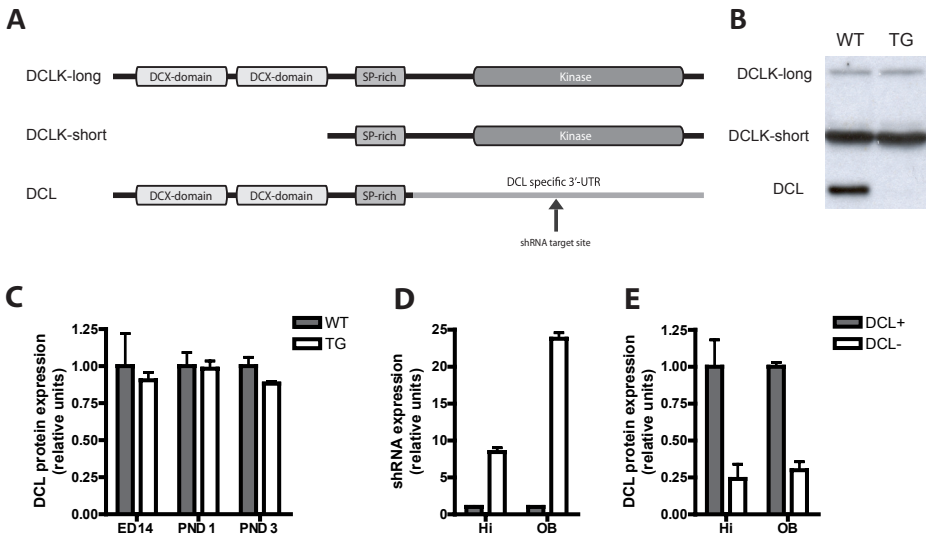

Figure 2: Specific knockdown of DCLK1 splice variant DCL. A) Overview of the three most important DCLK1 splice variants and their functional components. The shRNA target sequence resides in the 3'-UTR of DCL mRNA which is absent in DCLK-long and DCLK-short. B) Western blot analysis reveals splice variant 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.

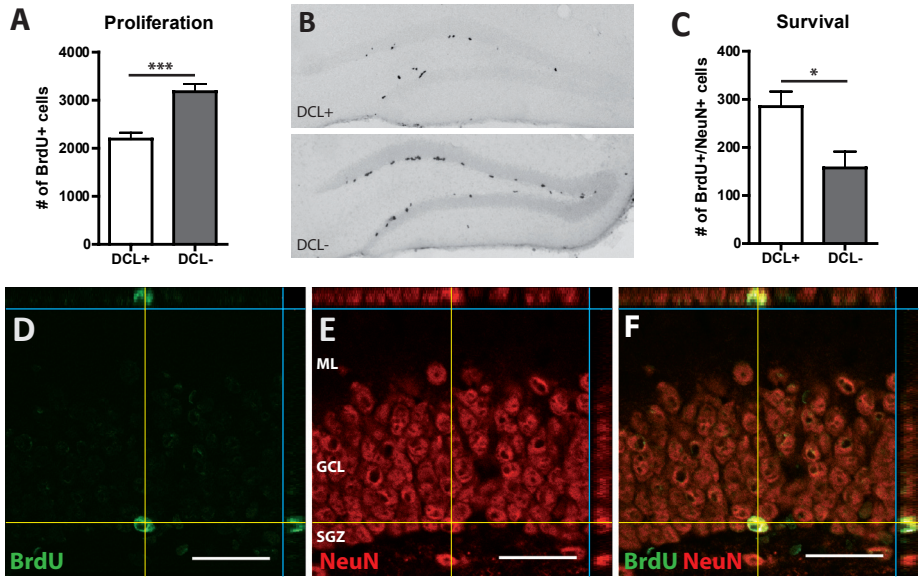

Figure 3: Adult neurogenesis measurement using BrdU labelling. A: 24 hours after a single BrdU injection, a highly significant ($p<0.05$, two-tailed) increase in BrdU positive cells was measured in dox induced transgenic animals (n=6) compared to non-induced transgenic littermates (n=6). B: Examples 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

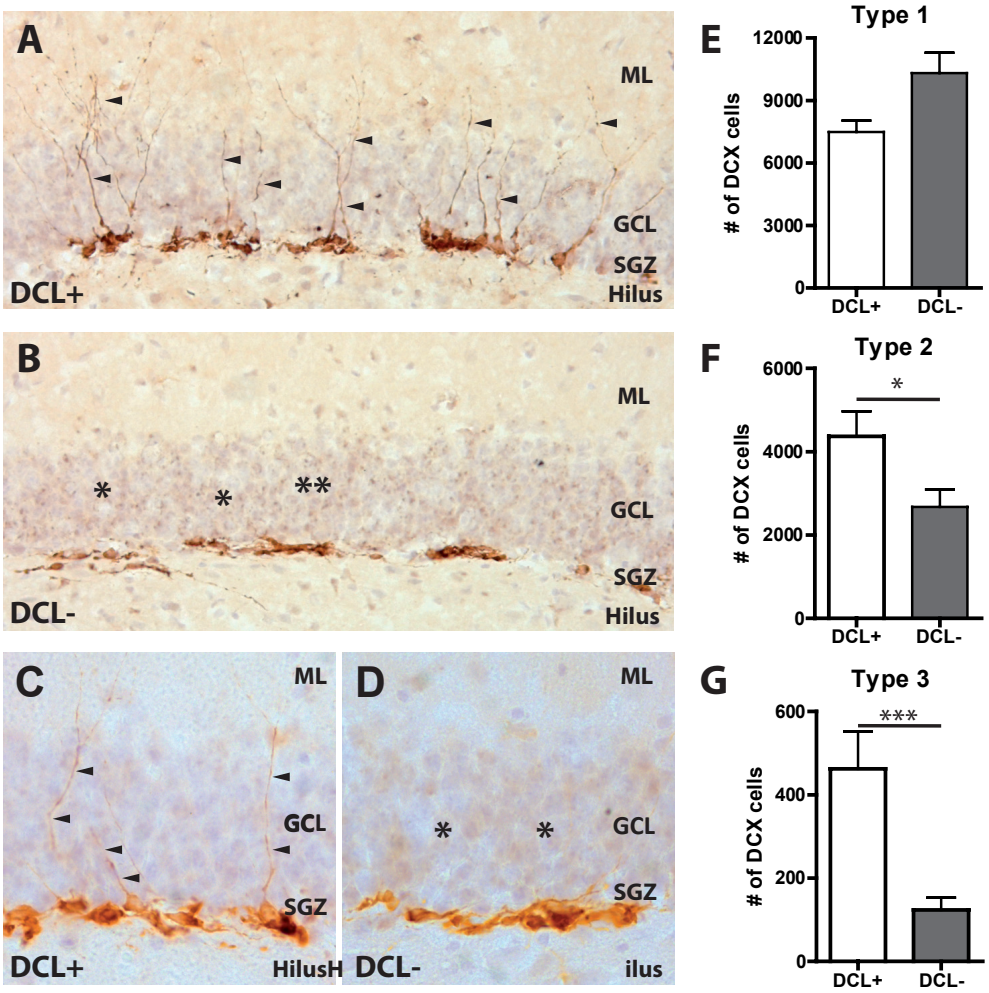

Figure 4: DCX cell morphology. A: DCX expressing cells in the hippocampal dentate gyrus of a transgenic animal on an control diet showing a normal DCX morphology with cell nuclei close to the subgranular zone (SGZ) and dendrites towards the molecular layer (ML). B: Hippocampal dentate gyrus of a dox 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-

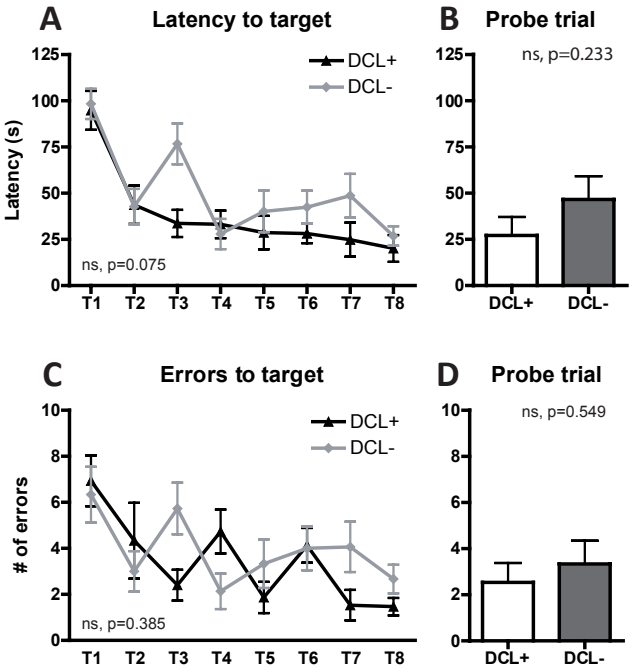

Figure 5: Spatial parameters measured on the circular hole board. DCL knockdown did not affect the latency to target during training sessions (A, GLM, F(1)=3.426, p=0.075) and probe trial (B, t-test, t(28)=1.219, p=0.233). Animals with DCL knockdown did not make more errors before reaching the target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

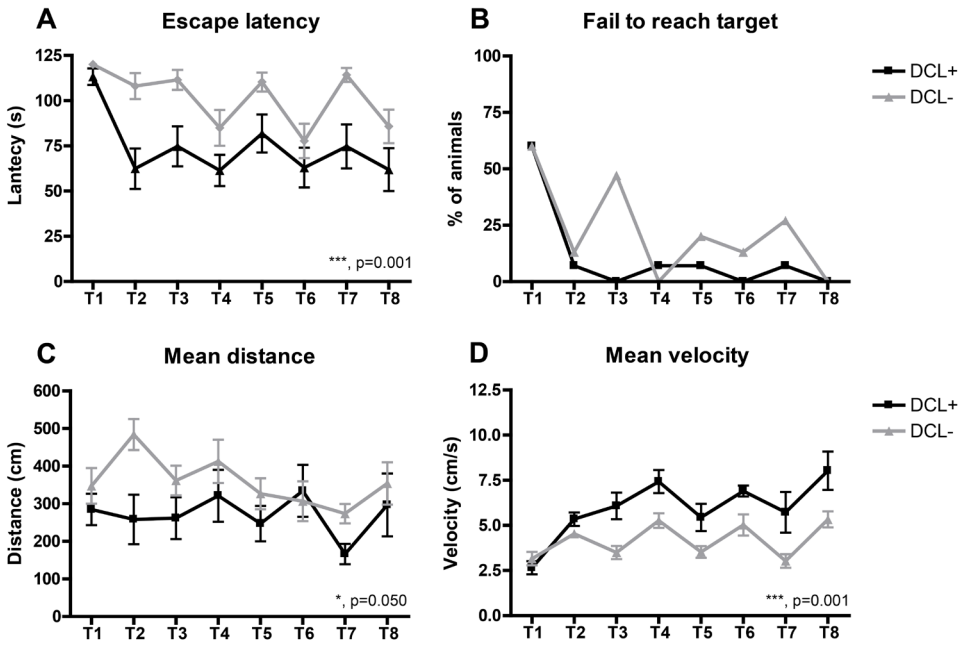

Figure 6: Motivational parameters measured on the circular hole board. A: DCL-KD animals showed a significant longer escape latency compared to DCL+ animals (GLM, F(1)=12.813, p=0.001). B: Percent of animals who did not reach the target within 120 seconds. C: Mean distance moved during each trial. DCL-KD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

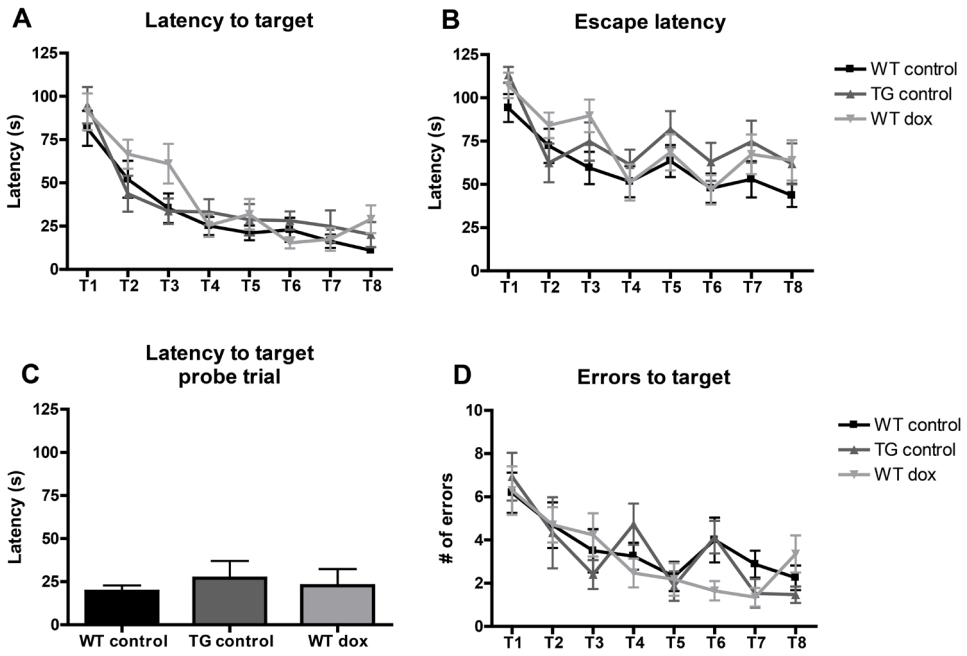

Figure 7: CHB score of all control groups. Wildtype and non-induced transgenic animals did not differ in latency to target (A), escape latency (B) and number of errors to target (D) during the training sessions. There is also no significant difference in latency to target during the probe trial (C).

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.

Acknowledgements

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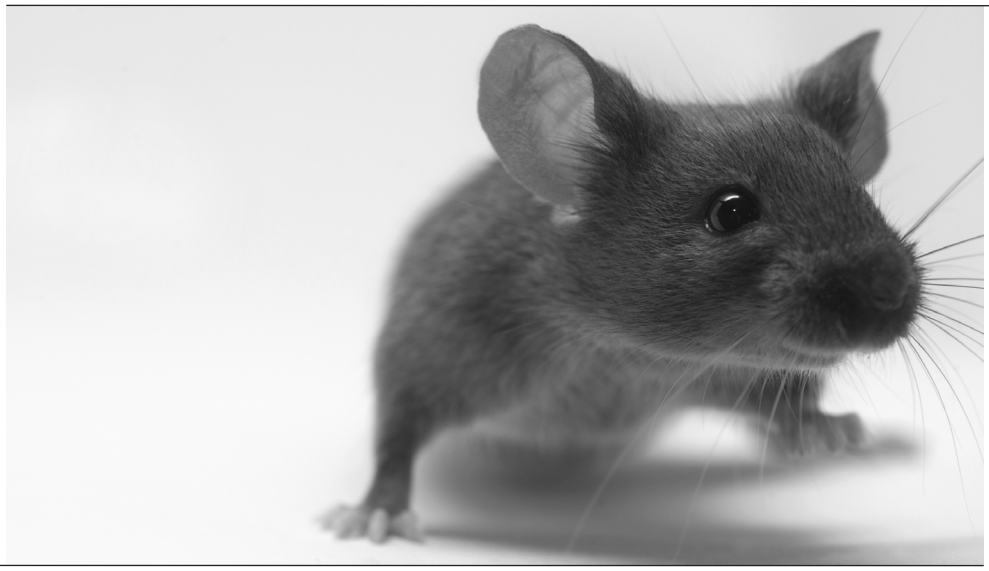
Chapter 4

Blockade of adult neurogenesis by Doublecortin-like knockdown does not affect contextual fear memory formation



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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. Previously, we have shown that DCL plays also an important role in adult neurogenesis. As adult neurogenesis has been implicated in anxiety, we have investigated the role of DCL in contextual fear memory formation using transgenic DCL knockdown (KD) mice producing inducible shRNA molecules that specifically target DCL. DCL-KD mice were tested in a contextual fear conditioning (CFC) paradigm. We found that DCL-KD and associated impaired neurogenesis does not abolish hippocampus-dependent contextual fear memory. However, DCL-KD animals show a significant stronger freezing response to the first cue where after they behave like wildtype littermates. In addition, DCL-KD mice exhibit significant reduced tail rattling behaviour during fear. Therefore, DCL-KD animals may form a valuable model to address the role of neurogenesis in the processing of fearful information by measuring more subtle aspects of context discrimination and pattern separation.

Introduction

Doublecortin-like (DCL), a splice-variant of the doublecortin-like kinase (DCLK) gene, encodes a neurogenesis-related microtubule associated protein (MAP) that shares a high amino acid sequence identity with doublecortin (DCX) over its entire length (Burgess and Reiner, 2002;Sossey-Alaoui and Srivastava, 1999;Vreugdenhil et al., 2001). During embryonic development, DCL is widely expressed in mitotic radial glial cells (RGs) and in radial processes. DCL functions as a microtubule stabilizing protein of mitotic spindles in vitro and in vivo (Boekhoorn et al., 2008;Vreugdenhil et al., 2007). In addition, DCL knockdown by RNA-interference technology leads to significantly reduced cell numbers in the inner proliferative zones and dramatically disrupted mostly radial processes (Vreugdenhil et al., 2007). In the adult brain, DCL is within the DG expressed in the neurogenic niche (Saaltink et al., 2012) and thought to be involved in suppression of spine maturation (Shin et al., 2013). Furthermore, DCL regulates fast retrograde transport of the glucocorticoid receptor (GR), a crucial mediator of the stress response, in neuronal progenitor cells (NPC's) (Fitzsimons et al., 2008). In the adult hippocampus, DCL knockdown resulted in increased proliferation of NPC's but in reduced numbers of post-mitotic NPC's and neuroblasts suggesting a key role for DCL in migration and maturation (chapter 3).

Neurogenesis occurring at adulthood in the subventricular zone (SVZ) of the ventricle walls and the hippocampal dentate gyrus (DG) (Kempermann, 2012;Ming and Song, 2011) is affected by a wide variety of conditions. For instance, neurogenesis is increased by exercise and environmental enrichment (Brown et al., 2003a;Farmer et al., 2004;Kempermann et al., 1997b;Kempermann et al., 1998a;Rhodes et al., 2003;van Praag et al., 1999b;van Praag et al., 1999a) whereas it can be inhibited by severe and chronic stress (Lucassen et al., 2010a;Schoenfeld and Gould, 2012). Since adult neurogenesis takes place in the hippocampus, it is thought that adult neurogenesis function is related to hippocampus dependent memory formation, like spatial and contextual memory. An often-used paradigm to test hippocampal function is contextual fear conditioning (CFC). Fear memory can be acquired during a training program using a cue (light and tone) as conditioned stimulus (CS) and a mild electrical shock as unconditioned stimulus (US). By presenting the CS to animals in the shock compartment (context), fear memory can be tested, which then is subject to extinction several days later. Fear for cue and context is processed in different brain areas. Context-related fear memory depends on the hippocampus and cue-related fear memory is amygdala dependent (Phillips and Ledoux, 1992). CFC appears susceptible to altered neurogenesis (Fitzsimons et al., 2013;Imayoshi et al., 2008;Latchney et al., 2013;Pan et al., 2012;Saxe et al., 2006;Tronel et al., 2012).

In chapter 3 we showed that inhibition of neurogenesis by DCL knockdown did not result in impaired hippocampus dependent spatial memory formation in a circular hole board paradigm. To validate this finding and to distinguish hippocampus dependent context memory formation from amygdala dependent fear memory formation, we tested our DCL-KD animals in the CFC paradigm. Although we observed subtle effects in mice with DCL knockdown-mediated neurogenesis inhibition, contextual fear memory formation is not affected.

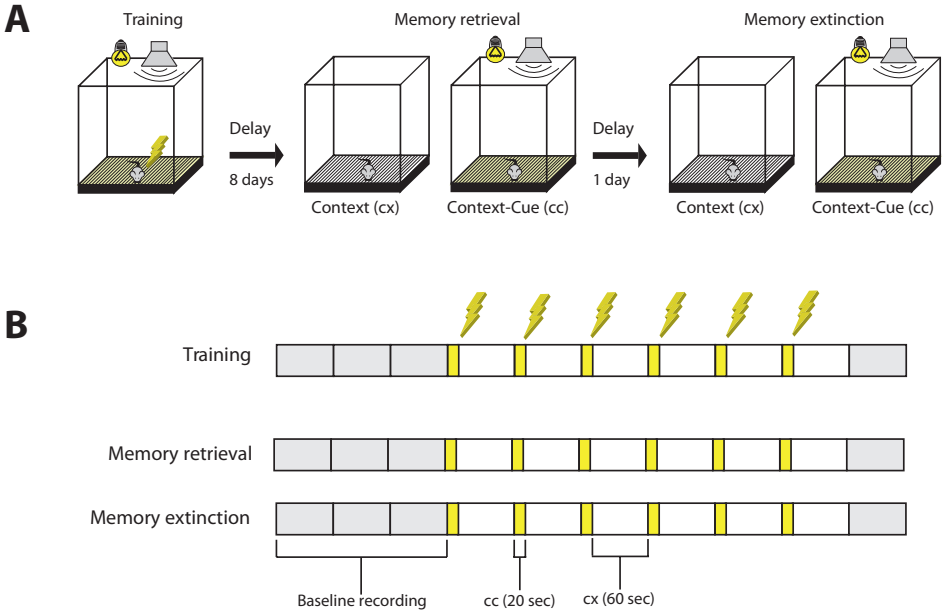

Figure 1: Contextual fear conditioning paradigm. A: Schematic overview of the experiment. During the training, an animal is put in a box with a metal grid. The animal is presented to a cue (tone (70dB) and light (260 lux)) functioning as conditioned stimulus (CS) which is followed by an unconditioned stimulus (US) represented by a shock (0.4 mA) via the grid. After 8 days, the animal is put in the same box to measure memory retrieval. During a context/cue (cc) period only the CS is presented for 20 seconds. 6 of these periods are interspersed with 1 minute of context (cx) without CS. 1 day after memory retrieval, the memory extinction is programmed in the same way. B: Program of the contextual fear conditioning. During the training, 3 minutes of baseline recording (grey) are followed up by a 20 seconds of cc (yellow) with CS together with a mild shock (lightening). The cc is followed by a cx (white) of 1 minute. Such a block is rehearsed 5 times and followed by a single post period of 1 minute (grey). Memory retrieval and extinction are programmed in the same way, however, without an electrical shock (US).

Methods

Animals

Three-month-old DCL KD transgenic (n=15) (Chapter 3) and wildtype (n=17) male mice were obtained from our outbred colony (derived from TaconicArtemis, Cologne, Germany). The animals were kept under a 12:12 light-dark cycle (lights on from 7:00 to 19:00 hours), in a temperature-controlled room (23°C). shRNA targeting DCL, was induced by doxycycline (dox) via dox containing food pellets (Dox Diet Sterile S3888, 200mg/kg, BioServ, New Jersey, USA). Water and food were available ad libitum. After 8 weeks of dox induction the animals were subject to contextual fear conditioning.

Apparatus

The fear conditioning paradigm was performed as described previously (Brinks et al., 2009). In short, a blinded Plexiglas chamber measuring 25x 25x 35 cm high was used as fear conditioning box. The floor consisted of a stainless steel grid connected to a shock generator (0.4 mA). In the wall a speaker was attached at 25 cm height and connected to a tone generator (70dB). A white light source (260 lux) and a camera were placed on top of the chamber (see also Fig. 1).

Procedure

Animals were placed in the chamber for 3 minutes baseline recording followed by 6 light/tone + shock pairings with 60 seconds interval. Light and tone were paired for 20 seconds. An electric shock was applied during the last 2 seconds of the light/tone pairing. 120 seconds after the last shock the animals returned to their home cage. Subsequently, to test memory (day 8) and memory extinction (day 9), this paradigm was repeated without the shock (see also Fig. 1).

Behavioural assessment

Video recorded behaviour was analyzed by an experimenter unaware of the genotype. Freezing behaviour was defined as immobility of the body including the head without any interaction with the environment. Also the number of tail rattles was scored. Behavioural scoring and analysis is performed with ObserverXT version 9.0 (Noldus BV, Wageningen, The Netherlands).

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) with repeated measurements. Tail rattling data is tested with an Univariate Analysis of Variance (UAV). Both tests are performed in SPSS statistical software version 20 (IBM, SPSS Inc. Chicago,IL).

Results

The fear conditioning paradigm consists of three tests. During training, the animals develop a fear for the electrical shock (US), which is associated with the presented cue (CS) and context. Fear memory is measured 8 days later during the memory retrieval test. Due to the lack of the painful US, this fear memory is partly extinguished. Fear memory extinction is measured during the third test at day 9. Analysis of all test series did not reveal significant effects of DCL knockdown on memory formation ($F(1)=0.047$, $p=0.830$), retrieval ($F(1)=0.276$, $p=0.603$) and extinction ($F(1)=1.026$, $p=0.319$). All DCL-KD animals performed similarly as wildtype littermates.

Fear memory training

During the initial training at day 1, the animals developed equal amounts of fear behaviour during both cue and context condition (Fig. 2A). Before each test series baseline behaviour was recorded for 3 minutes. Before training, animals were naïve and showed very low levels of fear behaviour (Fig. 2A). DCL-KD animals were not more or less naïve compared to wildtype littermates ($F(1)=5.770$, $p=0.242$). A GLM for repeated measurements showed a significant increase of freezing behaviour over time during cue and context alternations ($F(3.45)=34.784$, $p<0.001$). DCL knockdown did not affect the learning curve ($F(1)=0.047$, $p=0.830$). However, DCL-KD animals showed a more jagged learning curve with slightly more freezing behaviour in the cue-settings, compared to the subsequent context-setting (see Fig. 2A; ($F(1)=6.221$, $p=0.018$).

Fear memory retrieval

Eight days after training, fear memory was tested during the memory retrieval test (Fig. 2B). Over time animals did not freeze more or less ($F(5)=1.457$, $p=0.208$) but in the cue-setting animals showed more freezing behaviour compared to the context-setting ($F(1)=44.503$, $p<0.001$). DCL knockdown does not affect memory retrieval. However, the first cue elicited a much stronger freezing response in DCL-KD animals compared to wildtype littermates (Fig.

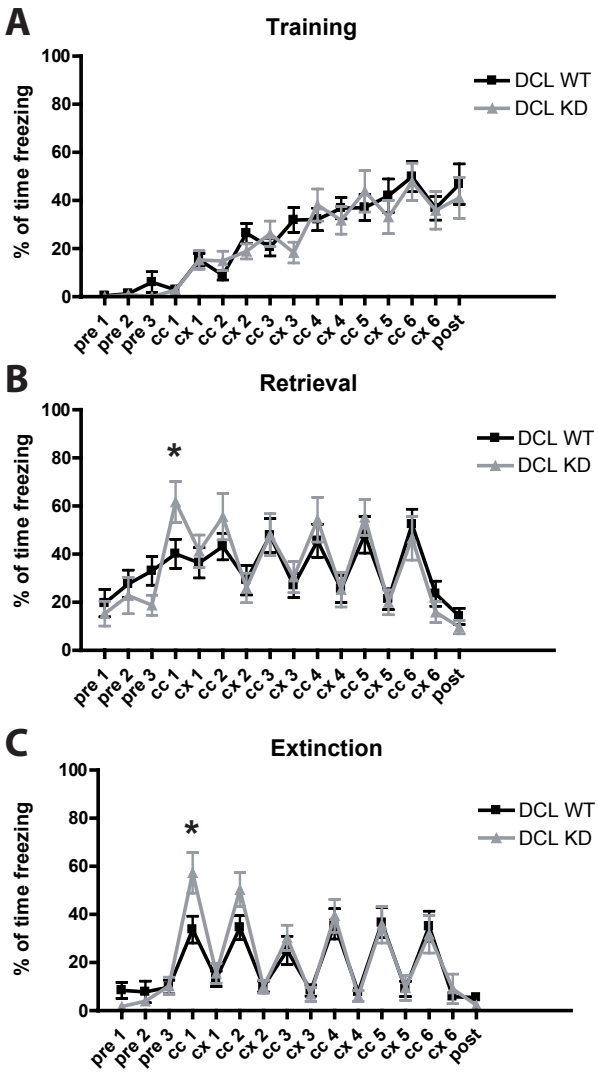

Figure 2: Fear behaviour during contextual fear conditioning. A: Percent of time the animals showed freezing behaviour during the training. DCL knockdown did not affect conditioning to the stimulus (F(1)=0.047, p=0.830). B: Percent of time the animals showed freezing behaviour during memory retrieval. DCL knockdown did not affect fear memory and retrieval (F(1)=0.276, p=0.603) except during the first cue (cc1, marked with an *, t(30)=-2.107, p=0.044). C: Percent of time the animals showed freezing behaviour during memory extinction. DCL knockdown did not affect fear memory extinction (F(1)=1.026, p=0.319) except during the first cue (cc1, marked with an *, t(30)=-2.413, p=0.022). CX: context CC: context-cue.

2B, cc1; t(30)=-2.107, p=0.044). Prior to the first cue, all animals showed an increase in freezing behaviour over the period of 3 minutes (F(2)=5.049, p=0.009), DCL knockdown had no effect on fear behaviour in this period (Fig. 2B; (F(1)=2.413, p=0.132).

Fear memory extinction

At day 9, fear memory was tested again (Fig. 2C). However, due to the previous test without an US, fear memory was slightly extinguished. All animals showed a decrease in freezing

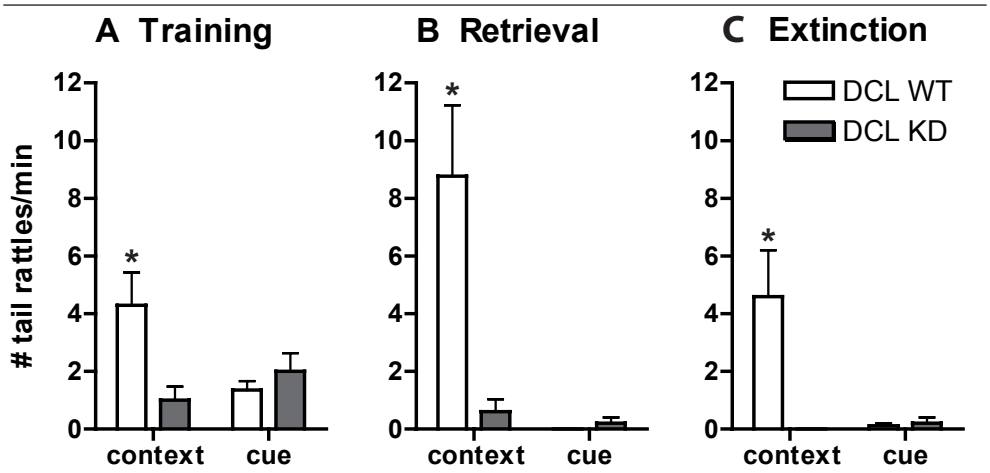

Figure 3: Total number of tail rattles per minute during contextual fear conditioning. In all tests, only in the context situation wildtype animals showed more tail rattling compared to DCL-KD littermates ($p<0.01$). A: During training, an effect for DCL knockdown was found ($F(1)=12.818$, $p=0.001$). B: During memory retrieval, the wildtype animals showed significant more tail rattling during context situation, compared to cue situation ($F(1)=11.024$, $p=0.002$). C: Like memory retrieval, the wildtype animals showed significant more tail rattling during context situation, compared to cue situation in the extinction test ($F(1)=6.439$, $p=0.014$).

behaviour over time in the alternating cue-context settings ($F(2.931)=4.089$, $p=0.010$). Like memory retrieval, animals showed less fear behaviour in the context-setting compared to the cue-setting ($F(1)=106.342$, $p<0.001$). DCL knockdown did not affect memory extinction except for the first cue (cc1). DCL-KD animals showed a much stronger freezing response to the first cue compared to wildtype littermates (fig. 2C, $t(30)=-2.413$, $p=0.022$). In the period prior to the cue, no strong increase of freezing behaviour over time was seen (fig. 2C, $F(1.581)=2.190$, $p=0.133$).

Tail rattling

Tail rattling represents ambivalent behaviour and is seen during territorial fights between male mice. In a fight, mice show this behaviour when they are at a certain distance from the opponent. It likely represents an internal conflict between approaching or avoiding the opponent (Grant E.C. and Mackintosh J.H., 1963;Scott, 1966). During the fear conditioning paradigm, we observed numerous times tail rattling behaviour (Fig. 3), in particular in the training sessions. 76% of the wildtype animals and 24% of the DCL-KD animals showed at least 1 time this behaviour during the context period (Table 1). When the cue with electrical shock was presented 65% (wildtype) and 41% (DCL-KD) of the animals showed tail rattling behaviour. Tail rattling was significantly reduced after DCL knockdown ($F(1)=12.818$, $p=0.001$). DCL-KD animals did not show different amounts of tail rattling behaviour in cue-

or context-setting (F(1)=0.725, p=0.398). In the memory retrieval test, the amount of tail rattling behaviour was reduced in DCL-KD animals (Fig. 3B). Only wildtype animals showed high amounts of tail rattling behaviour (F(1)=10.041, p=0.002) which was only observed in the context-setting (F(1)=11.024, p=0.002). Similar effects were found during the memory extinction test (Fig. 3C). Wildtype animals showed tail rattling behaviour in the context setting, DCL-KD animals did not (F(1)=7.357, p=0.009). Hardly any tail rattling behaviour was recorded during the presence of the cue resulting in a significant difference between context and cue settings (F(1)=5.330, p=0.024).

Table 1: Percent of animals showing tail rattling behaviour.

	Context		**Cue**	
	DCL WT	DCL KD	DCL WT	DCL KD
Training	76%	24%	65%	41%
Memory retrieval	59%	12%	0%	6%
Memory extinction	59%	0%	0%	6%

Discussion

In this study, we investigated the effect of impaired neurogenesis, induced by DCL knockdown, on behavioural aspects after contextual fear conditioning. As was observed previously in the circular hole board (see chapter 3), both wildtype and DCL knockdown mice exhibited similar learning curves in the CFC. Also, all mice are capable of to acquire, retrieve and extinguish fear memory, in a manner that is not affected by DCL knockdown. However, during training, DCL-KD animals showed a stronger freezing response in the cue-setting compared to the context-setting. Wildtype littermates showed a smooth learning curve whereas in the DCL-KD animals learning became manifest in a see-saw pattern. Remarkably, in response to the first cue in both the memory retrieval and extinction phase DCL-KD mice demonstrated a strong freezing response, which was significantly different from that of wildtype mice. Furthermore, in a context setting, DCL-KD animals showed significant less tail rattling behaviour in the memory retrieval and extinction phase as compared to wildtype animals.

Recently, we showed that knockdown of DCL resulted in a strong reduction of newborn neurons, that concerned in particular post-mitotic type 3 DCX positive NPCs (see Chapter 3). Newborn neurons are known to be preferentially activated during learning tasks (Arruda-Carvalho et al., 2011;Gu et al., 2012;Kee et al., 2007;Arruda-Carvalho et al., 2011;Gu et al., 2012). However, we cannot exclude the possibility that not all NPCs are blocked by DCL knockdown and that CFC-induced learning is mediated by newborn type 3 cells that are not affected by DCL knockdown (see chapter 3, Fig. 3G). Also, newborn neurons are not exclusively activated during learning (Kee et al., 2007), which opens up the possibility that the

older and mature granule cells in the dentate gyrus did participate in CFC-induced learning in DCL-KD mice.

Since our results suggest that DCL-positive neurons do not seem to be implicated in processing of contextual fear memory, an alternative explanation might be that our CFC test was inappropriate to elucidate the biological relevance of adult hippocampal neurogenesis. Indeed, the role of adult neurogenesis in contextual fear conditioning is debated as studies so far showed contradicting results. Several studies showed reduced contextual fear memory after impaired or aberrant adult neurogenesis (Fitzsimons et al., 2013;Imayoshi et al., 2008;Latchney et al., 2013;Pan et al., 2012;Saxe et al., 2006) whereas other studies including the current one did not find such an effect (Clark et al., 2008;Drew et al., 2010;Dupret et al., 2008;Jaholkowski et al., 2009;Jedynak et al., 2012;Shors et al., 2002;Tronel et al., 2012;Zhang et al., 2008).

There are, however, small differences in the experimental design of the CFC task in the above mentioned studies that may explain the contradictory findings. While most studies used 3 tone/shock pairings during the training, the context in which the cue was presented during the memory retrieval phases appeared either familiar -as we did- or novel. However, after inspection of the individual studies it appeared that fear memory processes proceeded irrespective the nature of the context.

Another explanation for the lack of effect of DCL-induced impaired neurogenesis on CFC-induced learning might be the number cue-context pairings in the CFC paradigm used in our study. Recently, Drew and colleagues compared several fear conditioning paradigms in combination with UV-irradiation decreased adult neurogenesis (Drew et al., 2010). Like DCL knockdown, irradiation did not affect freezing behaviour after a series of tone-shock pairings. However, they found an effect when they delivered a single shock without an additional cue. In this experiment the animals received a shock after 3 minutes in the conditioning chamber. One day later, the animals were exposed again to the same context. Under these specific conditions the animals with reduced neurogenesis showed less freezing behaviour suggesting subtle involvement of newborn neurons in context recognition. Also, Tronel and colleagues (Tronel et al., 2012) concluded that adult born neurons are not required for acquisition of contextual fear memory. Instead, they found impaired adult neurogenesis to be deleterious for the ability to discriminate between changes introduced into context during extensive training. Therefore, further testing DCL-KD mice in behavioural paradigms addressing contextual discrimination, may shed light on the role of DCL in context recognition.

Next to changes in the test paradigms, also the experimental strategies to block adult neurogenesis differ in the studies mentioned above. These strategies range from destruction

of the dentate gyrus by UV radiation (Clark et al., 2008;Drew et al., 2010;Saxe et al., 2006) to the knockout of specific cell proliferation-related genes (Dupret et al., 2008;Imayoshi et al., 2008;Jaholkowski et al., 2009;Jedynak et al., 2012;Latchney et al., 2013;Tronel et al., 2012;Zhang et al., 2008). In our study, we used inducible RNA-interference technology to specifically knockdown DCL which is an approach that may be comparable with TLX (Zhang et al, 2008), BAX (Dupret et al, 2008) and D2 knockout mice (Jaholkowski et al., 2009;Jedynak et al., 2012). Similar as observed in our study, CFC-induced learning is not changed in these knockout mice with compromised adult neurogenesis.

We found a significant increased in freezing behaviour in the first cue and a striking and significant decrease in tail rattling after DCL knockdown in the retrieval and extinction phase. As these type of behaviours are anxiety-related, impaired maturation of type-3 progenitor cells induced by DCL knockdown might be involved in the neuronal networks regulating fear-related processes. Interestingly, adult hippocampal neurogenesis has been implicated in subtle context discrimination or pattern separation (Sahay et al, 2011; Clelland et al, 2009), a process, which is believed to underlie anxiety disorders such as panic disorders and the post-traumatic stress disorders (Kheirbek et al., 2013).

In conclusion

DCL-induced impaired neurogenesis does not abolish hippocampus-dependent contextual fear memory. DCL-KD animals show a stronger response to the first cue where after they behave like wildtype littermates except for tail rattling behaviour during fear. These results are highly significant. Therefore, DCL-KD animals seem a valuable model for further research aimed to address the role of neurogenesis in the processing of fearful information by measuring more subtle aspects of context discrimination and pattern separation.

Acknowledgements

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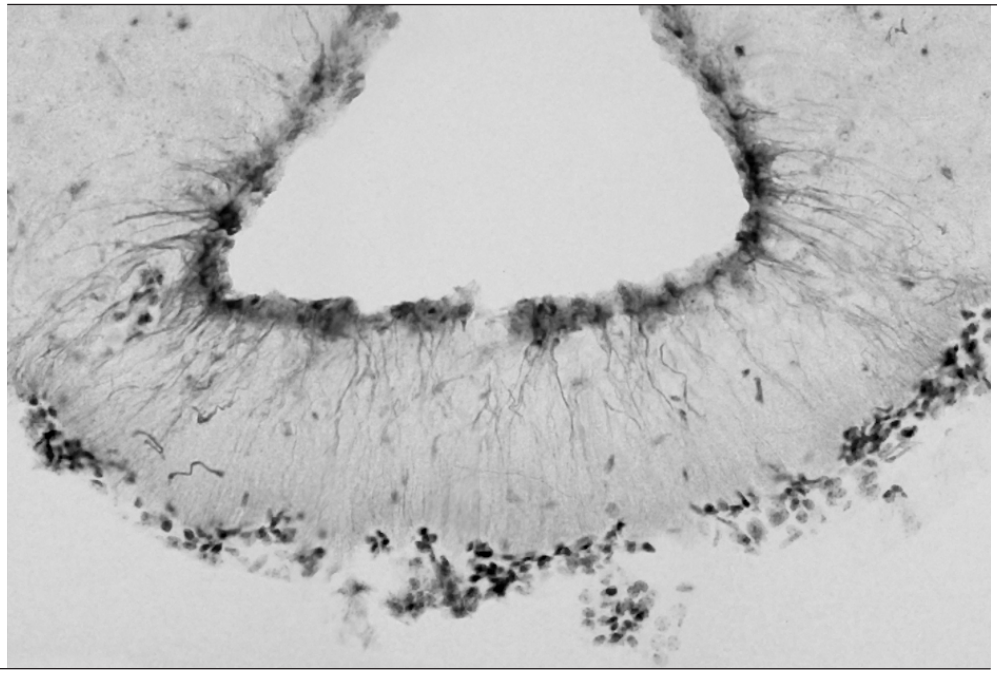
Chapter 5

Doublecortin-like knockdown in hypothalamic tanycytes induce subtle effects on bodyweight and Deiodinase 2 activity.



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Abstract

Doublecortin-like (DCL) is a microtubule-associated protein that is highly homologous to doublecortin and is crucially involved in adult neurogenesis in the hippocampus and forebrain. Previously, we showed high DCL expression in the hypothalamic tanycytes, a cell population involved in the production of thyroid hormones. Therefore, we address the question whether or not DCL is involved in thyroid hormone signalling. To this end, we measured bodyweight, serum T3 and T4 concentrations and D2 activity in hypothalamic tissue of DCL-knockdown (KD) mice and their littermate controls. Furthermore, we measured mRNA expression of TRH, NPY, D2 and D3 in hypothalamic punches containing the ARC-ME or the PVN. We observed a strong reduction in DCL expression in hypothalamic tanycytes, which was associated with reduced body weight growth and a significant increase in D2 activity, the enzyme metabolizing inactive T4 into active T3. However, serum levels of T4 and T3 did not differ between wildtype and DCL-KD animals and also the expression of TRH, NPY, D2 and D3 mRNA in the hypothalamus was not affected by DCL knockdown. Together, our data indicate a role for DCL in the regulation of D2 activity in hypothalamic tanycytes and a possible subtle role in thyroid signalling.

Introduction

The doublecortin-like kinase (DCLK) gene encodes multiple splice variants. One of these is Doublecortin-like (DCL), a microtubule associated protein (MAP) that shares a high amino acid sequence identity with doublecortin over its entire length (Burgess and Reiner, 2002;Sossey-Alaoui and Srivastava, 1999;Vreugdenhil et al., 2001). During embryonic development, DCL is widely expressed in mitotic cells, radial glial cells (RGs) and radial processes. DCL functions as a microtubule stabilizing protein of mitotic spindles in vitro and in vivo (Boekhoorn et al., 2008;Vreugdenhil et al., 2007). In addition, DCL knockdown by RNA-interference technology induces spindle collapse in vitro. DCL knockdown in vivo, by in utero electroporation, leads to a significantly reduced cell number in the inner proliferative zones and a dramatic disruption of most radial processes (Vreugdenhil et al., 2007). Furthermore, DCL is involved in suppression of spine maturation (Shin et al., 2013) and, in the postnatal brain, DCL regulates the transport of the glucocorticoid receptor (GR) in neuronal progenitor cells (NPC's) (Fitzsimons et al., 2008).

Previously we described DCL expression in the adult mouse brain (Saaltink et al., 2012). In the hippocampus and subventricular zone (SVZ) of the adult brain, DCL is expressed in a cell population which is also positive for the neurogenesis marker doublecortin (DCX; Saaltink et al., 2012) suggesting a role for DCL in neuronal progenitor cells (NPCs) (Couillard-Despres et al., 2005;Plumpe et al., 2006;Rao and Shetty, 2004). Indeed, conditional knockdown of DCL in adult mice inhibits newborn neurons in the hippocampal dentate gyrus (DG) to mature (chapter 3). DCL is also expressed in other brain areas like suprachiasmatic nucleus (SCN), islands of Calleja (ICj) and hypothalamic tanycytes, but these areas traditionally do not show adult neurogenesis. Since most DCL-related studies showed involvement in neurogenesis, the question comes up what the function of DCL is in these apparent non-neurogenic brain areas.

Although there are several studies suggesting neurogenesis within the hypothalamus (Haan et al., 2013;Lee et al., 2012;Xu et al., 2005), DCL-expressing hypothalamic tanycytes do probably not reflect a population of newborn neurons since DCL is in particular expressed in nearly all β-tanycytes (Saaltink et al., 2012). These β-tanycytes reside in the ventricle wall along the median eminence, which may be considered as a peri- or circumventricular organ (CVO). Tanycytes have a radial glia-like phenotype and express beside DCL, several markers of neural stem cells like nestin (Baroncini et al., 2007;Barrett et al., 2006;Chouaf-Lakhdar et al., 2003;Xu et al., 2005), vimentin (Baroncini et al., 2007;Bolborea et al., 2011;Chauvet et al., 1998;Kameda et al., 2003;Sidibe et al., 2010;Xu et al., 2005) and sox2 (Lee et al., 2012;Li et al., 2012). Beside the blood-brain barrier (BBB), CVO's form an alternative but semi-permeable barrier between the peripheral blood stream and the cerebrospinal fluid

(CSF) (Rodriguez et al., 2005;Rodriguez et al., 2010). Tanycytes play an important role as gatekeeper (Rodriguez et al., 2005) and are equipped with transport machinery to transfer substances from blood to CSF and vice versa (Rodriguez et al., 2010). Hypothalamic tanycytes are also thought to be part of the feedback mechanism that controls the set-point of the hypothalamic-pituitary-thyroid axis (HPT-axis; Coppola et al., 2007). Under influence of thyrotropin-releasing-hormone (TRH) produced in the neurons of the paraventricular nucleus of the hypothalamus (PVN) and released in the median eminence (ME) the thyrotrophes in the pituitary release thyroid-stimulating-hormone (TSH) into the systemic circulation. Circulating TSH stimulates the thyroid gland to produce the thyroid hormones (TH) thyroxine (T4) and tri-iodothyronine (T3). THs induces genomic and non-genomic effects in many tissues in the body (Bassett et al., 2003). T4 is the inactive hormone while T3 is the active form. T4 is converted locally into T3 by deiodinase enzymes. There are several types of deiodinase enzyme of which type 1 and 2 (D1 & D2) convert T4 into T3. D1 is mainly active in the liver and kidney whereas D2 is expressed in the central nervous system, the anterior pituitary, brown adipose tissue and to a lesser extent in skeletal muscle. Within the hypothalamus, D2 is mainly expressed in the arcuate nucleus (ARC) and hypothalamic tanycytes (Guadano-Ferraz et al., 1997;Kalsbeek et al., 2005;Tu et al., 1997). (Crantz et al., 1982;Fekete and Lechan, 2007). Since DCL is highly expressed in tanycytes and has transporting capacities (Fitzsimons et al., 2008), it's tempting to speculate about a role for DCL in tanycyte functioning within the HPT-axis.

In this study we address the question whether conditional DCL knockdown in mice affects tanycytes functioning in thyroid hormone signalling. To this end, we measured bodyweight, serum T3 and T4 concentrations and D2 activity in hypothalamic tissue of both mice with normal DCL expression and in mice with DCL knockdown. Furthermore we measured mRNA expression of TRH, NPY, D2 and D3 in hypothalamic punches containing the ARC-ME or the PVN.

Methods

Animals

Three-month-old DCL KD transgenic (n=12) (chapter 3) and wildtype (n=12) male mice were obtained from our outbred colony (derived from TaconicArtemis, Cologne, Germany). The animals were kept under a 12:12 light-dark cycle (lights on from 7:00 to 19:00 hours), in a temperature-controlled room (23°C). The shRNA system was induced by doxycycline (dox) via dox containing food pellets (Dox Diet Sterile S3888, 200mg/kg, BioServ, New Jersey, USA). Water and food were available ad libitum. After 5 weeks of dox induction the animals were decapitated and blood was collected. Bodyweight was measured regularly. Serum was

stored at -20°C until it was analyzed. The liver, pituitary and hypothalamus (defined rostrally by the optic chiasm, caudally by the mamillary bodies, laterally by the optic tract, and dorsally by the apex of the third ventricle) were isolated and stored immediately in liquid nitrogen. The tissue block containing the hypothalamus (n=6) was used for dissection of the periventricular area (PE) and the arcuate nucleus / median eminence region (ARC-ME). The PE consists of both paraventricular nuclei and the upper part of the ependymal lining of the third ventricle. This area was obtained by punching the hypothalamus with a hollow needle (diameter 1100µm) based on anatomical landmarks (the apex of the third ventricle Franklin K.B.J., 1997). The PE samples may include (part of) the dorsomedial nucleus (DMN) which –like the PVN- contains TRH neurons. The same instrument was used to obtain the ARC-ME samples (see Fig. 1). This experiment was approved by the Local Animal Welfare Committee of the University of Leiden, The Netherlands.

Immunohistochemistry

Wildtype and transgenic animals of 6 weeks old were put on a dox diet (n=6 per group). After 5 weeks the animals were killed and prepared for immunohistochemistry as described previously (Saaltink et al., 2012). In short, 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% para-formaldehyde 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.

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 antibody targeting DCL (Saaltink et al., 2012) was 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 (Alexa Fluor® 488 donkey anti-rabbit IgG) 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.)

RNA isolation and RT-PCR

Punches derived from the PE and ARC-ME were analysed as described earlier (Boelen et al., 2004). mRNA was isolated from hypothalamic brain tissue using the Magna Pure apparatus and the Magna Pure LC mRNA isolation kit II (tissue) (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. cDNA synthesis was performed with the 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche Molecular Biochemicals). Real Time PCR was performed using the Lightcycler480 and Lightcycler480SybrGreen I Master mix (Roche Molecular Biochemicals, Mannheim, Germany). Primer pairs for mouse hypoxanthine phosphoribosyl transferase (Hprt), D2 (Dio2), D3 (Dio3) and TRH have been previously described (Boelen et al., 2004). We designed primer pairs for mouse NPY (forward primer 5' -GGGCTGTGTGGACTGACCC-3', reverse primer 5'GGTACCCCTCAGCAGAATG-3'). Annealing temperature in the PCR reaction was 60ºC. Quantification was performed using the LinReg software (Ruijter et al., 2009). The mean of the efficiency was calculated for each assay, samples that had a greater difference than 0.05 of the efficiency mean value, were not taken into account (0-5%). mRNA levels were corrected for housekeeping gene (HPRT) expression.

Deiodinase type 2 activity

The hypothalamic block was homogenized on ice in 10 volumes of PED50 (0.1M sodium phosphate, 2 mM EDTA and 50 mM DTT pH 7.2) using a Polytron (Kinematica, Luzern, Switzerland). Homogenates were immediately processed for D2 measurement as previously described (Kwakkel et al., 2009). Protein concentration was measured with the Bio-Rad protein assay using bovine serum albumin (BSA) as the standard following the manufacturer's instructions (Bio-Rad Laboratories, Veenendaal, The Netherlands). D2 activity was measured in duplicate using 75 µl of homogenate. Samples were incubated for 4 hours, at 37 °C, in a final volume of 0,15 ml with 1 or 500 nM T4 (blank as high concentration of substrate (T4) saturates D2), 500 nM PTU (to block D1) and 2×105 cpm [3'5'- (125)I]T4. The reaction was stopped by cooling the samples on ice and adding 0.15 ml of ice-cold ethanol. After centrifugation, 0.125 ml of the supernatant was added to 0.125 ml 0.02 M ammonium acetate (pH 4), and 0.1 ml of the mixture was applied to 4.6 x 250 mm Symmetry C18 column connected to a Waters HPLC system (Model 600E pump, Model 717 WISP autosampler, Waters, Etten-Leur, The Netherlands).

Mobile phase A: 0.02 M ammonium acetate (pH 4.0), mobile phase B: acetonitril. The column was eluted with a linear gradient (28–42% B in 15 min) at a flow of 1.2 ml/min. The activity of T4, and T3 in the eluate was measured online using a FSA flow detector (150TR) van Perkin Elmer (Perkin Elmer, Groningen, The Netherlands). D2 activity was expressed

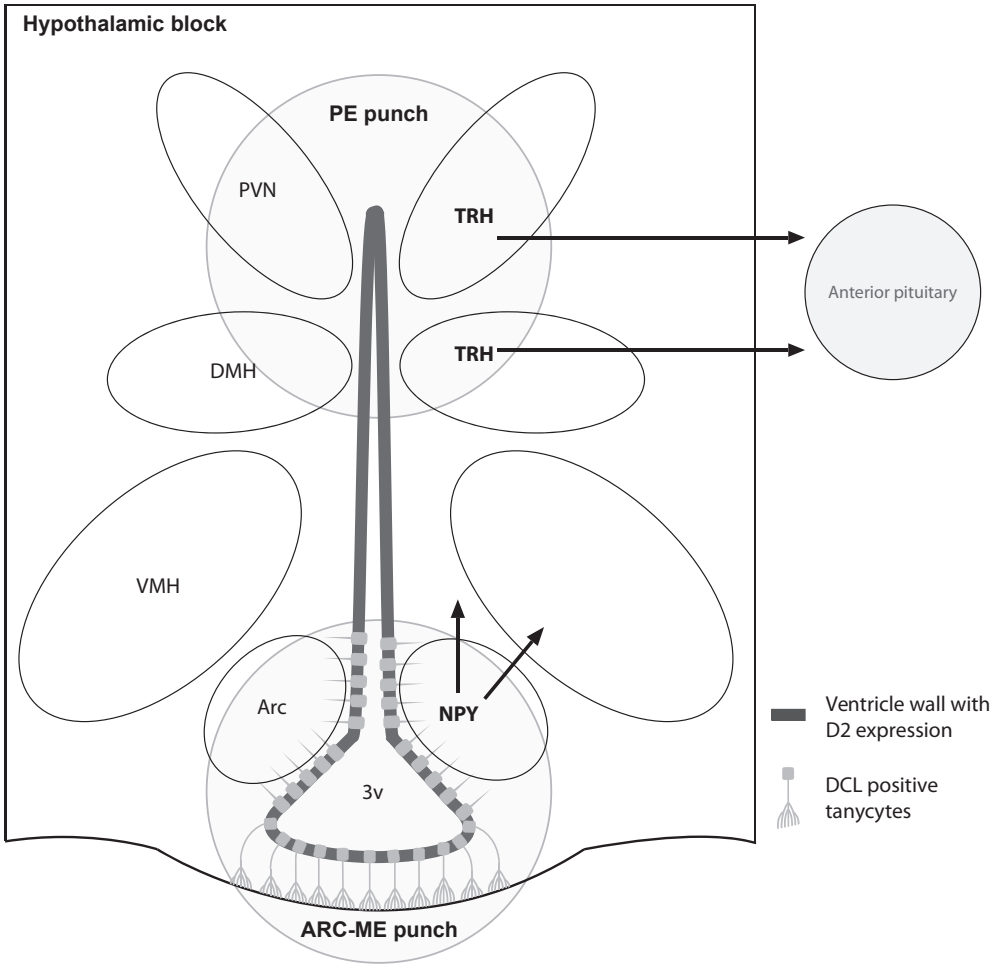

Figure 1: Schematic overview of the hypothalamic block from which the punches are derived. The periventricular area (PE) punch contains nuclei around the upper part of the wall of the third ventricle (3v) including the paraventricular nucleus (PVN) and parts of the dorsomedial hypothalamus (DMH). Both nuclei contain TRH producing neurons which stimulate the anterior pituitary to release TSH. The ARC-ME punch contains tissue from the arcuate nucleus (ARC) and the median eminence (ME) including the lower part of the ventricle wall. This part of the ventricle wall also contains DCL positive tanycytes. The ARC is characterized by the expression of NPY. The ventral medial hypothalamus (VMH) is not punched. D2 is expressed along the whole ventricle wall (red).

as fmol of generated T3 per minute per mg of tissue. The amount of generated T3 was calculated using the values of the 1nM T4 incubations minus the mean of the 500 nM T4 incubations.

Thyroid hormone levels

Serum T3 and T4 were measured with in-house radio immunoassays (RIAs) (Wiersinga and Chopra, 1982) as described before (Boelen et al., 2004). All samples of one experiment were measured within the same run (intra-assay variability T3: 3.6% and T4: 6.6%).

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). qPCR data is tested with a General Linear Model (GLM) for univariate analysis of variance in SPSS statistical software version 20 (IBM, SPSS Inc. Chicago,IL). P values less than 0.05 were considered as statistically significant.

Results

To knockdown DCL protein expression in hypothalamic tanycytes we put wildtype and DCL-KD animals on a dox diet. Five weeks later, mice were sacrificed and prepared for immunohistochemical analysis. DCL protein expression was mainly found in the lower part of the third ventricle wall close to the median eminence. In line with our previous findings (Saaltink et al, 2012) wildtype animals showed a clear DCL signal in both dendrites and cell bodies of hypothalamic tanycytes (Fig. 2 A&C). In contrast, DCL expression was strongly reduced in tanycyte dendrites and only a weak signal was left in the ventricle wall in DCL-KD littermates (Fig. 2 B&D).

Thyroid hormone signalling also affects energy balance and basal energy metabolism and therefore might affect bodyweight. We measured the bodyweight of the experimental animals before and after dox induction. At the start of the experiment there was a small, but significant, difference (1.23 ± 0.49 g, p=0.016) in bodyweight between wildtype (22.29 ± 0.29 g, N=24) and DCL-KD (21.05 ± 0.40 g, N=24) animals (Fig. 3A). This difference was increased after 5 weeks of dox induction (Fig. 3B). Wildtype animals (25.36 ± 0.44 g, N=24) were still significantly heavier than DCL-KD animals (23.04 ± 0.48g , N=24), but the difference was more pronounced (2.32 ± 0.65 g, p<0.001). Wildtype animals gained relative more bodyweight (13.8% ± 1.4, N=24) than their DCL-KD littermates (9.4% ± 0.9, N=24) (Fig.3C).

As tanycytes are involved in thyroid hormone signalling and express D2, we measured the activity of this enzyme. We found a marked increase in hypothalamic D2 activity in the hypothalamic block (Fig. 4C) of DCL-KD mice (0.074 fmol/mg/min ± 0.005, N=5) compared to wildtype littermates (0.030 fmol/mg/min ± 0.003, N=6). Despite the significant increase

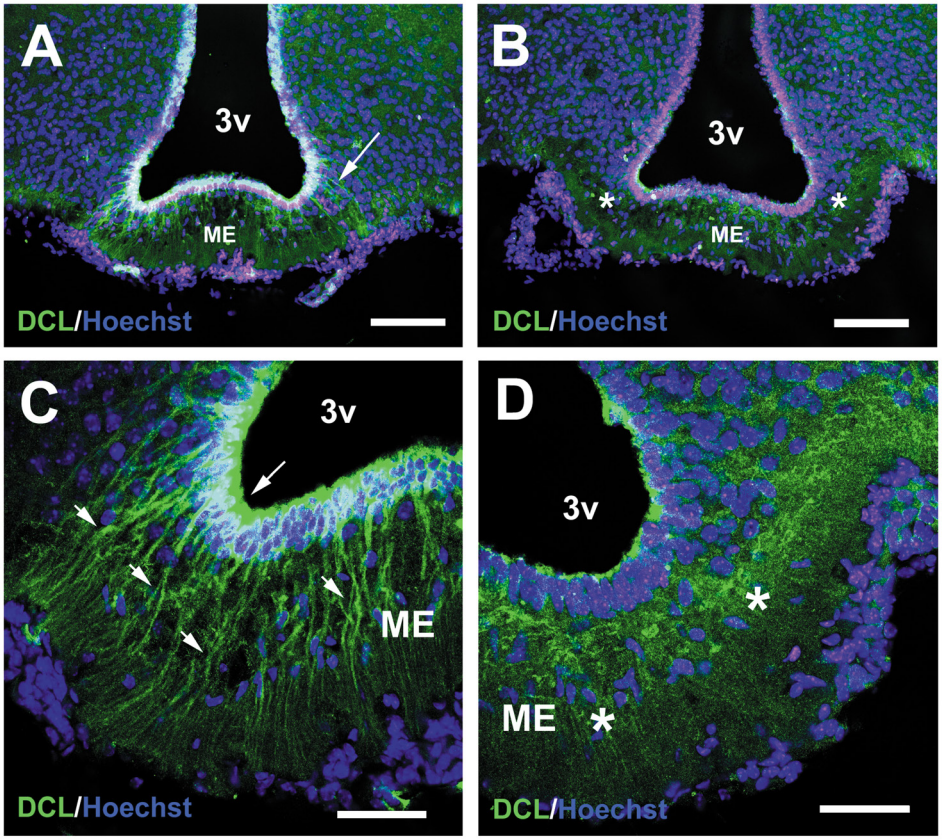

Figure 2: DCL-KD in hypothalamic tanycytes. A) Strong DCL expression in the wall of the third ventricle (3v). DCL positive projections in median eminence (ME) are clearly visible (arrows). B) DCL expression in the 3v wall is strongly reduced (asterix). C) Higher magnification of tanycytes close to the ME with DCL positive basal processes extending into the ME (arrows). D) Although some DCL signal is left, strong reduction of DCL expression is visible after siRNA mediated DCL knockdown. Scale bars measure in A and B 40µm, in C and D 100µm.

in D2 activity, DCL knockdown did not affect D2 mRNA expression in either the PE or the ARC-ME (Fig. 5A). Similar results were found for D3 mRNA; DCL knockdown did not affect D3 mRNA expression in PE and ARC-ME punches (Fig. 5B). Since hypothalamic D2 converts peripheral T4 into T3 and is thought to be involved in the set-point of the HPT-axis, we also measured serum T3 and T4 levels. DCL knockdown did not affect serum levels of either T3 or T4 (see Fig. 4A & B). In addition, DCL knockdown did not affect mRNA expression of several HPT-axis related genes in either the PE or ARC-ME punch (Fig. 5). However, both TRH and NPY mRNA levels did exhibit the expected area specificity with significant higher levels of TRH in the PE punch ($F(1)=60.53$, $p<0.001$, Fig. 5C) and a significantly higher NPY mRNA expression in the ARC-ME punch ($F(1)=43.50$, $p<0.001$).

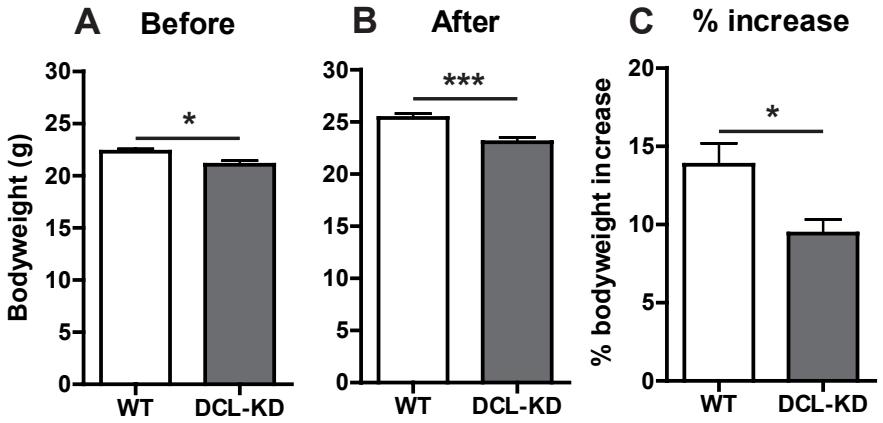

Figure 3: Bodyweight before and after doxycycline induction. A) Wildtype (WT) (22.29 ± 0.2887 g, N=24) and DCL-KD (21.05 ± 0.3996 g, N=24) littermates differed in bodyweight at the start of the experiment (1.233 ± 0.4930 g, p=0.016). B) After 5 weeks of dox induction, WT animals (25.36 ± 0.4381 g, N=24) were still significantly heavier than DCL-KD animals (23.04 ± 0.4774g , N=24), but the difference had increased (2.321 ± 0.6479 g, p<0.001). C) WT animals gained relative more bodyweight (13.82% ± 1.370, N=24) than their DCL-KD littermates (9.418 %± 0.9036, N=24).

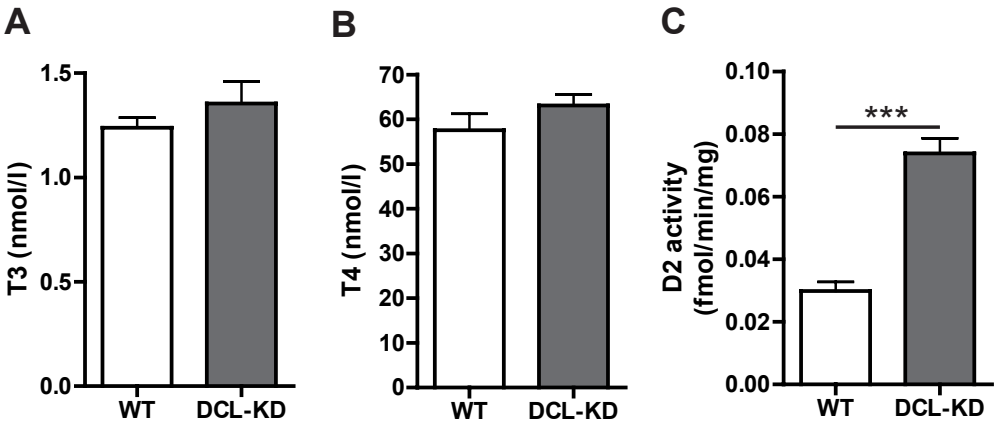

Figure 4: TH levels in blood plasma. A) T3 levels in peripheral blood serum did not differ between WT (1.238 ± 0.04816 N=13) and DCL-KD (1.355 ± 0.1047 N=11) animals. B) Even so did T4 levels not differ between WT (57.54 ± 3.681 N=13) and DCL-KD (63.09 ± 2.470 N=11) animals. C) DCL-KD animals have a more than two times higher D2 activity (0.074 fmol/mg/min ± 0.005, N=5) compared to wildtype littermates (0.030 fmol/mg/min ± 0.003, N=6).

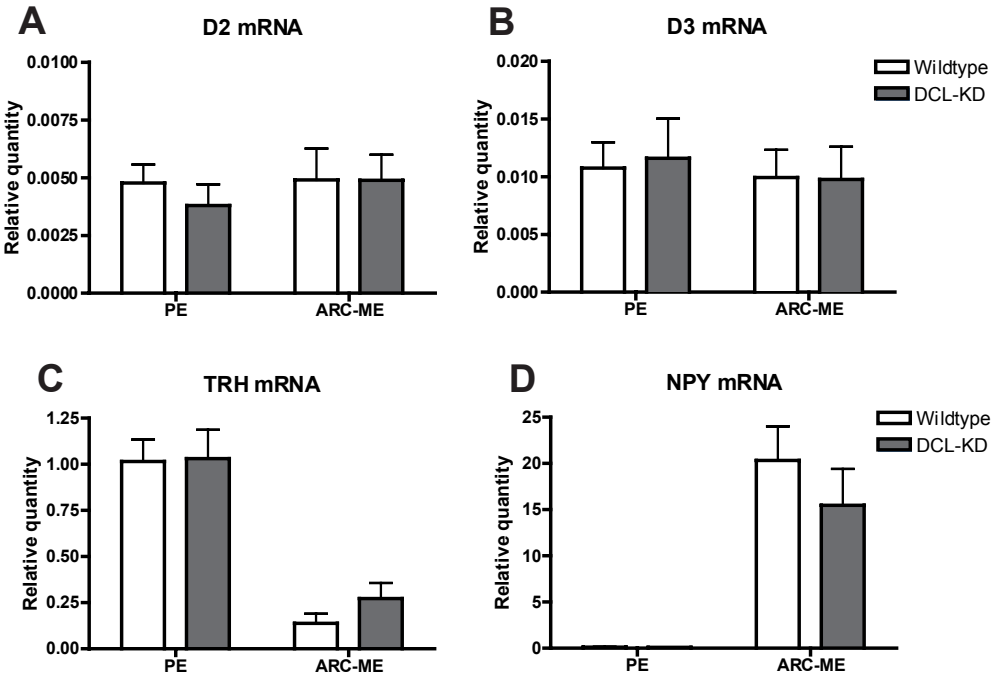

Figure 5: mRNA levels of 4 genes involved in the HPT-axis. A) Despite the significant increase in D2 activity, DCL knockdown did not affect D2 mRNA expression in all hypothalamic tissue ($F(1)=0.191$, $p>0.05$). Furthermore, levels of D2 mRNA did not differ between both PVN and ARC/median eminence ($F(1)=0.294$, $p>0.05$). B) Similar results were found for D3 mRNA; DCL knockdown did not affect D3 mRNA expression between wildtype and DCL-KD animals ($F(1)=0.0.013$, $p>0.05$) and between both PVN and ARC/median eminence (($F(1)=0.201$, $p>0.05$). C) DCL knockdown did not affect TRH mRNA expression levels ($F(1)=0.502$, $p>0.05$) within the hypothalamus. However, a difference between both punches show area specificity since PVN and ARC/median eminence differ in TRH mRNA levels ($F(1)=60.53$, $p<0.001$). D) NPY mRNA expression is characteristic for the arcuate nucleus. A strong significant difference was found between both area's in favour of ARC in all animals ($F(1)=43.50$, $p<0.001$). DCL knockdown did not affect NPY mRNA expression ($F(1)=0.816$, $p>0.05$).

Discussion

Here, we have studied the possible role of DCL in hypothalamic tanycytes in thyroid hormone metabolism by using genetically modified mice with a knockdown of DCL after dox administration in food pellets. We observed a strong reduction in DCL expression in hypothalamic tanycytes which was associated with reduced body weight growth and a significant increase in D2 activity, the enzyme metabolizing inactive T4 into active T3. However, serum levels of T4 and T3 did not differ between wildtype and DCL-KD animals and also the expression of TRH, NPY, D2 and D3 mRNA in the hypothalamus was not affected by DCL knockdown. Toge-

ther, our data suggest a subtle role for DCL in the regulation of D2 in hypothalamic tanycytes which is however not affecting the set-point of the HPT-axis.

We observed a small, but significant difference in bodyweight between wildtype and DCL-KD littermates. After the dox-induced knockdown of DCL, the transgenic mice showed a smaller increase of bodyweight compared to the wild types. The observed difference in hypothalamic D2 activity between DCL-KD and wildtype mice cannot explain the reduced increase in bodyweight in DCL-KD animals since serumT4 and T3 levels were unaffected. A possible explanation for the observed differences in body weight might be leakage of the siRNA system. Leakage of the tet repression function has been reported and might lead to reduced DCL expression which is further decreased by dox administration. However, whether or not leakage affected early bodyweight development in our mice is presently unknown. Another possible explanation for reduced bodyweight increase might be aberrant glucose sensing. Glucose sensing is also an important function of tanycytes and ATP plays an important role in tanycytes mediated glucose sensing within the hypothalamus (Frayling et al., 2011). Interestingly, DCL has recently been shown to reside in mitochondria where DCL knockdown results in decreased cytochrome C activity and ATP production (Verissimo et al., 2013) suggesting that DCL knockdown might affect glucose sensing by reducing ATP production. However, if glucose sensing is affected and if impaired glucose sensing affects bodyweight remains to be elucidated.

D2 in the hypothalamic tanycytes converts inactive T4 into active T3. DCL knockdown animals displayed higher hypothalamic D2 activity than WT littermates. Although D2 activity is increased after DCL knockdown, D2 mRNA expression is not affected in both hypothalamic PE and ARC-ME punches suggesting post-transcriptional regulation. D2 activity is amongst others regulated by ubiquitination via an ATP-dependent process (Gereben et al., 2000;Steinsapir et al., 2000). As DCL knockdown leads to reduction of ATP production (Verissimo et al., 2013), increased D2 activity in DCL knockdown mice might be the result of a blockade of ubiquitination-mediated D2 degradation.
Higher amounts of D2 activity would be expected to result in an increased production of local T3 and thereby lower hypothalamic TRH expression. This is however not the case, despite higher amounts of D2 activity, TRH mRNA expression in the PE area is similar in WT and DCL-KD mice.

Serum thyroid hormone levels were not affected by DCL knockdown either. Determination of hypothalamic T3 levels might be necessary in order to establish the function of the tanycytes with respect to local T3 production by D2. Since tanycytes in the ME create a private milieu within the hypothalamus by functioning as a barrier between peripheral blood and hypothalamus, T3 levels can be different between peripheral blood and hypothalamic

tissue (Rodriguez et al., 2010). Also measurement of TSH hormone levels might provide more useful information about the consequences of increased D2 activity on the HPT-axis function.

All together, DCL knockdown affects tanycytes functioning in thyroid hormone signalling. However, this effect seems to be limited to hypothalamic D2 activity. Although D2 activity is increased, THR mRNA expression and serum thyroid hormone concentrations are not affected by DCL knockdown. Whether increased D2 activity is a direct or indirect result of DCL knockdown remains to be elucidated.

Acknowledgements

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Chapter 6

General discussion

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1 Introduction

The main objective of this thesis was to study the role of the DCLK1 splice variant DCL in the brain, in particular in adult hippocampal neurogenesis. To map DCL in the adult brain, a DCL-specific antibody was designed and experiments were performed to validate a new genetic mouse model that is based on knockdown of DCL by doxycycline-induced sh-RNA molecules, called the DCL-KD mouse. First we reviewed the existing literature about developmental and adult neurogenesis in chapter 1. In the same chapter we reviewed the role of doublecortin and doublecortin-like 1 in this process and discussed several animal models to study adult neurogenesis. In chapter 2 we developed a DCL specific antibody and mapped the expression of DCL in the adult brain. As expected, we identified DCL in neurogenic areas like the SVZ and SGZ of the dentate gyrus where it co localized with DCX. DCL protein was also found in several non-neurogenic areas which raised novel questions about the function of DCL in these areas. In chapter 3, the DCL-KD mouse model was validated by showing specific DCL knockdown after doxycycline administration. Furthermore, the effect of DCL knockdown was studied on adult hippocampal neurogenesis using stereological techniques. Evidence was found for a role of DCL in the cell cycle exit of proliferating NPCs. In addition, we studied the effect of DCL knockdown on hippocampus-dependent behaviour. Additional evidence for the effect of DCL knockdown on hippocampus-dependent learning using a contextual fear conditioning paradigm is discussed in chapter 4. We describe the possible function of DCL in the hypothalamic tanycytes in chapter 5. In the latter chapter, we studied the question if DCL is involved in the Hypothalamic-Pituitary-Thyroid-axis (HPT-axis) and if DCL knockdown can affect thyroid hormone signalling via deiodinase type 2 activity in hypothalamic tanycytes.

2 DCL expression in the adult brain

Doublecortin-like kinase 1 (DCLK1) is, In contrast to doublecortin (DCX), a multiple splice-variants encoding gene containing at least four different splice variants (Burgess and Reiner, 2002;Sossey-Alaoui and Srivastava, 1999;Vreugdenhil et al., 2001). Each of the splice variants has its own functional and expression pattern (Burgess and Reiner, 2002;Engels et al., 2004;Vreugdenhil et al., 2001). Up to now, differentiation between DCLK1 splice variants by immunohistochemical methods was difficult since available antibodies did not differentiate between DCLK1 splice variants (Femia et al., 2013;Kikuchi et al., 2010;Kruidering et al., 2001). There are several other biochemical methods to separate DCLK proteins based upon their length or sequence, but in an immunohistochemical staining no distinction can be visualized under the microscope with a-specific DCLK antibodies. Based on studies in embryonic and postnatal brain tissue it is known that the DCLK splice-variant doublecortin-like (DCL) is expressed in radial glia cells and immature neurons during embryonic and early

postnatal neurogenesis (Boekhoorn et al., 2008;Vreugdenhil et al., 2007). Furthermore, DCL is also expressed in adult hippocampal tissue (Vreugdenhil et al., 2001) but the exact cellular location in this heterogeneous brain region is unknown. As DCL shares a number of biophysical properties with DCX and is also highly homologous to DCX (Vreugdenhil et al., 2007), a well known marker of adult neurogenesis, we hypothesized that DCL, like DCX, might be expressed in the population of migrating immature neurons in the hippocampal dentate gyrus. However, other DCLK1 splice variants like DCLK-long and DCLK-short are also expressed in the hippocampal dentate gyrus (DG) (Vreugdenhil et al., 2001) and may cross-react with antibodies targeting different DCLK1 splice-variants and are not suitable to study DCL. Therefore, we developed a novel antibody targeting specifically DCL and no other DCLK1 splice variants. This antibody appears to be highly specific for DCL and does not stain other DCLK1 splice variants or the highly homologous DCX (Saaltink et al., 2012).

2.1 DCL expression in neurogenic area's like hippocampus and olfactory bulb.

As expected, we found DCL expression in the subgranular zone of the DG, the subventricular zone (SVZ), the rostral migratory stream (RMS) and the olfactory bulb (OB)(chapter 2). In all these neurogenic areas, DCL co localizes with DCX confirming several studies showing a functional interaction between both DCX and DCLK1 (Deuel et al., 2006;Koizumi et al., 2006;Tanaka et al., 2006;Tuy et al., 2008). In the embryonic brain (Boekhoorn et al., 2008), DCL is slightly earlier expressed compared to DCX. Within the adult brain such a temporal difference in expression seems to be absent although more detailed studies are needed to support this statement. This overlap in expression suggests a similar role as DCX for DCL in adult neurogenesis (Brown et al., 2003b;Couillard-Despres et al., 2005;Couillard-Despres et al., 2006;Rao and Shetty, 2004). Also during migration via the RMS and within the OB, DCL and DCX are strongly co localised. Only in the periglomerular cells (PGC's) in the granule cell layer (GL) of the OB some single positive DCX cells are found, suggesting a different temporal expression pattern of DCL compared to DCX as found earlier (Boekhoorn et al., 2008).
Images of DCL and DCX double staining show another remarkable fact. Whereas a DCX staining results in a rather empty cell nucleus and densely stained cytosol including dendritic branches, DCL staining exhibits a more speckled pattern without a densely filled cytosol (see Chapter 2, Fig. 5). This suggests different sub-cellular functions for DCL and DCX. Techniques with higher resolutions than confocal laser scanning microscopy are needed to visualize the exact location of DCL in the cell.

2.2 DCL expression outside the neurogenic areas.

Beside the neurogenic DG, SVZ, RMS and OB, DCL expression was found in several other brain regions like the suprachiasmatic nucleus (SCN), islands of Calleja (ICj) and hypotha-

lamic tanycytes (chapter 2). The majority of studies show DCL and DCLK to be involved in neurogenesis and neuronal development. During embryonic development, DCL is widely expressed in mitotic neuronal progenitor cells, radial glial cells (RGs) and radial processes and functions as a microtubule stabilizing protein of mitotic spindles in vitro and in vivo (Boekhoorn et al., 2008;Vreugdenhil et al., 2007). Furthermore, DCL is involved in spine maturation (Shin et al., 2013). These findings raise the question why DCL is expressed in the SCN, in the ICj and in tanycytes, brain areas and cell populations in which neurogenesis has not been established.

As reported in chapter 2, DCL expression in the ICj shows strong resemblance with expression in the OB. DCL is mainly expressed in neuropil or extra-nuclear cytosol. Both OB and ICj consist of granule cells and are part of the olfactory system since ICj are located within the olfactory tubercle. Like the OB, the ICj are formed during brain development via cells born in the SVZ. Whereas the OB is supplied via the RMS, are the ICj supplied with granule cells via the ventral migratory mass (VMM) (De Marchis et al., 2004). The RMS continues with streaming neurons from the SVZ to the OB, whereas the VMM ceases in the adolescent mouse brain. However, 11 days after BrdU injection Shapiro and colleagues found BrdU/NeuN positive cells in the ICj of mice 2 months of age, suggesting some neurogenesis is still present in the adult ICj (Shapiro et al., 2009). Since DCL is expressed in the majority of ICj granule cells, its function in this area might be broader than neurogenesis alone and might reflect a form of plasticity or intracellular transport along the neuronal fibres (see Chapter 1).
DCL expression in the SCN might also be related to plasticity and fast transport along microtubules. The SCN exhibits daily structural rearrangements within the SCN (Girardet et al., 2010a;Meijer et al., 2010), which may demand high levels of neuronal plasticity. DCL might be involved in SCN plasticity, as has been reported for polysialic acid (PSA) and neural cell adhesion molecule (NCAM) (Bonfanti et al., 1992;Glass et al., 2003;Prosser et al., 2003;Shen et al., 1997). PSA/NCAM is thought to play a key role in the daily structural rearrangement of the SCN (Bonfanti et al., 1992;Girardet et al., 2010a;Glass et al., 2003;Prosser et al., 2003) and is thus crucial for the circadian organization of behaviour (Fedorkova et al., 2002;Prosser et al., 2003;Shen et al., 1997). In line with DCL expression in the SCN, PSA/NCAM is also a well-known marker for neurogenesis in the adult brain and is often co expressed with DCX (Bonfanti, 2006;Nacher et al., 2001). Up to now, no neurogenesis in the SCN has been reported and therefore, a functional role of DCL in SCN plasticity is most obvious. However, besides neurogenesis, DCL is involved in fast microtubule-guided retrograde transport of signalling molecules in neuroblasts and neuroblastoma cells (Fitzsimons et al., 2008) and thus might also play such a role in the SCN. Possible implications of DCL as intracellular transport factor will be discussed further below (see paragraph 6.8).
An increasing number of studies show (condition-dependent) neurogenesis in the hypothalamus (Haan et al., 2013;Lee et al., 2012;Xu et al., 2005). Although hypothalamic tanycytes

do have DCL expression, these DCL positive tanycytes do probably not reflect continuous hypothalamic neurogenesis like in the SVZ and DG. The hypothalamus is a highly dynamic region, which plays a crucial role in neuro-endocrine signalling between body and brain (Rodriguez et al., 2010). Several processes are regulated by seasonal time cues, which induce physiological adaptation (Hazlerigg and Loudon, 2008). Like in the SCN, such dynamic processes need cellular rearrangements in which DCL might play a role. DCL might also play a similar role in tanycytes as it does in neuronal progenitor cells (NPC's) were it is involved in sub cellular transport of the glucocorticoid receptor (GR) (Fitzsimons et al., 2008). Like DCX (Friocourt et al., 2001), DCL is equipped with a serine-proline rich C-terminus which functions as an anchor point for interaction with other proteins.

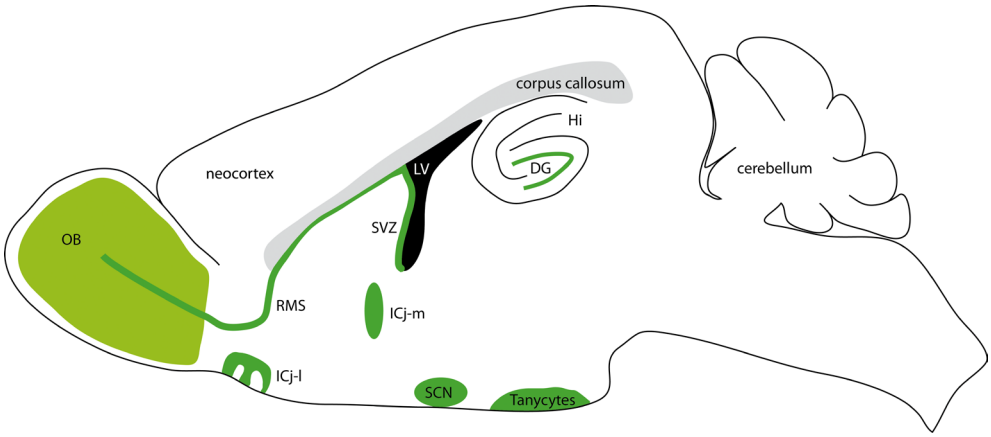


Figure 1: Schematic overview of all DCL expression sites (green) in the adult mouse brain. Within the hippocampus (Hi) DCL is expressed in the dentate gyrus (DG). DCL is highly expressed along the walls of the lateral ventricles (LV). From this subventricular zone (SVZ), DCL positive immature neurons migrate along the rostral migratory stream (RMS) towards the olfactory bulb (OB) were DCL is also expressed in the neuropil. Other sites of DCL expression are the major Islands of Calleja (ICj-m), the lateral Islands of Calleja (ICj-l), suprachiasmatic nucleus (SCN) and the hypothalamic tanycytes.

In summary, DCL expression in non-neurogenic brain areas does probably not reflect neurogenesis, but might be an indication of cellular plasticity. The ICj show great resemblance with the OB and therefore, DCL might play a similar role in both these areas. The hypothalamic SCN and tanycytes are areas with functional neuronal plasticity and dendritic rearrangements in which DCL might play a role. Further studies on DCL have to reveal its function within these non-neurogenic brain areas.

3 A transgenic mouse model to study DCL in the brain: application of in vivo RNA-interference

The main objective of this thesis is to investigate the role of DCLK1 splice variant DCL in adult neurogenesis. The technical focus in this study is the validation of an in vivo model in which DCL is targeted by RNA interference technology. We hypothesized that DCL is functionally involved in adult neurogenesis. Therefore, by knocking down DCL, we expect to disrupt the integration of neurons into neuronal networks, which will affect the behavioural output. Since DCL plays a crucial role in embryonic corticogenesis (Boekhoorn et al., 2008;Vreugdenhil et al., 2007) and its analogue DCX may compensate for the loss of DCL during development, a conditional model which can be activated on demand in adult life is required. As described in chapter 3, we designed a transgenic mouse model harbouring an short hairpin DNA construct in its germ line of which the expression is blocked by a TET-repressor (TETr). Expression of the shRNA targeting DCL can induced by doxycycline (Seibler et al., 2007).

Firstly, we have investigated induction of the DCL-shRNA expression by doxycycline administration. We found a strong up regulation of shRNA expression after doxycycline (dox) induction. Although this was a 10-fold up regulation in the hippocampus and 25-fold up regulation in the olfactory bulb, we also found some shRNA expression in non-induced transgenic animals. Theoretically, TETr should block the expression of the DCL targeting shRNA. However, incomplete blockade may result in shRNA expression (leakage) and subsequent reduction of DCL levels which might be compensated by DCX. Indeed, our initial experiments indicate reduced DCL expression in the adult brain of non-induced transgenic mice. Therefore, we inspected embryonic and early postnatal tissue to explore possible premature DCL knockdown. However, despite little hairpin leakage, the developing brain was unaffected and no significant reduction of DCL protein was found at this stage of development. Our data indicate normal brain development in which DCL performs like in a wildtype animal without DCX compensation. This finding is important since DCL knockdown at an adult stage might be ineffective when DCX compensates for DCL during brain development (Deuel et al., 2006;Koizumi et al., 2006;Tanaka et al., 2006;Tuy et al., 2008). In contrast to other studies with shRNA mice (Acehan et al., 2011;He et al., 2010;Out et al., 2011), we report the presence of leakage. However, we only observe a clear phenotype after doxycycline administration, indicating that leakage did not affect brain development and brain function.

A major advantage of the DCL shRNA mouse model is the specificity of the hairpin. Since DCL is one of the 10 splice variant of the DCLK1 gene (Burgess and Reiner, 2002;Sossey-Alaoui and Srivastava, 1999;Vreugdenhil et al., 2001), specific knockdown of DCL, but not of other DCLK splice variants, is a necessary step to study DCL function in the adult brain. Indeed, Western Blot analysis of induced wildtype and transgenic brain tissue shows a highly specific

knockdown of DCL whereas DCLK-short and DCLK-long are unaffected. Especially the distinction between DCL and DCLK-long is important since DCL shares most of its functional protein domains with DCLK-long. Furthermore, DCL and DCLK-short appeared to precisely regulate postsynaptic functions, because these variants have distinct roles in postsynaptic density (PSD) protein accumulation and spine morphogenesis (Shin et al., 2013). Therefore, our mouse model seems also an excellent tool to study the role of DCL in spine morphogenesis.

4 Effect of DCL knockdown on neurogenesis in the adult brain.

The first studies with BrdU revealed an unexpected increase of proliferation after DCL knockdown. The DCX domains in the DCX and DCLK1 genes are thought to function as anti-catastrophe factor by stabilizing microtubules (Moores et al., 2004;Moores et al., 2006). Previous observations in our lab showed a function for DCL in mitotic spindle formation and thus a role in cell mitosis (Vreugdenhil et al., 2007). Furthermore, combination treatment of microtubule disrupting agents such as vinca alkaloids and siRNA targeting DCL and DCLK1-long induced apoptosis in neuroblastoma cells in vitro (Verissimo et al., 2010a). However, these studies used siRNA targeting both DCL and DCLK-long. Since the study in chapter 3 used specific constructs targeting DCL only, it seems likely that only DCLK-long might be involved in cell proliferation. However, both DCLK-long and DCL contain microtubules binding domains, which stabilize microtubules. Which role each of the two DCLK1 splice variants plays in proliferation is still unknown. Knocking down both DCLK-long and DCL leads to apoptosis and microtubules destabilization both in vitro and in vivo (Shu et al., 2006;Verissimo et al., 2010a;Vreugdenhil et al., 2007). Specific knockdown of DCL in vivo leads to increased proliferation. Although DCL is expressed in mitotic spindle formations within the embryonic brain, we have no indications that DCL is also expressed in stem cells in the adult brain (unpublished data). Therefore, DCL knockdown might have an indirect effect on proliferation. One explanation might be a feedback mechanism within the hypothalamus. When the flow of immature neurons reaching the network is reduced due to DCL knockdown, the stem cells might be triggered to increase their production of new immature neurons. However, the mechanism of such a feedback system is unknown.

Another possibility is the involvement of DCL in transporting signalling proteins affecting the fate of neuronal progenitor cells such as the glucocorticoid receptor (GR; (Fitzsimons et al., 2008). Stress is a well known inhibitor of neurogenesis (Lucassen et al., 2010a;Schoenfeld and Gould, 2012) with strong effects on both proliferation and differentiation/maturation. Stress is known to increase circulating levels of glucocorticoids (cortisol in humans and corticosterone in rodents) which bind to and activate GRs. The increased corticosterone concentration drives in the dentate gyrus progenitor cells the cell nuclear localization of GR that in turn can modulate gene transcription and suppress cell proliferation (Veenema et al., 2007).

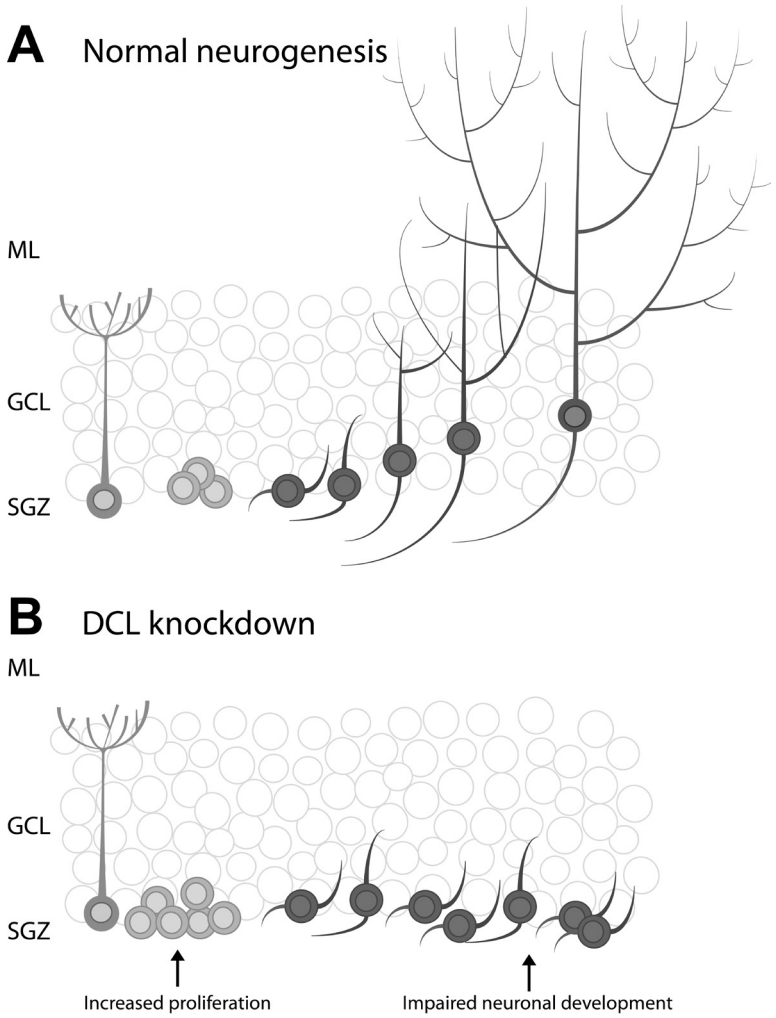


Figure 2: Effect of DCL knockdown on hippocampal adult neurogenesis. A: In the subgranular zone (SGZ) reside stem cells which generate neural progenitor cells (NPC's; pink). These new born cells develop into immature neurons (green) which migrate into the granule cell layer (GCL). The immature neurons make contact with the existing network in the molecular layer (ML) and develop into mature neurons (blue). B: After DCL knockdown, proliferation is increased, but immature neurons do not migrate and develop into mature neurons.

Therefore, the increased proliferation of these progenitor cells after DCL knockdown might reflect lower GR transport to the nucleus. Also here, we have no data that actually show DCL expression in neurogenic stem cells.

The first glimpse of a striking DCX phenotype in our DCL-KD mice became visible when hippocampal sections were inspected under the fluorescent microscope (Chapter 3). Nearly

all DCX positive cells reside in the subgranular zone (SGZ) of the DG and show hardly any elongate dendrites towards the granular cell layer (GCL) and molecular layer (ML) suggesting an arrest in neuronal development. Cells seem to stop their development at the point where they have to migrate into the GC and develop elongated dendrites towards the ML. This arrest suggests that DCL might play a key role in dendritic outgrow. Since both DCL and DCLK-short are thought to regulate postsynaptic density (PSD) accumulation and spine morphogenesis (Shin et al., 2013), knockdown of DCL might affect these processes. This seems to be the case since DCL knockdown results in reduced dendritic development in the population of DCX positive cells. DCLK-short is one of the genes which is highly up-regulated after NGF-induced differentiation of PC12 cells (Dijkmans et al., 2008). After NGF induction, PC12 cells develop neurites and differentiate into neurons. DCLK-short has been shown to repress CREB-mediated transcription (Ohmae et al., 2006;Silverman et al., 1999), which is an important transcription factor in NGF-induced processes like PC12 differentiation (Finkbeiner et al., 1997). However, whether DCLK-short is expressed in NPC's is unknown. The question remains whether we look at a cell cycle arrest, or impaired dendritic outgrow. Since type-1 DCX positive cells in DCL-KD mice show small to medium processes (as describe by (Plumpe et al., 2006)) it is likely that immature neurons do not receive the right signals to develop further into mature granule cells. When they fail to integrate into the network, the cells might go into apoptosis and disappear.

DCL is highly homologues to DCX (Vreugdenhil et al., 2007) and both DCLK1 and DCX are thought to compensate each other during development in knockout conditions (Deuel et al., 2006;Koizumi et al., 2006;Tanaka et al., 2006;Tuy et al., 2008). Evidently, this compensation does not occur when DCL knockdown is applied during adulthood. It is quite remarkable that micro (mi)RNA-mediated retroviral knockdown of DCX does not alter adult neurogenesis in either adult neurogenic niche (Merz and Lie, 2013). Although DCX is a well known marker for adult neurogenesis (Brown et al., 2003b;Couillard-Despres et al., 2005;Couillard-Despres et al., 2006;Rao and Shetty, 2004) its function in adult neurogenesis is unknown. Apparently, DCX is dispensable for adult neurogenesis. This study is in contrast with findings in the developing embryo in which RNAi mediated knockdown of DCX had an effect on developmental neurogenesis (Koizumi et al., 2006). The explanation for these opposite findings might be technical. A DCX-KD mouse like the DCL-KD mouse would provide useful information regarding the function of DCX during adult neurogenesis.

In conclusion, DCL knockdown results in aberrant neuronal development and maturation within the hippocampal neurogenic niche. In line with the expected function of DCL, immature neurons do not migrate into the granule cell layer and do hardly develop dendrites reaching the hippocampal network in molecular layer after DCL knockdown (Fig. 2B). Unexpected is the increased proliferation seen in vivo in hippocampal dentate gyrus where

DCL knockdown in vitro in PC12 cells leads to a reduction of cell proliferation. A feedback mechanism involving DCL in glucocorticoid signalling might explain this unexpected finding in vivo in the hippocampus.

5 The effect of DCL knockdown on hippocampus-dependent memory tasks.

In the past decades hippocampal adult neurogenesis gained considerable attention in the fields of psychiatric and degenerative brain disorders. Neurogenesis is affected by severe stressors and might form a potential cure for brain degeneration. The hippocampus is involved in higher cognitive brain functions like learning and memory formation. Especially spatial and contextual memory formation is thought to be hippocampus dependent (Marin-Burgin and Schinder, 2012). Adult neurogenesis might play a key role in these hippocampal functions (Kee et al., 2007;Kempermann et al., 1998a;Raber et al., 2004). However, the hippocampus is a complex and heterogeneous brain area in which neurogenesis is just a part of the story. One of the first techniques to target adult neurogenesis was the use of x-ray radiation showing effective blockade of adult neurogenesis (Santarelli et al., 2003). Using this technique, Saxe et al. (Saxe et al., 2006) showed an effect of reduced neurogenesis on a hippocampus-dependent behavioural task measuring contextual fear memory. Using a genetic manipulation strategy, Immayoshi and colleagues (Imayoshi et al., 2008) observed that neurogenesis inhibition affects hippocampus-dependent spatial memory formation using a circular hole board paradigm. A range of studies followed with varying results. We applied similar tests to our mouse model (chapter 3 and 4) and did not find an effect of impaired adult neurogenesis on spatial and contextual memory formation.
In a circular hole board paradigm, DCL-KD animals are capable of learning to find the exit hole like wildtype littermates. Also contextual fear memory formation is unaffected by DCL knockdown. However, some subtle behavioural differences are found. For example, DCL-KD animals have a significant longer escape latency compared to wildtype littermates. In the fear conditioning paradigm, DCL-KD animals show hardly any tail rattling behaviour which suggests reduced levels of basal anxiety. Thus, DCL knockdown affects behavioural output but it seems that the behavioural paradigms, used in our studies, are not suitable tests. However, the most important finding is the normal spatial and contextual fear memory performance despite the impairment of adult neurogenesis.

There are several explanations for our findings. In the first place, we make use of a specific knockdown strategy. Although we see a clear phenotype with respect to adult neurogenesis, neurogenesis is not completely blocked. Proliferation is even increased and we still measured some BrdU/NeuN double positive cells. Neurogenesis blockade by X-ray radiation is less specific and potentially affects the whole hippocampus including the non-neurogenic cell population. In support of the outcome of our approach several other techniques targeting

adult neurogenesis specifically did not result in a change of hippocampus-dependent spatial memory formation (Jaholkowski et al., 2009;Jedynak et al., 2012;Martinez-Canabal et al., 2013;Urbach et al., 2013). These studies raised the question whether adult neurogenesis is involved in hippocampus-dependent learning and memory formation (Frankland, 2013).

Recently, adult hippocampal neurogenesis have been implicated in pattern separation (Aimone et al., 2011;Sahay et al., 2011b;Aimone et al., 2011;Sahay et al., 2011b;Tronel et al., 2012). Pattern separation is the capacity to distinct between two highly similar input patterns (Treves et al., 2008). The process of pattern separation is suggested to be associated with post traumatic stress disorders and with panic disorders (Kheirbek et al., 2012). However, the studies described in this thesis are not suitable for studying pattern separation performance. Therefore, the effect of DCL knockdown on subtle context recognition remains to be speculative.

Another factor of interest is neurogenesis in the olfactory bulb. Although we did not study the OB in detail, the aberrant morphology of DCX positive cells seen in the hippocampus, seems to be absent in the SVZ, RMS and OB. Adult neurogenesis in both OB and hippocampus might play a similar role in spatial and contextual discrimination (Konefal et al., 2013) but seems to be regulated independently (Belnoue et al., 2013). Impairment of neurogenesis in both brain areas might increase the effect on spatial and context discrimination. Due to our limited observations on the SVZ, RMS and OB, we can only speculate about the effect of DCL knockdown on adult neurogenesis in the olfactory system.

It remains the question why the DCL-KD mice take more time to enter their home cage despite the fact they know how to find the exit hole. Unlike rats, mice have some problems with navigating in the Barnes maze (Koopmans et al., 2003). Therefore mice are habituated to the tunnels 1 week before the free exploration trial (FET). Mice with reduced DCL expression might not benefit from this training. Also during the training sessions, DCL-KD mice perform better in the second training of the day suggesting a limited habituation memory. This might be an effect of reduced pattern recognition; the animals fail to recognize the tunnel as safe passageway in a novel context (circular hole board). Why DCL-KD animals fail to remember the tunnel training, but succeed in spatial memory formation remains to be elucidated.

One may also reason the other way around. The behavioural parameters affected by DCL knockdown, i.e. prolonged escape latency and tail rattling, point towards reduced anxiety. Since cue fear memory is thought to be amygdala dependent, the strong freezing response of DCL-KD animals during the first cue presented at the memory retrieval trials is rather difficult to explain from the perspective of impaired neurogenesis. However, the interplay

between amygdala and hippocampus might be affected. In chapter 4 we discussed the fear conditioning paradigm in more detail. A series of multiple cue/shock pairings might be too strong. A fear conditioning paradigm with a delayed or immediate single context-shock pairing might provide a more precise answer on hippocampus related fear memory formation with involvement of the amygdala.

The significant differences in tail rattling and increased freezing behaviour in the first cue suggest an effect of DCL knockdown on trait anxiety. Trait anxiety is defined as an enduring level of anxiety, independent from environmental stimuli whereas state anxiety is temporary anxiety induced by environmental stimuli (Lister, 1990). Both forms of anxiety seem to have genetic components which are differentially rooted in the genetic background of laboratory mouse strains (Avgustinovich et al., 2000). DCL knockdown might not affect state anxiety, which explains the normal fear conditioning response, but might reduce trait anxiety, explaining the reduction in tail rattling behaviour and increased escape latency. Though high levels of trait anxiety are correlated to reduced adult neurogenesis (Earnheart et al., 2007;Sah et al., 2012), we find opposite effects in our DCL-KD animals. Although the numbers of BrdU/NeuN double positive cells are strongly reduced in DCL-KD animals, their proliferation is significantly increased (see chapter 3). Since stress is deleterious for proliferation, increased proliferation in our DCL-KD animals might reflect reduced trait anxiety. DCL is involved in sub cellular transport of the GR (Fitzsimons et al., 2008) which might explain the mechanism underlying this increased proliferation. Exposing DCL-KD animals to more anxiety related behavioural paradigms like novel object exploration in combination with familiar and unfamiliar context, might provide answers to the questions raised here.

In conclusion, DCL-induced impaired neurogenesis does not affect hippocampus-dependent spatial memory and contextual fear memory. DCL-KD animals need equal amount of time to find the exit as their litter mate controls, but their latency to escape the board and enter their home cage is increased. Furthermore, they show a stronger freezing response to the first cue in CFC where after they behave like wildtype littermates except for tail rattling behaviour during fear. These results are highly significant. These findings suggest a role for DCL in anxiety in which DCL facilitates the stress-induced corticosterone signal. Therefore, DCL-KD animals seem a valuable model for further research aimed to address the role of neurogenesis in the processing of fearful information by measuring more subtle aspects of context discrimination and pattern separation.

6 DCL in the hypothalamus

Mapping studies with the novel DCL antibody revealed unexpected expression sites within the brain. One of these sites are hypothalamic tanycytes. Tanycytes reside at a crossroad

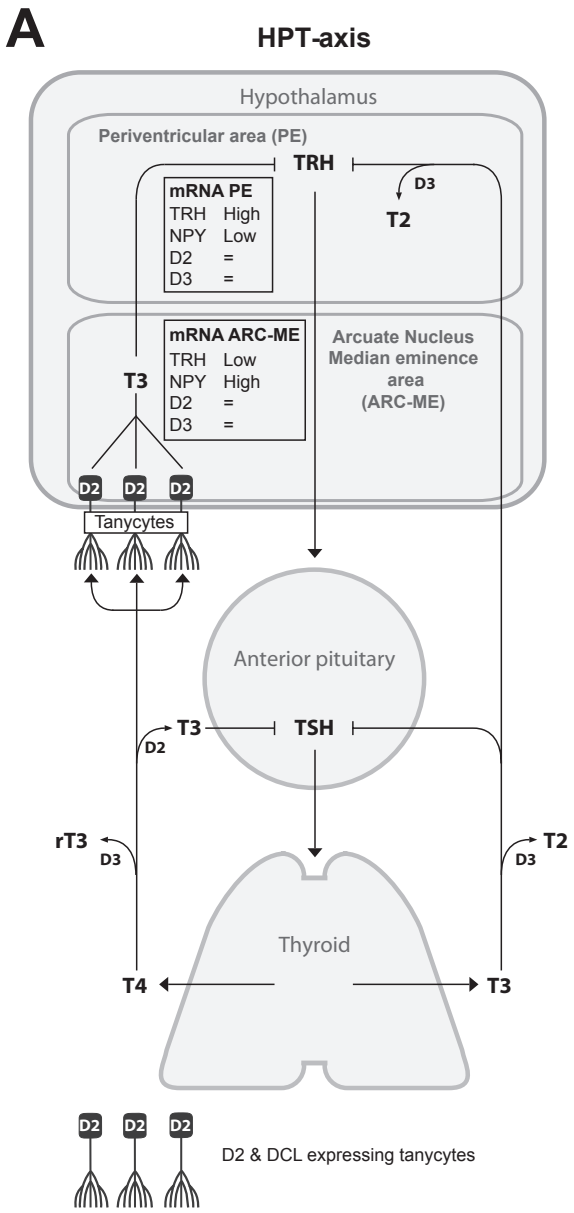
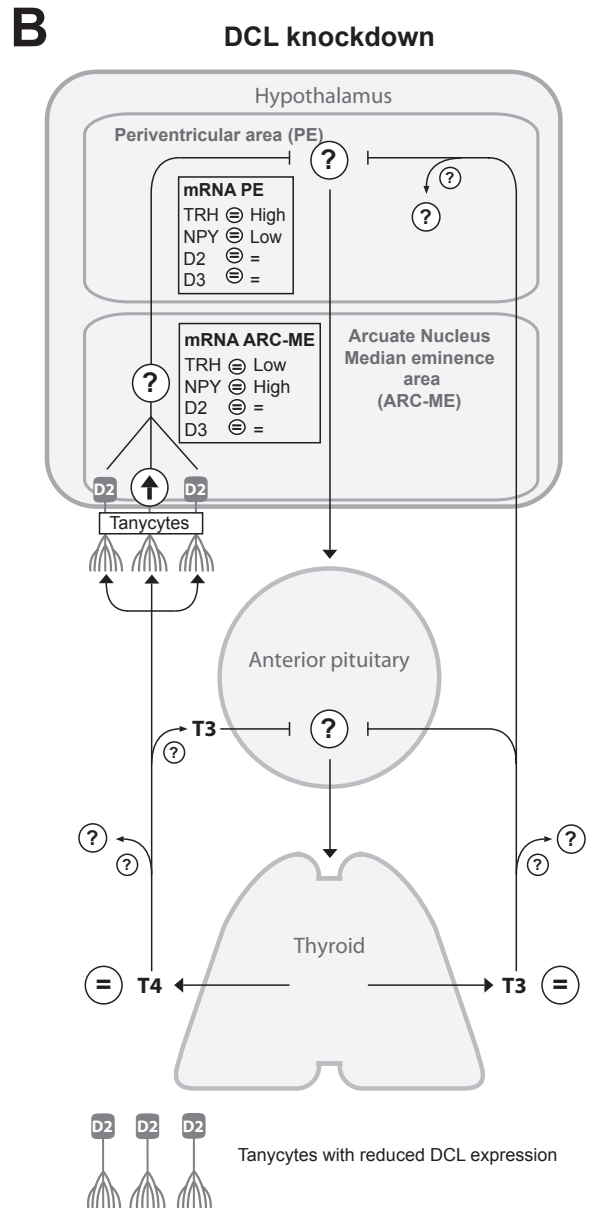


Figure 3: Schematic overview of HPT-axis and the effect of DCL knockdown. **A:** Normal condition in which Thyrotropin-releasing hormone (TRH) is released from the periventricular area (PE) of the hypothalamus. TRH stimulates the anterior pituitary to release Thyroid-stimulating hormone (TSH). TSH stimulates the thyroid to release thyroid hormone in 2 variants; inactive T4 and active T3. Deiodinase enzymes D2 and D3 convert respectively T3 into inactive T2 and T4 into active T3. In the Arcuate Nucleus and Median eminence area (ARC-ME) reside DCL positive tanycytes which also contain high levels of D2.



B: After DCL knockdown, D2 activity whithin the ARC-ME region is increased, but has no effect on peripheral levels T3 and T4. However, from many parameters it is still unknown what happens after DCL knockdown. Brain areas and organs involved in the HPT-axis are written in grey.

between cerebrospinal fluid (CSF), brain and peripheral bloodstream which may be considered as a peri- or circumventricular organ (CVO). Beside the blood-brain barrier (BBB), CVO's form an alternative route between the peripheral blood stream and the CSF (Rodriguez et al., 2005;Rodriguez et al., 2010).

Tanycytes play an important role as gatekeeper (Rodriguez et al., 2005) and are equipped with transport machinery to transfer substances from blood to CSF and vice versa (Rodriguez et al., 2010). Tanycytes are also the major source of deiodinase type 2 (D2) expression in the adult brain (Guadano-Ferraz et al., 1997;Kalsbeek et al., 2005;Tu et al., 1997). The enzyme D2 converts inactive thyroid hormone T4 into active T3. Within the hypothalamus, thyroid hormone signalling is involved in the regulation of energy balance and lipid metabolism (Bernal, 2002;Murphy and Ebling, 2011). In chapter 5, we studied the effect DCL knockdown on thyroid hormone signalling. Thyroid hormone levels in the peripheral bloodstream were not affected by DCL knockdown. Similarly, mRNA expression levels of several HPT-axis related genes within the hypothalamus are not changed. Interestingly, D2 activity within the hypothalamus was significantly increased. Up to now, we cannot state whether DCL knockdown affects D2 activity directly or indirectly. A possible mechanism is DCL-mediated post-translational modification of D2 proteins, which is independent from transcriptional regulation of the D2 gene. One post-translational modification mechanism that regulate D2 activity is ubiquitination via an ATP-dependent process (Gereben et al., 2000;Steinsapir et al., 2000). As DCL knockdown leads to reduction of ATP production (Verissimo et al., 2013), increased D2 activity might be the result of a blockade of its ubiquitination-mediated degradation in DCL knockdown mice.

Another possibility is the involvement of DCL in hypothalamic neurogenesis. Tanycytes are thought to represent a population of stem cells which can give birth to new neurons (Haan et al., 2013;Kokoeva et al., 2005;Kokoeva et al., 2007;Lee et al., 2012;Perez-Martin et al., 2010;Xu et al., 2005; for reviews see Bolborea and Dale, 2013;Cheng, 2013;Lee and Blackshaw, 2012). Like the SVZ and SGZ, the wall of the third ventricle in the hypothalamus might form a third neurogenic niche although the rate of neurogenesis is low and is context dependent (food availability). Based on morphological characteristics, the third ventricle can be divided into three zones (I, II & III) by (Perez-Martin et al., 2010). The ventral part of the wall is mainly inhabited by multiciliated cubic ependyma cells together with subependymal astrocytes. These cells also form the upper part of zone II in which also tanycytes reside. Zone III consists entirely of hypothalamic tanycytes. Only subependymal astrocytes and tanycytes are thought to be part of the neurogenic niche (Perez-Martin et al., 2010). Since DCL is mainly expressed in zone III near the median eminence, is limited expressed in zone II and is absent in zone I, its expression might be restricted to tanycytes (chapter 2). Moreover, blockade of neurogenesis by X-ray irradiation in the hypothalamus results in a

reduction of weight gain and in increase of energy expenditure and total activity (Lee et al., 2012). In chapter 5, we also report a reduction in weight gain which might be due to DCL knockdown-induced aberrant hypothalamic neurogenesis. Therefore it might be interesting to address the relationship between DCL and neurogenesis in the hypothalamus in future studies. Whether DCL-KD animals show more activity remains also to be determined though DCL-KD animals show increased proliferation which is also induced in animals exposed to exercise (van Praag et al., 1999a;van Praag et al., 1999b).

In conclusion, DCL knockdown in hypothalamic tanycytes results in increased activity of hypothalamic D2 without affecting peripheral thyroid hormone levels and hypothalamic mRNA expression of several HPT-axis related genes (Fig. 3). A possible mechanism is DCL mediated post-translational modification of D2 proteins, which might regulate D2 activity by ubiquitination via an ATP-dependent process. Furthermore, it may be interesting to study the possible relationship between DCL and hypothalamic neurogenesis in future studies.

7 The use of a conditional siRNA expressing mouse model to study mental disorders.

In this paragraph I will discuss the sh-RNA DCL mouse as a model for mental disorders. Furthermore, what are the advantages and disadvantages of the use of siRNA technology?

7.1 siRNA technology in disease models

There are several genetic modified mouse models with inducible techniques affecting adult neurogenesis; for an overview see (Dhaliwal and Lagace, 2011;Imayoshi et al., 2011). The majority of these models used inducible site-specific recombinases like Cre which can recognize LoxP sites and cuts out the DNA of interest. In the case of adult neurogenesis targeting models, Cre recombinases are often under control of neuronal stem cell specific promoters, like nestin and GFAP. It is deleterious for adult neurogenesis to flox several stem cell marker genes like Cdk5 (Lagace et al., 2008), Notch1 (Ables et al., 2010), NeuroD1 (Gao et al., 2009) and Sox2 (Favaro et al., 2009). In all studies, the gene of interest is cut out the DNA at a specific time point after brain development. In this respect, these techniques also circumvent knockout possible compensation effects during embryogenesis by homologous genes, e.g. DCX and DCL. Compared to these studies, our DCL-KD animals show comparable effects of DCL knockdown on adult neurogenesis. The population of immature neurons is decreased as well as the number of BrdU/NeuN positive cells. Remarkably, proliferation is increased in DCL-KD animals, though this finding is likely due to DCL knockdown and not the use of siRNA technology.

The strength of the conditional siRNA technique is the specificity: individual splice-variants are targeted by the shRNA, leaving the expression of other splice-variants unaltered. In our model, shRNA targets very precisely DCL without affecting DCLK-long and DCLK-short. The conditional aspect of shRNA expression is of great importance in particularly our DCLK1 study. Embryonic neocortical development DCX (Corbo et al., 2002) and DCLK1 knockout mice is normal, strongly suggesting compensational effects of both DCX and DCLK1 in these transgenic animals (Deuel et al., 2006;Pramparo et al., 2010;Tanaka et al., 2006;Tuy et al., 2008). Conditional knockdown of DCL in adult life circumvents the inactivation of DCLK1 function by DCX compensation. Although some leakage is found in adult animals, studies on embryo's and early postnatal pups in chapter 3 revealed no significant effect of leakage on DCL protein expression suggesting normal development of non-induced DCL-KD animals (data not shown).

siRNA-mediated DCL knockdown is systemic, i.e. after induction by doxycycline, DCL-shRNA is expressed in every cell of the animal. Beside the neurogenic hippocampus and forebrain (SVZ, RMS & OB) DCL is expressed in several other brain areas like ICj, SCN and hypothalamic tanycytes (chapter 2). One technique to specifically hit adult neurogenesis in the dentate gyrus is the use of stereotactic injections of viral vectors with siRNA constructs. Two studies applied this technique on genes closely related to DCL, DCX (Merz and Lie, 2013) and GR (Fitzsimons et al., 2013). In the case of GR, NPC's were targeted using lentiviral vectors with GFP and siRNA targeting the GR. The viral vectors were applied locally into the dentate gyrus and GR knockdown resulted in hyper active neurogenesis with more elaborate dendritic arborisation and increased migration (Fitzsimons et al., 2013). The cellular phenotype resulted in a reduction of contextual fear memory. In contrast, a study (Merz and Lie, 2013) in which DCX was targeted in the dentate gyrus using retroviral vectors did not affect adult neurogenesis. Although DCX knockdown in NPCs appeared successful, new-born cells developed normally suggesting that DCX is dispensable for adult neurogenesis. Retro- or lentiviral vector mediated gene transfer seems a good technique to target neurons within the hippocampus, but stereotactical brain surgery might be associated with substantial mechanical damage and discomfort. Transgenic mice, engineered with shRNA expressing constructs in their germ line, like DCL-KD mice, do not suffer from these side effects but offer similar opportunities to target genes involved in adult neurogenesis.

7.2 Impaired neurogenesis as phenotype

The initial aim of this project was to explore the possibilities to develop a model for Major Depressive Disorder (MDD) since neurogenesis is inhibited in acute and chronically stressed animals. Also the effect of many antidepressant drugs and electroconvulsive therapy seemed to be dependent on adult neurogenesis (Duman, 2004). Furthermore, adult neurogenesis

was thought to underlie the hippocampal volume decrease in depressed patients (Sheline et al., 1996). Although there is a strong correlation between neurogenesis and MDD, there are several arguments to support the idea that MMD and hippocampal volume decrease are independent from adult neurogenesis (Czeh and Lucassen, 2007;Sapolsky, 2001;Sapolsky, 2004). Also, cognitive impairment, as observed in MDD patients, is not correctly modelled in mice with inhibited adult neurogenesis (Frankland, 2013). This raised the question to what extend adult neurogenesis is involved in hippocampus functioning.

The selection of our behavioural studies was based on the idea that adult neurogenesis plays an important role in hippocampal related cognition like spatial memory and contextual fear conditioning. However, despite the significant impairment of adult neurogenesis, DCL-KD mice did not show impaired spatial and contextual fear memory formation. Therefore, DCL-KD mice are likely not a suitable model to study cognitive and emotional features of MDD. Nevertheless, adult neurogenesis is significantly affected and therefore the shRNA DCL-KD model is suitable to study the role of adult neurogenesis in other subtle hippocampal functions like context discrimination and possibly pattern recognition (Clelland et al., 2009;Sahay et al., 2011b). These functions are thought to underlie diseases like anxiety disorders, panic disorders and post traumatic stress disorder (PTSD; Kheirbek et al., 2012).

8 Perspectives

The studies reported in this thesis provided answers about the role of DCL in hippocampal adult neurogenesis. DCL is expressed in the neurogenic niche in the olfactory forebrain and the hippocampus. DCL knockdown has a deleterious effect on the population of DCX positive immature neurons and the number of BrdU/NeuN double positive neurons. Furthermore, DCL is also expressed in areas of which neurogenic capacity is less well studied. The function within these brain areas remains to be determined.

These findings raise several novel questions and create possibilities for further research. In the first place, the fate of neurons in a DCL knockdown environment is not precisely determined. Stereotactic injections with viral vectors containing green fluorescent protein (GFP) can label and trace new-born neurons (van Hooijdonk et al., 2009). GFP labelling of hippocampal tissue in DCL-KD animals might shed light on the fate of immature neurons in DCL-KD animals. How many of new-born neurons integrate into the network? Can new-born neurons develop into mature neurons with healthy dendrites or is there stagnation in the developmental process?

Another point of interest is the increased proliferation after DCL knockdown. Such a profile is seen in animals with increased exercise or after removal of the adrenals. When adre-

nalectomy (ADX) is applied, the level of circulating glucocorticoids is reduced and proliferation is boosted (Gould et al., 1992;Cameron and Gould, 1994). Increased glucocorticoid signalling by injection of corticosterone or after exposure to a stressor reduces proliferation (Cameron et al., 1998;Wong and Herbert, 2004). How does DCL affect proliferation? In vitro, in PC12 cells DCL knockdown inhibits proliferation of the neuroblastoma cells and also in vivo, neuroblastoma tumor growth is slowed down (Verissimo et al., 2013). A similar effect was expected regarding proliferation within the hippocampus, but we found the opposite. Since proliferation is strongly suppressed by glucocorticoid signalling and DCL is involved in intracellular GR transport (Fitzsimons et al., 2008), DCL knockdown might promote proliferation indirectly via abolished glucocorticoid signalling. Hence, this hypothesis can be tested in an experiment correlating different concentrations of circulating glucocorticoid with its cell nuclear localization in the proliferated cells using the DCL knockdown mouse model.
In hypothalamic tanycytes, DCL knockdown affects D2 activity, but other parameters within the hypothalamic-pituitary-thyroid hormone axis are unaffected. This mild outcome raises the question whether DCL might be involved in other hypothalamic functions as well. The first function to be affected by DCL knockdown may be hypothalamic neurogenesis. Since reduced neurogenesis in the hypothalamus was reported to affect bodyweight, energy expenditure and total activity (Lee et al., 2012), measuring daily activity of DCL-KD animals might provide clues about hypothalamic neurogenesis. Increased daily activity might explain the increase in proliferation. However, intracerebroventricular (icv) infusion of BrdU into the third ventricle is necessary to study hypothalamic neurogenesis properly.

Another interesting hypothalamic site of DCL expression is the suprachiasmatic nucleus (SCN). Within the SCN, DCL is mainly expressed in the area in which also arginine-vasopressin (AVP) is expressed. The SCN is the only source from which AVP is rhythmically released into the extracellular space (Schwartz and Reppert, 1985). In voles, the loss of rhythmic AVP protein expression is correlated to behavioural arrhythmicity in constant darkness. When voles are subject to constant darkness, their behavioural activity is organized by their internal clock. Some of the voles loose this rhythm and become arrhythmic. In these arrhythmic animals, AVP is still expressed, but no longer released resulting in accumulation of AVP protein in SCN neurons (Jansen et al., 2000;Jansen et al., 2007). Preliminary data suggest a similar pattern in DCL-KD animals. After DCL knockdown, the number of AVP positive cells seems to be increased. Since DCL is involved in intracellular transport along microtubules, DCL knockdown might affect AVP transport and hamper subsequent release. Whether this has an effect on normal daily rhythms remains to be determined although it is more likely that DCL knockdown affects adaptation towards transitions in novel environmental cues or “Zeitgebers” that mimic seasonal changes in day length. Also, the hypothalamic tanycytes are involved in seasonal changes (Bratincsak et al., 2007;Hazlerigg and Loudon, 2008) which makes our DCL-KD animals an interesting model to study hypothalamic function in daily

and seasonal regulation of behaviour. However, several parameters need to be studied in DCL-KD animals first, like the circadian rhythm of AVP protein expression, constant registration of daily activity and adaptation towards novel light/dark regimen.

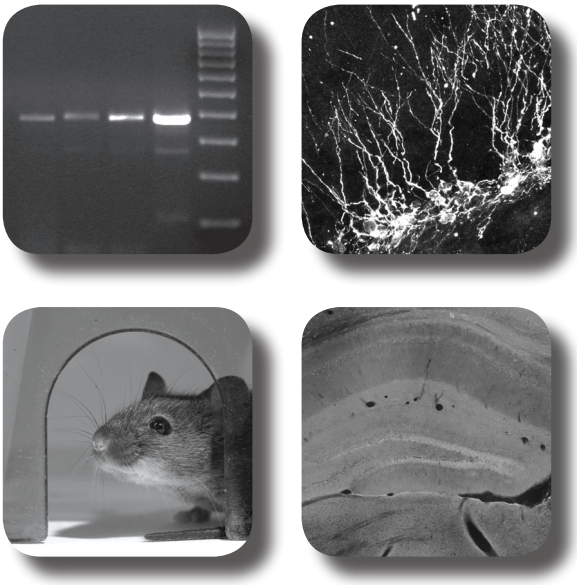
Finally, the DCL-KD model is suitable to study ageing and cognitive impairment. There is a steep decline in the number of new-born neurons during the lifespan, which coincides with a decline in cognitive, sensory and motor functions (Andrews-Hanna et al., 2007;Hof and Morrison, 2004). Although there are still outstanding questions about the role of adult neurogenesis in olfaction and contextual recognition (Lazarini and Lledo, 2010), the olfactory tubercle is subject to changes over time. The DCL expressing Islands of Calleja (ICj) are part of the olfactory tubercle. During lifetime, the number of granule cells inhabiting the ICj decreases and the volume and location of the ICj change (Adjei et al., 2013). The function of DCL in the neuropil and neurogenesis in both OB and ICj remains to be determined. One of the intriguing questions is: does DCL knockdown affect ICj plasticity over time and thereby olfactory function? Altogether, DCL-KD mice may be used for a number of studies related to brain plasticity in the field of cognition, stress, energy metabolism and olfaction.

9 General conclusions

The results in this thesis showed for the first time DCL-specific expression in the adult mouse brain. Besides the expected regions with the capacity to generate new neurons (hippocampus and olfactory forebrain), DCL expression was found in three novel brain areas namely hypothalamic tanycytes, suprachiasmatic nucleus (SCN) and Islands of Calleja (ICj). A state of the art conditional shRNA expressing mouse model was used to target DCL mRNA. The analysis of these DCL knockdown animals using qPCR and Western blot revealed strong reduction of DCL protein expression. Subsequent stereological analysis using BrdU and several stem cell and neuronal markers revealed increased progenitor proliferation, but impaired neurogenesis in the hippocampus. This impaired neurogenesis was associated, however, with an apparent normal spatial and contextual fear memory formation in circular hole board and in a contextual fear conditioning paradigm. Therefore, DCL-regulated adult neurogenesis seems not crucial for hippocampus-dependent learning. However, more subtle functions like pattern separation and context distinction might be regulated by DCL. DCL knockdown also increased D2 activity within the hypothalamus. Further studies are needed to reveal the role of DCL in hypothalamus function. Altogether, the DCL-KD mouse seems a good working model to study adult neurogenesis and the role of DCL in this process. Furthermore, this model offers novel opportunities to study several hypothalamic processes like energy metabolism and the circadian rhythm.

Chapter 7

Summary/Samenvatting



Summary

Adult neurogenesis is a process in which neuronal stem cells in the adult brain give birth to new neurons. Two locations within the brain do show adult neurogenesis; the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles. Within the DG neuronal progenitor cells (NPC's) develop into mature neurons within the same DG. They migrate over a short distance within the granule cell layer. NPC's born in the SVZ migrate over a longer distance along the rostral migratory stream (RMS) towards the olfactory bulb (OB) were they develop into mature neurons. Doublecortin (DCX) and the highly homologous Doublecortin-like (DCL) play crucial roles during embryonic development. However, for DCL it is unknown whether it is also involved in adult neurogenesis. To study the role of DCL in the adult brain and specifically in adult neurogenesis we developed a DCL specific antibody and an inducible siRNA expressing mouse targeting DCL.

In **chapter 2** we described a DCL specific antibody and mapped the expression of DCL in the adult brain. DCL is encoded by the complex DCLK1 gene generating several splice variants like DCLK-long, DCLK-short and DCL. Since conventional DCLK1 antibodies do not distinguish between all splice variants, a novel, DCL specific antibody was designed. As expected, we identified DCL in neurogenic areas like the SVZ and SGZ of the dentate gyrus where it co localized with DCX. These findings indicate that DCL plays an important role in adult neurogenesis. Interestingly, DCL protein was also found in several non-neurogenic areas like the Islands of Calleja (ICj), Suprachiasmatic nucleus (SCN) and hypothalamic tanycytes. These novel findings raised questions about the function of DCL in these areas.

In **chapter 3**, the DCL-KD mouse model was validated by showing specific DCL knockdown after doxycycline administration. Doxycycline in food pellets resulted in a strong up-regulation of shRNA targeting DCL. Western blot analysis showed a strong reduction in DCL protein expression whereas DCLK-long and DCLK-short were not affected. Furthermore, the effect of DCL knockdown was studied on adult hippocampal neurogenesis using stereological techniques. Bromodeoxyuridine (BrdU) labelling studies showed an increase in proliferation and a reduction in cell survival. Also the number of DCX positive immature neurons was strongly reduced. In addition, we studied the effect of DCL knockdown on hippocampus-dependent behaviour. In a circular hole board paradigm the spatial memory performance of normal and DCL-KD mice was tested. Impaired neurogenesis does not affect spatial memory formation, DCL-KD animals performed equally compared to non-induced littermates. Interestingly, although DCL-KD mice reached the exit hole in the same time as their non-induced littermates, they showed a much longer escape latency.

In **chapter 4**, the findings described in chapter 3 were confirmed. The effect of DCL knockdown on hippocampus-dependent learning was measured using a contextual fear conditioning paradigm. During 6 training sessions, mice were put in a conditioning chamber

were they received a mild electric shock during the presence of a light and tone cue. In 6 memory retrieval sessions, freezing behaviour was recorded in the same conditioning box with the presence of the cue but without the shock. Hippocampus-dependent learning was not affected after DCL knockdown since DCL-KD animals (compared to wildtype littermates) showed equal amounts of freezing behaviour during the periods in the context. Also during the presence of the cue, freezing behaviour of DCL-KD animals was similar as the behaviour of their littermate controls. However, subtle differences were found. During memory retrieval, the first out of six presented cue's induced s significant stronger freezing response in DCL-KD animals. Also the amount of tail rattling behaviour was strongly reduced after DCL knockdown.

A rather unexpected finding was the presence of DCL in hypothalamic tanycytes as reported in chapter 2. Hypothalamic tanycytes are part of the Hypothalamic-Pituitary-Thyroid axis (HPT-axis), which is involved in energy metabolism. Therefore, we address the question whether or not DCL is involved in thyroid hormone signalling in **chapter 5**. We measured bodyweight, serum T3 and T4 concentrations and D2 activity in hypothalamic tissue of DCL-knockdown (KD) mice and their littermate controls. Furthermore, we measured mRNA expression of TRH, NPY, D2 and D3 in hypothalamic punches containing the ARC-ME or the PVN. We observed a strong reduction in DCL expression in hypothalamic tanycytes, which was associated with reduced body weight growth and a significant increase in D2 activity, the enzyme metabolizing inactive T4 into active T3. However, serum levels of T4 and T3 did not differ between wildtype and DCL-KD animals and also the expression of TRH, NPY, D2 and D3 mRNA in the hypothalamus was not affected by DCL knockdown. Together, our data indicate a role for DCL in the regulation of D2 activity in hypothalamic tanycytes and a possible subtle role in thyroid signalling.

In **chapter 6**, we discussed our DCL findings in the adult mouse brain. First of all, for the first time, we show immunohistochemically the presence of DCL in the adult brain. As expected, DCL is expressed in the neurogenic regions of the hippocampus and forebrain. The presence of DCL in other brain areas raises the question to what extend these brain areas have a neurogenic or neuroplastic nature. The generation of a doxycycline-inducible DCL-KD mouse model was validated and DCL knockdown resulted in impaired adult hippocampal neurogenesis. However, hippocampus-dependent learning is not affected by DCL knockdown. DCL may play a role in hypothalamus-regulated energy metabolism since D2 activity is increased after DCL knockdown. In conclusion, The DCL-KD mouse model seems a suitable model to study DCL in the adult brain also by circumventing possible developmental compensation by DCX. This model may be instrumental to elucidate the role of DCL in neurogenesis and neuronal plasticity in brain areas like hippocampus, hypothalamus and forebrain.

Samenvatting

Neurogenese in het volwassen brein is een proces waarbij neuronale stam cellen zich ontwikkelen naar nieuwe neuronen. In twee hersengebieden van het volwassen brein worden nieuwe neuronen geboren; de hippocampale dentate gyrus (DG) en de subventriculaire zone (SVZ) van de laterale ventrikels. In de DG groeien neuronale voorloper cellen (NVC's) uit tot volwassen neuronen die hun functie vervullen in de DG. NVC's die in de SVZ geboren worden migreren over een langere afstand naar hun doel gebied. Via de rostrale migratie stroom (RMS) bewegen deze cellen zich naar de bulbus olfactorius waar zij zich ontwikkelen tot volwassen neuronen. De eiwitten Doublecortin (DCX) en het sterk homologe Doublecortin-like (DCL) spelen een belangrijke rol tijdens de embryonale ontwikkeling van het brein. In het volwassen brein komt ook DCX tot expressie in met name de gebieden met neurogenese. Of DCL ook tot expressie komt in het volwassen brein is onbekend. Daarom hebben we met een nieuw DCL-antibody onderzocht of DCL tot expressie komt in het volwassen brein. Ook hebben we gekeken naar de rol die DCL speelt in het volwassen brein middels een transgene muis welke een induceerbare siRNA construct tot expressie brengt dat DCL eiwit niveaus sterk reduceert.

In **hoofdstuk 2** beschrijven we DCL expressie in het volwassen brein. DCL is een onderdeel van het complexe DCLK1 gen dat onder andere ook de eiwitten DCLK-long en DCLK-short tot expressie brengt. Omdat de huidige antibodies tegen DCLK1 geen onderscheid maken tussen de verschillende DCLK1 eiwit varianten hebben we een nieuw DCL specifiek antibody ontwikkeld. Zoals verwacht komt DCL voor in de populatie cellen in de hippocampus en SVZ, RMS en bulbus olfactorius, die ook bij neurogenese betrokken zijn. Deze resultaten laten zien dat DCL nauw betrokken is bij het neurogenese proces in het volwassen brein. Ook hebben we DCL aangetroffen in hersengebieden waar we dat niet hadden verwacht. Zo komt DCL tot expressie in de eilanden van Calleja (ICj), suprachiasmatische nucleus (SCN) en de tanycyten in de hypothalamus. Omdat deze hersengebieden niet bekend staan als gebieden met neurogenese roepen onze resultaten de vraag op welke rol DCL wel speelt in deze gebieden.

In **hoofdstuk 3** hebben we de DCL knockdown (DCL-KD) muis onderzocht en we laten zien dat DCL eiwit expressie sterk is gereduceerd na toediening van doxycycline. Doxycycline werd via het voer aan de dieren gegeven en resulteerde in een hoge expressie van short hairpin RNA (shRNA) tegen DCL. De daarop volgende Western blot analyse liet een sterke reductie zien van DCL eiwit expressie terwijl de eiwit varianten DCLK-long en DCLK-short niet waren aangetast. Vervolgens hebben we het effect van DCL knockdown op cel morfologie bekeken door gebruik te maken van stereologische technieken. Bromodeoxyuridine (BrdU) labelling liet een toename zien in cel proliferatie in de hippocampus terwijl er minder BrdU gelabelde cellen het volwassen stadium bereikten. DCX positieve cellen lieten een sterk afwijkend morfologisch fenotype zien waarbij er nauwelijks uitlopers werden gevormd

die richting het bestaande netwerk in de moleculaire laag van de hippocampus groeiden. Omdat DCL knockdown een sterk effect had op neurogenese in de hippocampus hebben we hippocampus afhankelijk leergedrag onderzocht bij de DCL-KD muizen. Middels een 'circular hole board' taak is het hippocampus afhankelijke ruimtelijk geheugen getest. Het ruimtelijk geheugen van de DCL-KD muizen verschilde niet van normale muizen uit hetzelfde nest. Wel gingen DCL-KD muizen significant veel later hun thuiskooi in.

In **hoofdstuk 4** hebben we de gedragsresultaten uit hoofdstuk 3 bevestigd middels een tweede hippocampus afhankelijke leertaak. Tijdens een 'contextual fear conditioning' test kregen de dieren tijdens 6 trainingsessies een stimulus aangeboden met daarbij een milde, elektrische schok. In de daar op volgende geheugen test werden de dieren opnieuw blootgesteld aan de stimulus, maar zonder schok. DCL-KD dieren vertoonden in dezelfde mate angstig gedrag als hun wildtype nestgenoten. Opvallend was echter de sterke angstresponse bij het zien en horen van de eerste stimulus in de serie van 6 geheugen testen. Bij die eerste stimulus vertoonden DCL-KD dieren significant meer angstgedrag. Daarnaast vertoonden de DCL-KD muizen veel minder 'tail rattling' gedrag.

Een onverwachte vondst betrof DCL expressie in hypothalamische tanycyten zoals beschreven in hoofdstuk 2. Deze tanycyten maken deel uit van de 'Hypothalamic-Pituitary-Thyroid-as' (HPT-axis) en zijn betrokken bij de regulatie van de energie huishuiding. Daarom hebben we de rol van DCL in schildklierhormoon regulatie onderzocht en beschreven in **hoofdstuk 5**. We hebben lichaamsgewicht, serum concentratie T3 en T4 en deiodinase 2 (D2) enzym activiteit gemeten in weefsel uit de hypothalamus van DCL-KD muizen en hun wildtype nestgenoten. Daarnaast hebben we mRNA expressie gemeten van TRH, NPY, D2 en D3 in puches van de hypothalamus met daarbij weefsel uit de ARC-ME en PVN kernen. DCL expressie was sterk gereduceerd in de tanycyten. Dit ging gepaard met een verminderde toename van het lichaamsgewicht en een significante toename van D2 activiteit. D2 is een enzym dat inactieve T4 omzet in actieve T3. Daarom hebben we ook gekeken naar de T3 en T4 concentraties in het bloed. Ondanks de verhoogde D2 activiteit hebben we geen verschillen gevonden in T3 of T4 serum concentraties. Ook is er geen verschil gevonden in mRNA expressie van TRH, NPY, D2 en D3 in hypothalamus weefsel van DCL-KD en wildtype muizen. Deze resultaten laten zien dat DCL een rol speelt in de regulatie van D2 activiteit en daarbij een mogelijke rol heeft in de schildklierhormoon huishuiding in de hypothalamus.

In **hoofdstuk 6** worden alle bevinding aangaande DCL in het volwassen muizenbrein in dit proefschrift bediscussiëerd. Ten eerste laten we met immunohistochemie zien dat DCL tot expressie komt in het volwassen brein. Zoals verwacht komt DCL tot expressie in de hippocampus, SVZ, RMS en bulbus olfactorius. Het feit dat DCL ook in andere hersengebieden tot expressie komt roept de vraag op in welke mate deze hersengebieden in staat zijn tot

plasticiteit en neurogenese. De validatie van het doxycycline induceerbare DCL-KD muismodel resulteerde in een reductie van DCL eiwit expressie en verstoorde neurogenese. Hippocampus gerelateerd leervermogen was echter niet aangetast na DCL knockdown. Naast neurogenese lijkt DCL een rol te spelen in D2 activiteit in de hypothalamus. Resumerend, het DCL-KD muismodel is een bruikbaar model om DCL te bestuderen in het volwassen brein zonder dat DCL reductie wordt gecompenseerd door DCX. Met dit model in de hand kan de rol van DCL in neurogenese en neuroplasticiteit verder worden onderzocht in de hippocamous, hypothalamus en frontale brein.

Chapter 8

Dankwoord
Curriculum vitae
Publicatie lijst

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Curriculum vitae

Dirk-Jan Saaltink werd geboren op 6 december 1980 te Heerenveen. In 1999 behaalde hij zijn HAVO diploma aan het Carmel College Salland te Raalte waarna hij in datzelfde jaar startte met de opleiding Laboratorium Techniek aan de Internationale Agrarische Hogeschool Larenstein te Velp.

Na het behalen van zijn propedeuse in 2000 is hij in Groningen Biologie gaan studeren aan de Rijksuniversiteit Groningen. Daar bestudeerde hij onder andere circadiaan gedrag van veldmuizen bij prof. dr. Menno Gerkema, agressieve huismuizen bij dr. Sietse de Boer en ritmische expressie van klokgenen bij de mens in het laboratorium van prof. dr. Urs Albrecht te Fribourg, Zwitserland. In 2006 behaalde hij zijn doctoraal Biologie met als specialisatie gedrags & neurowetenschappen.

In 2007 is hij begonnen als assistent in opleiding in het laboratorium van prof. dr. Ron de Kloet en dr. Erno Vreugdenhil. Daar hield hij zich bezig met het eiwit DCL en neurogenese in het volwassen brein van de muis.

Saaltink, Hubens, Ninaber & Vreugdenhil. Doublecortin-like is implicated in hippocampal neurogenesis but not in hippocampus-dependent memory formation. *Submitted*

Saaltink, Belegri, Boelen, Kalsbeek & Vreugdenhil. Doublecortin-like knockdown in hypothalamic tanycytes induce subtle effects on bodyweight and D2 activity. *In preparation*

Saaltink & Vreugdenhil 2014. Stress, glucocorticoid receptors and adult neurogenesis: a balance between excitation and inhibition? *Cellular & Molecular Life Sciences*.

Verissimo, Elands, Cheng, **Saaltink**, Ter Horst, Alme, Pont, Van de Water, Håvik, Fitzsimons & Vreugdenhil 2013. Silencing doublecortin-like results in decreased mitochondrial activity and delayed neuroblastoma tumor growth. *Plos One*. 8(9):e75752.

Fitzsimons, van Hooijdonk, Brinks, Schouten, **Saaltink**, Dijkmans, Verbeek, de Kloet, Karst, Joels, Oitzl and Vreugdenhil 2013. Knockdown of the Glucocorticoid Receptor Accelerates Functional Integration of Newborn Neurons in the Adult Hippocampus. *Molecular Psychiatry*, 18(9):993-1005.

Saaltink, Havik, Verissimo, Lucassen and Vreugdenhil 2012. Doublecortin and Doublecortin-like are expressed in overlapping and non-overlapping neuronal cell population: implications for neurogenesis. *Journal of Comparative Neurology*, 520(13):2805-23.

Boelen, Beeren, Vos, Surovtseva, **Saaltink**, Vreugdenhil, Kalsbeek, Kwakkel, and Fliers 2012. Leptin administration restores the fasting induced increase of hepatic type 3 deiodinase expression in mice. *Thyroid*, 22(2):192-9.

Van der Veen, **Saaltink** & Gerkema 2011. Behavioral responses to combinations of timed light, food availability, and ultradian rhythms in the common vole (Microtus arvalis).
Chronbiology International 28(7): 563-71.

Jud, Chappuis, Revell, Sletten, **Saaltink**, Cajochen, Skene & Albrecht 2009. Age-dependent alterations in human PER2 levels after early morning blue light exposure.
Chronobiology International 26(7): 1462-1469.

Natarajan, de Vries, **Saaltink**, de Boer & Koolhaas 2008. Delineation of violence from functional aggression in mice: an ethological approach. *Behavior Genetics* 39(1): 73-90.

Van de Pol, Bakker, **Saaltink** & Verhulst 2006. Rearing conditions determine offspring survival independent of egg quality: a cross-foster experiment with Oystercatchers Haematopus ostralegus. *Ibis* 148: 203-210.

Chapter 9

References

References

Ables JL, Decarolis NA, Johnson MA, Rivera PD, Gao Z, Cooper DC, Radtke F, Hsieh J, Eisch AJ (2010) Notch1 is required for maintenance of the reservoir of adult hippocampal stem cells. J Neurosci 30:10484-10492.

Abrous DN, Koehl M, Le Moal M (2005) Adult neurogenesis: From precursors to network and physiology. Physiological Reviews 85:523-569.

Abrous DN, Wojtowicz JM (2008) Neurogenesis and hippocampal memory system. In: Adult Neurogenesis (Gager FH, Kempermann G, Song H, eds), pp 445-462. New York: Cold Spring Harbor Laboratory Press.

Acehan D, Vaz F, Houtkooper RH, James J, Moore V, Tokunaga C, Kulik W, Wansapura J, Toth MJ, Strauss A, Khuchua Z (2011) Cardiac and skeletal muscle defects in a mouse model of human Barth syndrome. J Biol Chem 286:899-908.

Adjei S, Houck AL, Ma K, Wesson DW (2013) Age-dependent alterations in the number, volume, and localization of islands of Calleja within the olfactory tubercle. Neurobiol Aging 34:2676-2682.

Aimone JB, Deng W, Gage FH (2010) Adult neurogenesis: integrating theories and separating functions. Trends in Cognitive Sciences 14:325-337.

Aimone JB, Deng W, Gage FH (2011) Resolving New Memories: A Critical Look at the Dentate Gyrus, Adult Neurogenesis, and Pattern Separation. Neuron 70:589-596.

Aimone JB, Wiles J, Gage FH (2006) Potential role for adult neurogenesis in the encoding of time in new memories. Nature Neuroscience 9:723-727.

Airan RD, Meltzer LA, Roy M, Gong YQ, Chen H, Deisseroth K (2007) High-speed Imaging reveals neurophysiological links to behavior in an animal model of depression. Science 317:819-823.

Altman J (1963) Autoradiographic investigation of cell proliferation in the brains of rats and cats. Anat Rec 145:573-591.

Altman J (1969) Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J Comp Neurol 137:433-457.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.

Alvarez-Buylla A, Lim DA (2004) For the long run: maintaining germinal niches in the adult brain. Neuron 41:683-686.

Alvarez-Buylla A, Ling CY, Yu WS (1994) Contribution of neurons born during embryonic, juvenile, and adult life to the brain of adult canaries: regional specificity and delayed birth of neurons in the song-control nuclei. J Comp Neurol 347:233-248.

Amaral DG, Scharfman HE, Lavenex P (2007) The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). Prog Brain Res 163:3-22.

Amrein I, Slomianka L, Lipp HP (2004a) Granule cell number, cell death and cell proliferation in the dentate gyrus of wild-living rodents. European Journal of Neuroscience 20:3342-3350.

Amrein I, Slomianka L, Poletaeva II, Bologova NV, Lipp HP (2004b) Marked species and age-dependent differences in cell proliferation and neurogenesis in the hippocampus of wild-living rodents. Hippocampus 14:1000-1010.

Andrews-Hanna JR, Snyder AZ, Vincent JL, Lustig C, Head D, Raichle ME, Buckner RL (2007) Disruption of large-scale brain systems in advanced aging. Neuron 56:924-935.

Anthony TE, Klein C, Fishell G, Heintz N (2004) Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 41:881-890.

Arruda-Carvalho M, Sakaguchi M, Akers KG, Josselyn SA, Frankland PW (2011) Posttraining ablation of adult-generated neurons degrades previously acquired memories. J Neurosci 31:15113-15127.

Avgustinovich DF, Lipina TV, Bondar NP, Alekseyenko OV, Kudryavtseva NN (2000) Features of the genetically defined anxiety in mice. Behav Genet 30:101-109.

Ayala R, Shu TZ, Tsai LH (2007) Trekking across the brain: The journey of neuronal migration. Cell 128:29-43.

Bai JL, Ramos RL, Ackman JB, Thomas AM, Lee RV, LoTurco JJ (2003) RNAi reveals doublecortin is required for radial migration in rat neocortex. Nature Neuroscience 6:1277-1283.

Bain MJ, Dwyer SM, Rusak B (2004) Restraint stress affects hippocampal cell proliferation differently in rats and mice. Neuroscience Letters 368:7-10.
Baroncini M, Allet C, Leroy D, Beauvillain JC, Francke JP, Prevot V (2007) Morphological evidence for direct interaction between gonadotrophin-releasing hormone neurones and astroglial cells in the human hypothalamus. J Neuroendocrinol 19:691-702.

Bassett JH, Harvey CB, Williams GR (2003) Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. Mol Cell Endocrinol 213:1-11.

Bauer S, Hay M, Amilhon B, Jean A, Moyse E (2005) In vivo neurogenesis in the dorsal vagal complex of the adult rat brainstem. Neuroscience 130:75-90.

Becquet D, Girardet C, Guillaumond F, Francois-Bellan AM, Bosler O (2008) Ultrastructural plasticity in the rat Suprachiasmatic nucleus. Possible involvement in clock entrainment. Glia 56:294-305.

Bedard A, Gravel C, Parent A (2006) Chemical characterization of newly generated neurons in the striatum of adult primates. Exp Brain Res 170:501-512.

Bedard A, Levesque M, Bernier PJ, Parent A (2002) The rostral migratory stream in adult squirrel monkeys: contribution of new neurons to the olfactory tubercle and involvement of the antiapoptotic protein Bcl-2. Eur J Neurosci 16:1917-1924.

Belnoue L, Grosjean N, Ladeveze E, Abrous DN, Koehl M (2013) Prenatal stress inhibits hippocampal neurogenesis but spares olfactory bulb neurogenesis. PLoS One 8:e72972.

Belvindrah R, Nissant A, Lledo PM (2011) Abnormal Neuronal Migration Changes the Fate of Developing Neurons in the Postnatal Olfactory Bulb. Journal of Neuroscience 31:7551-7562.

Berke JD, Paletzki RF, Aronson GJ, Hyman SE, Gerfen CR (1998) A complex program of striatal gene expression induced by dopaminergic stimulation. J Neurosci 18:5301-5310.

Bernal J (2002) Action of thyroid hormone in brain. J Endocrinol Invest 25:268-288.

Bernier PJ, Bedard A, Vinet J, Levesque M, Parent A (2002) Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc Natl Acad Sci U S A 99:11464-11469.

References

Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA, Almeida OF, Sousa N (2008) The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. Mol Psychiatry.

Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA, Almeida OFX, Sousa N (2009) The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. Molecular Psychiatry 14:764-773.

Biancardi VC, Campos RR, Stern JE (2010) Altered Balance of gamma-Aminobutyric Acidergic and Glutamatergic Afferent Inputs in Rostral Ventrolateral Medulla-Projecting Neurons in the Paraventricular Nucleus of the Hypothalamus of Renovascular Hypertensive Rats. Journal of Comparative Neurology 518:567-585.

Biebl M, Cooper CM, Winkler J, Kuhn HG (2000) Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. Neurosci Lett 291:17-20.

Bloch J, Kaeser M, Sadeghi Y, Rouiller EM, Redmond DE, Jr., Brunet JF (2011) Doublecortin-positive cells in the adult primate cerebral cortex and possible role in brain plasticity and development. J Comp Neurol 519:775-789.

Boekhoorn K, Sarabdjitsingh A, Kommerie H, de PK, 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 507:1639-1652.

Bolborea M, Dale N (2013) Hypothalamic tanycytes: potential roles in the control of feeding and energy balance. Trends Neurosci 36:91-100.

Bolborea M, Laran-Chich MP, Rasri K, Hildebrandt H, Govitrapong P, Simonneaux V, Pevet P, Steinlechner S, Klosen P (2011) Melatonin controls photoperiodic changes in tanycyte vimentin and neural cell adhesion molecule expression in the Djungarian hamster (Phodopus sungorus). Endocrinology 152:3871-3883.

Boldrini M, Underwood MD, Hen R, Rosoklija GB, Dwork AJ, Mann JJ, Arango V (2009) Antidepressants increase neural progenitor cells in the human hippocampus. Neuropsychopharmacology 34:2376-2389.

Bonfanti L (2006) PSA-NCAM in mammalian structural plasticity and neurogenesis. Progress in Neurobiology 80:129-164.

Bonfanti L, Olive S, Poulain DA, Theodosis DT (1992) Mapping of the Distribution of Polysialylated Neural Cell-Adhesion Molecule Throughout the Central-Nervous-System of the Adult-Rat - An Immunohistochemical Study. Neuroscience 49:419-436.

Bonfanti L, Peretto P (2011) Adult neurogenesis in mammals--a theme with many variations. Eur J Neurosci 34:930-950.

Branda CS, Dymecki SM (2004) Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. Developmental Cell 6:7-28.

Bratincsak A, McMullen D, Miyake S, Toth ZE, Hallenbeck JM, Palkovits M (2007) Spatial and temporal activation of brain regions in hibernation: c-fos expression during the hibernation bout in thirteen-lined ground squirrel. J Comp Neurol 505:443-458.

Bremner JD, Narayan M, Anderson ER, Staib LH, Miller HL, Charney DS (2000) Hippocampal volume reduction in major depression. American Journal of Psychiatry 157:115-117.

Brinks V, Berger S, Gass P, de Kloet ER, Oitzl MS (2009) Mineralocorticoid receptors in control of emotional arousal and fear memory. Horm Behav 56:232-238.

Brown J, Cooper-Kuhn CM, Kempermann G, van Praag H, Winkler J, Gage FH, Kuhn HG (2003a) Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. European Journal of Neuroscience 17:2042-2046.

Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG (2003b) Transient expression of doublecortin during adult neurogenesis. Journal of Comparative Neurology 467:1-10.

Burgess HA, Martinez S, Reiner O (1999) KIAA0369, doublecortin-like kinase, is expressed during brain development. Journal of Neuroscience Research 58:567-575.

Burgess HA, Reiner O (2001) Cleavage of doublecortin-like kinase by calpain releases an active kinase fragment from a microtubule anchorage domain. J Biol Chem 276:36397-36403.

Burgess HA, Reiner O (2000) Doublecortin-like kinase is associated with microtubules in neuronal growth cones. Molecular and Cellular Neuroscience 16:529-541.

Burgess HA, Reiner O (2002) Alternative splice variants of doublecortin-like kinase are differentially expressed and have different kinase activities. Journal of Biological Chemistry 277:17696-17705.
Cai Y, Xiong K, Chu Y, Luo DW, Luo XG, Yuan XY, Struble RG, Clough RW, Spencer DD, Williamson A, Kordower JH, Patrylo PR, Yan XX (2009) Doublecortin expression in adult cat and primate cerebral cortex relates to immature neurons that develop into GABAergic subgroups. Exp Neurol 216:342-356.

Cameron HA, Gould E (1994) Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. Neuroscience 61:203-209.

Cameron HA, Tanapat P, Gould E (1998) Adrenal steroids and N-methyl-D-aspartate receptor activation regulate neurogenesis in the dentate gyrus of adult rats through a common pathway. Neuroscience 82:349-354.

Chauvet N, Prieto M, Alonso G (1998) Tanycytes present in the adult rat mediobasal hypothalamus support the regeneration of monoaminergic axons. Exp Neurol 151:1-13.

Cheng MF (2013) Hypothalamic neurogenesis in the adult brain. Front Neuroendocrinol 34:167-178.

Cierpicki T, Kim MH, Cooper DR, Derewenda U, Bushweller JH, Derewenda ZS (2006) The DC-module of doublecortin: dynamics, domain boundaries, and functional implications. Proteins 64:874-882.

Cifuentes M, Perez-Martin M, Grondona JM, Lopez-Avalos MD, Inagaki N, Granados-Duran P, Rivera P, Fernandez-Llebrez P (2011) A comparative analysis of intraperitoneal versus intracerebroventricular administration of bromodeoxyuridine for the study of cell proliferation in the adult rat brain. J Neurosci Methods 201:307-314.

Clark PJ, Brzezinska WJ, Thomas MW, Ryzhenko NA, Toshkov SA, Rhodes JS (2008) Intact neurogenesis is required for benefits of exercise on spatial memory but not motor performance or contextual fear conditioning in C57BL/6J mice. Neuroscience 155:1048-1058.

Clelland CD, Choi M, Romberg C, Clemenson GD, Jr., Fragniere A, Tyers P, Jessberger S, Saksida LM, Barker RA, Gage FH, Bussey TJ (2009) A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science 325:210-213.

Coppola A, Liu ZW, Andrews ZB, Paradis E, Roy MC, Friedman JM, Ricquier D, Richard D, Horvath TL, Gao XB, Diano S (2007) A central thermogenic-like mechanism in feeding regulation: an interplay between arcuate nucleus T3 and UCP2. Cell Metab 5:21-33.

Coquelle FM, Levy T, Bergmann S, Wolf SG, Bar-El D, Sapir T, Brody Y, Orr I, Barkai N, Eichele G, Reiner O (2006) Common and divergent roles for members of the mouse DCX superfamily. Cell Cycle 5:976-983.

References

Corbo JC, Deuel TA, Long JM, LaPorte P, Tsai E, Wynshaw-Boris A, Walsh CA (2002) Doublecortin is required in mice for lamination of the hippocampus but not the neocortex. Journal of Neuroscience 22:7548-7557.

Couillard-Despres S, Winner B, Karl C, Lindemann G, Schmid P, Aigner R, Laemke J, Bogdahn U, Winkler J, Bischofberger J, Aigner L (2006) Targeted transgene expression in neuronal precursors: watching young neurons in the old brain. European Journal of Neuroscience 24:1535-1545.

Couillard-Despres S, Winner B, Schaubeck S, Aigner R, Vroemen M, Weidner N, Bogdahn U, Winkler J, Kuhn HG, Aigner L (2005) Doublecortin expression levels in adult brain reflect neurogenesis. Eur J Neurosci 21:1-14.

Crantz FR, Silva JE, Larsen PR (1982) An analysis of the sources and quantity of 3,5,3'-triiodothyronine specifically bound to nuclear receptors in rat cerebral cortex and cerebellum. Endocrinology 110:367-375.

Curtis MA, Kam M, Faull RL (2011) Neurogenesis in humans. Eur J Neurosci 33:1170-1174.

Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C, Holtas S, van Roon-Mom WM, Bjork-Eriksson T, Nordborg C, Frisen J, Dragunow M, Faull RL, Eriksson PS (2007) Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science 315:1243-1249.

Curtis MA, Penney EB, Pearson AG, van Roon-Mom WM, Butterworth NJ, Dragunow M, Connor B, Faull RL (2003) Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain. Proc Natl Acad Sci U S A 100:9023-9027.

Czeh B, Lucassen PJ (2007) What causes the hippocampal volume decrease in depression? : Are neurogenesis, glial changes and apoptosis implicated? Eur Arch Psychiatry Clin Neurosci 257:250-260.

Czeh B, Michaelis T, Watanabe T, Frahm J, de Biurrun G, van Kampen M, Bartolomucci A, Fuchs E (2001) Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. Proceedings of the National Academy of Sciences of the United States of America 98:12796-12801.

Dalm S, Schwabe L, Schachinger H, Oitzl MS (2009) Post-training self administration of sugar facilitates cognitive performance of male C57BL/6J mice in two spatial learning tasks. Behav Brain Res 198:98-104.

David DJ, Samuels BA, Rainer Q, Wang JW, Marsteller D, Mendez I, Drew M, Craig DA, Guiard BP, Guilloux JP, Artymyshyn RP, Gardier AM, Gerald C, Antonijevic IA, Leonardo ED, Hen R (2009) Neurogenesis-Dependent and -Independent Effects of Fluoxetine in an Animal Model of Anxiety/Depression. Neuron 62:479-493.

David DJ, Wang JW, Samuels BA, Rainer Q, David I, Gardier AM, Hen R (2010) Implications of the Functional Integration of Adult-Born Hippocampal Neurons in Anxiety-Depression Disorders. Neuroscientist 16:578-591.

Dayer AG, Cleaver KM, Abouantoun T, Cameron HA (2005) New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol 168:415-427.

de Kloet ER, Joels M, Holsboer F (2005) Stress and the brain: From adaptation to disease. Nature Reviews Neuroscience 6:463-475.

de Kloet ER, Oitzl MS, Joels M (1999) Stress and cognition: are corticosteroids good or bad guys? Trends Neurosci 22:422-426.

de Kloet ER, Vreugdenhil E, Oitzl MS, Joels M (1998) Brain corticosteroid receptor balance in health and disease. Endocrine Reviews 19:269-301.

De Marchis S, Fasolo A, Puche AC (2004) Subventricular zone-derived neuronal progenitors migrate into the subcortical forebrain of postnatal mice. Journal of Comparative Neurology 476:290-300.

De MS, Fasolo A, Puche AC (2004) Subventricular zone-derived neuronal progenitors migrate into the subcortical forebrain of postnatal mice. J Comp Neurol 476:290-300.
Deacon RM, Croucher A, Rawlins JN (2002) Hippocampal cytotoxic lesion effects on species-typical behaviours in mice. Behav Brain Res 132:203-213.

Dehmelt L, Halpain S (2004) Actin and microtubules in neurite initiation: Are MAPs the missing link? Journal of Neurobiology 58:18-33.

Dehmelt L, Halpain S (2007) Neurite outgrowth: A flick of the wrist. Current Biology 17:R611-R614.

Deng W, Aimone JB, Gage FH (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci.

Denny CA, Burghardt NS, Schachter DM, Hen R, Drew MR (2012) 4- to 6-week-old adult-born hippocampal neurons influence novelty-evoked exploration and contextual fear conditioning. Hippocampus 22:1188-1201.

des Portes V, Pinard JM, Billuart P, Vinet MC, Koulakoff A, Carrie A, Gelot A, Dupuis E, Motte J, Berwald-Netter Y, Catala M, Kahn A, Beldjord C, Chelly J (1998) A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. Cell 92:51-61.

Deuel TAS, Liu JS, Corbo JC, Yoo SY, Rorke-Adams LB, Walsh CA (2006) Genetic interactions between doublecortin and doublecortin-like kinase in neuronal migration and axon outgrowth. Neuron 49:41-53.

Dhaliwal J, Lagace DC (2011) Visualization and genetic manipulation of adult neurogenesis using transgenic mice. Eur J Neurosci 33:1025-1036.

Dickmeis T, Foulkes NS (2011) Glucocorticoids and circadian clock control of cell proliferation: At the interface between three dynamic systems. Molecular and Cellular Endocrinology 331:11-22.

Dietrich MO, Horvath TL (2012) Fat incites tanycytes to neurogenesis. Nat Neurosci 15:651-653.

Dijkmans TF, van Hooijdonk LW, Fitzsimons CP, Vreugdenhil E (2010) The doublecortin gene family and disorders of neuronal structure. Cent Nerv Syst Agents Med Chem 10:32-46.

Dijkmans TF, van Hooijdonk LW, Schouten TG, Kamphorst JT, Vellinga AC, Meerman JH, Fitzsimons CP, de Kloet ER, Vreugdenhil E (2008) Temporal and functional dynamics of the transcriptome during nerve growth factor-induced differentiation. J Neurochem 105:2388-2403.

Dityatev A, Dityateva G, Sytnyk V, Delling M, Toni N, Nikonenko I, Muller D, Schachner M (2004) Polysialylated neural cell adhesion molecule promotes remodeling and formation of hippocampal synapses. Journal of Neuroscience 24:9372-9382.

Dobrossy MD, Drapeau E, Aurousseau C, Le MM, Piazza PV, Abrous DN (2003) Differential effects of learning on neurogenesis: learning increases or decreases the number of newly born cells depending on their birth date. Mol Psychiatry 8:974-982.

Doetsch F, GarciaVerdugo JM, AlvarezBuylla A (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. Journal of Neuroscience 17:5046-5061.

Dong H, Goico B, Martin M, Csernansky CA, Bertchume A, Csernansky JG (2004) Modulation of hippocampal cell proliferation, memory, and amyloid plaque deposition in APPsw (Tg2576) mutant mice by isolation stress. Neuroscience 127:601-609.

Drew MR, Denny CA, Hen R (2010) Arrest of adult hippocampal neurogenesis in mice impairs single- but not multiple-trial contextual fear conditioning. Behav Neurosci 124:446-454.

Duan X, Chang JH, Ge S, Faulkner RL, Kim JY, Kitabatake Y, Liu XB, Yang CH, Jordan JD, Ma DK, Liu CY, Ganesan S, Cheng HJ, Ming GL, Lu B, Song H (2007) Disrupted-In-Schizophrenia 1 Regulates Integration of Newly Generated Neurons in the Adult Brain. Cell 130:1146-1158.

Duan X, Kang EC, Liu CY, Ming GL, Song HJ (2008) Development of neural stem cell in the adult brain. Current Opinion in Neurobiology 18:108-115.

Duman RS (2004) Depression: A case of neuronal life and death? Biological Psychiatry 56:140-145.

Dupret D, Fabre A, Dobrossy MD, Panatier A, Rodriguez JJ, Lamarque S, Lemaire V, Oliet SH, Piazza PV, Abrous DN (2007) Spatial learning depends on both the addition and removal of new hippocampal neurons. PLoS Biol 5:e214.

Dupret D, Revest JM, Koehl M, Ichas F, De GF, Costet P, Abrous DN, Piazza PV (2008) Spatial relational memory requires hippocampal adult neurogenesis. PLoS ONE 3:e1959.

Earnheart JC, Schweizer C, Crestani F, Iwasato T, Itohara S, Mohler H, Luscher B (2007) GABAergic control of adult hippocampal neurogenesis in relation to behavior indicative of trait anxiety and depression states. J Neurosci 27:3845-3854.

Edelman AM, Kim WY, Higgins D, Goldstein EG, Oberdoerster M, Sigurdson W (2005) Doublecortin kinase-2, a novel doublecortin-related protein kinase associated with terminal segments of axons and dendrites. Journal of Biological Chemistry 280:8531-8543.

Encinas JM, Vaahtokari A, Enikolopov G (2006) Fluoxetine targets early progenitor cells in the adult brain. Proc Natl Acad Sci U S A 103:8233-8238.
Engels BM, Schouten TG, van Dullemen J, Gosens I, Vreugdenhil E (2004) Functional differences between two DCLK splice variants. Molecular Brain Research 120:103-114.

Ennis M, Hamilton KA, Hayar A (2007) Neurochemistry of the Main Olfactory System. In: Handbook of Neurochemistry and Molecular Neurobiology; Sensory Neurochemistry (Johnson, Lajtha, eds), pp 137-204. Springer Science.

Epp JR, Barker JM, Galea LA (2009) Running wild: neurogenesis in the hippocampus across the lifespan in wild and laboratory-bred Norway rats. Hippocampus 19:1040-1049.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nature Medicine 4:1313-1317.

Esposito MS, Piatti VC, Laplagne DA, Morgenstern NA, Ferrari CC, Pitossi FJ, Schinder AF (2005) Neuronal differentiation in the adult hippocampus recapitulates embryonic development. J Neurosci 25:10074-10086.

Falconer EM, Galea LAM (2003) Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. Brain Research 975:22-36.

Fallon JH (1983) The Islands of Calleja Complex of Rat Basal Forebrain .2. Connections of Medium and Large Sized Cells. Brain Research Bulletin 10:775-793.

Fallon JH, Loughlin SE, Ribak CE (1983) The Islands of Calleja Complex of Rat Basal Forebrain .3. Histochemical Evidence for A Striatopallidal System. Journal of Comparative Neurology 218:91-120.

Fallon JH, Riley JN, Sipe JC, Moore RY (1978) Islands of Calleja - Organization and Connections. Journal of Comparative Neurology 181:375-394.

Farmer J, Zhao X, van Praag H, Wodtke K, Gage FH, Christie BR (2004) Effects of voluntary exercise on synaptic plasticity and gene expression in the dentate gyrus of adult male Sprague-Dawley rats in vivo. Neuroscience 124:71-79.

Faulkner RL, Jang MH, Liu XB, Duan X, Sailor KA, Kim JY, Ge S, Jones EG, Ming GL, Song H, Cheng HJ (2008) Development of hippocampal mossy fiber synaptic outputs by new neurons in the adult brain. Proc Natl Acad Sci U S A 105:14157-14162.

Favaro R, Valotta M, Ferri AL, Latorre E, Mariani J, Giachino C, Lancini C, Tosetti V, Ottolenghi S, Taylor V, Nicolis SK (2009) Hippocampal development and neural stem cell maintenance require Sox2-dependent regulation of Shh. Nat Neurosci 12:1248-1256.

Fediuc S, Campbell JE, Riddell MC (2006) Effect of voluntary wheel running on circadian corticosterone release and on HPA axis responsiveness to restraint stress in Sprague-Dawley rats. J Appl Physiol 100:1867-1875.

Fedorkova L, Rutishauser U, Prosser R, Shen FM, Glass JD (2002) Removal of polysialic acid from the SCN potentiates nonphotic circadian phase resetting. Physiology & Behavior 77:361-369.

Fekete C, Lechan RM (2007) Negative feedback regulation of hypophysiotropic thyrotropin-releasing hormone (TRH) synthesizing neurons: role of neuronal afferents and type 2 deiodinase. Front Neuroendocrinol 28:97-114.

Femia AP, Dolara P, Salvadori M, Caderni G (2013) Expression of LGR-5, MSI-1 and DCAMKL-1, putative stem cell markers, in the early phases of 1,2-dimethylhydrazine-induced rat colon carcinogenesis: correlation with nuclear beta-catenin. BMC Cancer 13:48.

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME (1997) CREB: a major mediator of neuronal neurotrophin responses. Neuron 19:1031-1047.

Fitzsimons CP, Ahmed S, Wittevrongel CF, Schouten TG, Dijkmans TF, Scheenen WJ, Schaaf MJ, de Kloet ER, Vreugdenhil E (2008) The microtubule-associated protein doublecortin-like regulates the transport of the glucocorticoid receptor in neuronal progenitor cells. Mol Endocrinol 22:248-262.

Fitzsimons CP, van Hooijdonk LW, Schouten M, Zalachoras I, Brinks V, Zheng T, Schouten TG, Saaltink DJ, Dijkmans T, Steindler DA, Verhaagen J, Verbeek FJ, Lucassen PJ, de Kloet ER, Meijer OC, Karst H, Joels M, Oitzl MS, Vreugdenhil E (2013) Knockdown of the glucocorticoid receptor alters functional integration of newborn neurons in the adult hippocampus and impairs fear-motivated behavior. Mol Psychiatry 18:993-1005.

Fowler CD, Johnson F, Wang ZX (2005) Estrogen regulation of cell proliferation and distribution of estrogen receptor-alpha in the brains of adult female prairie and meadow voles. Journal of Comparative Neurology 489:166-179.

Fowler CD, Liu Y, Ouimet C, Wang Z (2002) The effects of social environment on adult neurogenesis in the female prairie vole. Journal of Neurobiology 51:115-128.
Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N, Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P, Chelly J (1999) Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron 23:247-256.

Frankland PW (2013) Neurogenic evangelism: comment on Urbach et al. (2013). Behav Neurosci 127:126-129.

Franklin K.B.J. aPG (1997) The Mouse Brain in Stereotaxic Coordinates. San Diego: Academic Press.

Frayling C, Britton R, Dale N (2011) ATP-mediated glucosensing by hypothalamic tanycytes. J Physiol 589:2275-2286.

Frielingsdorf H, Schwarz K, Brundin P, Mohapel P (2004) No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A 101:10177-10182.

Friocourt G, Chafey P, Billuart P, Koulakoff A, Vinet MC, Schaar BT, McConnell SK, Francis F, Chelly J (2001) Doublecortin interacts with mu subunits of clathrin adaptor complexes in the developing nervous system. Mol Cell Neurosci 18:307-319.

References

Friocourt G, Koulakoff A, Chafey P, Boucher D, Fauchereau F, Chelly J, Francis F (2003) Doublecortin functions at the extremities of growing neuronal processes. Cerebral Cortex 13:620-626.

Friocourt G, Liu JS, Antypa M, Rakic S, Walsh CA, Parnavelas JG (2007) Both doublecortin and doublecortin-like kinase play a role in cortical interneuron migration. Journal of Neuroscience 27:3875-3883.

Fukuda S, Kato F, Tozuka Y, Yamaguchi M, Miyamoto Y, Hisatsune T (2003) Two distinct subpopulations of nestin-positive cells in adult mouse dentate gyrus. J Neurosci 23:9357-9366.

Fuss J, Ben Abdallah NMB, Hensley FW, Weber KJ, Hellweg R, Gass P (2010) Deletion of Running-Induced Hippocampal Neurogenesis by Irradiation Prevents Development of an Anxious Phenotype in Mice. Plos One 5.

Gao Z, Ure K, Ables JL, Lagace DC, Nave KA, Goebbels S, Eisch AJ, Hsieh J (2009) Neurod1 is essential for the survival and maturation of adult-born neurons. Nat Neurosci 12:1090-1092.

Garcia A, Steiner B, Kronenberg G, Bick-Sander A, Kempermann G (2004) Age-dependent expression of glucocorticoid- and mineralocorticoid receptors on neural precursor cell populations in the adult murine hippocampus. Aging Cell 3:363-371.

Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 439:589-593.

Geoghegan D, Carter DA (2008) A novel site of adult doublecortin expression: neuropeptide neurons within the suprachiasmatic nucleus circadian clock. Bmc Neuroscience 9.

Gereben B, Goncalves C, Harney JW, Larsen PR, Bianco AC (2000) Selective proteolysis of human type 2 deiodinase: a novel ubiquitin-proteasomal mediated mechanism for regulation of hormone activation. Mol Endocrinol 14:1697-1708.

Gerritsen L, Comijs HC, van der Graaf Y, Knoops AJG, Penninx BWJH, Geerlings MI (2011) Depression, Hypothalamic Pituitary Adrenal Axis, and Hippocampal and Entorhinal Cortex Volumes-The SMART Medea Study. Biological Psychiatry 70:373-380.

Gheusi G, Cremer H, McLean H, Chazal G, Vincent JD, Lledo PM (2000) Importance of newly generated neurons in the adult olfactory bulb for odor discrimination. Proc Natl Acad Sci U S A 97:1823-1828.

Girardet C, Becquet D, Blanchard MP, Francois-Bellan AM, Bosler O (2010a) Neuroglial and synaptic rearrangements associated with photic entrainment of the circadian clock in the suprachiasmatic nucleus. European Journal of Neuroscience 32:2133-2142.
Girardet C, Blanchard MP, Ferracci G, Leveque C, Moreno M, Francois-Bellan AM, Becquet D, Bosler O (2010b) Daily changes in synaptic innervation of VIP neurons in the rat suprachiasmatic nucleus: contribution of glutamatergic afferents. European Journal of Neuroscience 31:359-370.

Glass JD, Watanabe M, Fedorkova L, Shen H, Ungers G, Rutishauser U (2003) Dynamic regulation of polysialylated neural cell adhesion molecule in the suprachiasmatic nucleus. Neuroscience 117:203-211.

Glavan G, Sket D, Zivin M (2002) Modulation of neuroleptic activity of 9,10-didehydro-N-methyl-(2-propynyl)-6-methyl-8-aminomethylergoline bimaleinate (LEK-8829) by D1 intrinsic activity in hemi-parkinsonian rats. Mol Pharmacol 61:360-368.

Gleeson JG, Allen KM, Fox JW, Lamperti ED, Berkovic S, Scheffer I, Cooper EC, Dobyns WB, Minnerath SR, Ross ME, Walsh CA (1998) doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. Cell 92:63-72.

Gleeson JG, Lin PT, Flanagan LA, Walsh CA (1999) Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23:257-271.

Gold PW, Chrousos GP (2002) Organization of the stress system and its dysregulation in melancholic and atypical depression: high vs low CRH/NE states. Molecular Psychiatry 7:254-275.

Goldman SA, Nottebohm F (1983) Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci U S A 80:2390-2394.

Gomez-Climent MA, Castillo-Gomez E, Varea E, Guirado R, Blasco-Ibanez JM, Crespo C, Martinez-Guijarro FJ, Nacher J (2008) A population of prenatally generated cells in the rat paleocortex maintains an immature neuronal phenotype into adulthood. Cereb Cortex 18:2229-2240.

Gonczy P, Bellanger JM, Kirkham M, Pozniakowski A, Baumer K, Phillips JB, Hyman AA (2001) zvg-8, a gene required for spindle positioning in C-elegans, encodes a doublecortin-related kinase that promotes microtubule assembly. Developmental Cell 1:363-375.

Gould E (2007) Opinion - How widespread is adult neurogenesis in mammals? Nature Reviews Neuroscience 8:481-488.

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999a) Learning enhances adult neurogenesis in the hippocampal formation. Nature Neuroscience 2:260-265.

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999b) Learning enhances adult neurogenesis in the hippocampal formation. Nature Neuroscience 2:260-265.

Gould E, Cameron HA, Daniels DC, Woolley CS, Mcewen BS (1992) Adrenal Hormones Suppress Cell-Division in the Adult-Rat Dentate Gyrus. Journal of Neuroscience 12:3642-3650.

Gould E, Mcewen BS, Tanapat P, Galea LAM, Fuchs E (1997) Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. Journal of Neuroscience 17:2492-2498.

Gould E, Reeves AJ, Graziano MSA, Gross CG (1999c) Neurogenesis in the neocortex of adult primates. Science 286:548-552.

Gould E, Tanapat P, Mcewen BS, Flugge G, Fuchs E (1998) Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proceedings of the National Academy of Sciences of the United States of America 95:3168-3171.

Grant E.C., Mackintosh J.H. (1963) A Comparison of the Social Postures of Some Common Laboratory Rodents. Behaviour 21:246-259.

Gu Y, rruda-Carvalho M, Wang J, Janoschka SR, Josselyn SA, Frankland PW, Ge S (2012) Optical controlling reveals time-dependent roles for adult-born dentate granule cells. Nat Neurosci 15:1700-1706.

Guadano-Ferraz A, Obregon MJ, St Germain DL, Bernal J (1997) The type 2 iodothyronine deiodinase is expressed primarily in glial cells in the neonatal rat brain. Proc Natl Acad Sci U S A 94:10391-10396.

Gupta A, Tsai LH, Wynshaw-Boris A (2002) Life is a journey: a genetic look at neocortical development. Nat Rev Genet 3:342-355.

Guzman-Marin R, Suntsova N, Bashir T, Szymusiak R, McGinty D (2007) Cell proliferation in the dentate gyrus of the adult rat fluctuates with the light-dark cycle. Neuroscience Letters 422:198-201.

References

Haan N, Goodman T, Najdi-Samiei A, Stratford CM, Rice R, El AE, Bellusci S, Hajihosseini MK (2013) Fgf10-expressing tanycytes add new neurons to the appetite/energy-balance regulating centers of the postnatal and adult hypothalamus. J Neurosci 33:6170-6180.

Hack MA, Saghatelyan A, de CA, Pfeifer A, shery-Padan R, Lledo PM, Gotz M (2005) Neuronal fate determinants of adult olfactory bulb neurogenesis. Nat Neurosci 8:865-872.

Hazlerigg D, Loudon A (2008) New insights into ancient seasonal life timers. Current Biology 18:R795-R804.

He G, Luo W, Li P, Remmers C, Netzer WJ, Hendrick J, Bettayeb K, Flajolet M, Gorelick F, Wennogle LP, Greengard P (2010) Gamma-secretase activating protein is a therapeutic target for Alzheimer's disease. Nature 467:95-98.

Heine VM, Maslam S, Joels M, Lucassen PJ (2004a) Prominent decline of newborn cell proliferation, differentiation, and apoptosis in the aging dentate gyrus, in absence of an age-related hypothalamus-pituitary-adrenal axis activation. Neurobiol Aging 25:361-375.

Heine VM, Maslam S, Zareno J, Joels M, Lucassen PJ (2004b) Suppressed proliferation and apoptotic changes in the rat dentate gyrus after acute and chronic stress are reversible. European Journal of Neuroscience 19:131-144.

Herrera DG, Garcia-Verdugo JM, varez-Buylla A (1999) Adult-derived neural precursors transplanted into multiple regions in the adult brain. Ann Neurol 46:867-877.

Hof PR, Morrison JH (2004) The aging brain: morphomolecular senescence of cortical circuits. Trends Neurosci 27:607-613.

Holick KA, Lee DC, Hen R, Dulawa SC (2008) Behavioral effects of chronic fluoxetine in BALB/cJ mice do not require adult hippocampal neurogenesis or the serotonin 1A receptor. Neuropsychopharmacology 33:406-417.

Holsboer F, Ising M (2010) Stress Hormone Regulation: Biological Role and Translation into Therapy. Annual Review of Psychology 61:81-109.

Horesh D, Sapir T, Francis F, Wolf SG, Caspi M, Elbaum M, Chelly J, Reiner O (1999) Doublecortin, a stabilizer of microtubules. Human Molecular Genetics 8:1599-1610.

Huang GJ, Bannerman D, Flint J (2008) Chronic fluoxetine treatment alters behavior, but not adult hippocampal neurogenesis, in BALB/cJ mice. Molecular Psychiatry 13:119-121.

Huang L, DeVries GJ, Bittman EL (1998) Photoperiod regulates neuronal bromodeoxyuridine labeling in the brain of a seasonally breeding mammal. J Neurobiol 36:410-420.

Ibi D, Takuma K, Koike H, Mizoguchi H, Tsuritani K, Kuwahara Y, Kamei H, Nagai T, Yoneda Y, Nabeshima T, Yamada K (2007) Social isolation rearing-induced impairment of the hippocampal neurogenesis is associated with deficits in spatial memory and emotion-related behaviors in juvenile mice. J Neurochem ..

Imayoshi I, Ohtsuka T, Metzger D, Chambon P, Kageyama R (2006) Temporal regulation of Cre recombinase activity in neural stem cells. Genesis 44:233-238.

Imayoshi I, Sakamoto M, Kageyama R (2011) Genetic methods to identify and manipulate newly born neurons in the adult brain. Front Neurosci 5:64.

Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 11:1153-1161.

Jacobs BL, van Praag H, Gage FH (2000) Adult brain neurogenesis and psychiatry: a novel theory of depression. Molecular Psychiatry 5:262-269.

Jacobson L, Sapolsky R (1991) The Role of the Hippocampus in Feedback-Regulation of the Hypothalamic-Pituitary-Adrenocortical Axis. Endocrine Reviews 12:118-134.

Jaenisch R, Mintz B (1974) Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. Proc Natl Acad Sci U S A 71:1250-1254.

Jaholkowski P, Kiryk A, Jedynak P, Ben Abdallah NM, Knapska E, Kowalczyk A, Piechal A, Blecharz-Klin K, Figiel I, Lioudyno V, Widy-Tyszkiewicz E, Wilczynski GM, Lipp HP, Kaczmarek L, Filipkowski RK (2009) New hippocampal neurons are not obligatory for memory formation; cyclin D2 knockout mice with no adult brain neurogenesis show learning. Learn Mem 16:439-451.

Jansen K, Van der Zee EA, Gerkema MP (2007) Vasopressin immunoreactivity, but not vasoactive intestinal polypeptide, correlates with expression of circadian rhythmicity in the suprachiasmatic nucleus of voles. Neuropeptides 41:207-216.

Jansen K, Van der Zee EA, Gerkema MP (2000) Being circadian or not: vasopressin release in cultured SCN mirrors behavior in adult voles. Neuroreport 11:3555-3558.

Jayatissa MN, Henningsen K, West MJ, Wiborg O (2009) Decreased cell proliferation in the dentate gyrus does not associate with development of anhedonic-like symptoms in rats. Brain Research 1290:133-141.

Jedynak P, Jaholkowski P, Wozniak G, Sandi C, Kaczmarek L, Filipkowski RK (2012) Lack of cyclin D2 impairing adult brain neurogenesis alters hippocampal-dependent behavioral tasks without reducing learning ability. Behav Brain Res 227:159-166.

Jessberger S, Romer B, Babu H, Kempermann G (2005) Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. Exp Neurol 196:342-351.

Jessberger S, Zhao C, Toni N, Clemenson GD, Jr., Li Y, Gage FH (2007) Seizure-associated, aberrant neurogenesis in adult rats characterized with retrovirus-mediated cell labeling. J Neurosci 27:9400-9407.

Kalsbeek A, Buijs RM, van SR, Kaptein E, Visser TJ, Doulabi BZ, Fliers E (2005) Daily variations in type II iodothyronine deiodinase activity in the rat brain as controlled by the biological clock. Endocrinology 146:1418-1427.

Kameda Y, Arai Y, Nishimaki T (2003) Ultrastructural localization of vimentin immunoreactivity and gene expression in tanycytes and their alterations in hamsters kept under different photoperiods. Cell and Tissue Research 314:251-262.

Kannangara TS, Webber A, Gil-Mohapel J, Christie BR (2009) Stress Differentially Regulates the Effects of Voluntary Exercise on Cell Proliferation in the Dentate Gyrus of Mice. Hippocampus 19:889-897.

Kaplan MS (1981) Neurogenesis in the 3-month-old rat visual cortex. J Comp Neurol 195:323-338.

Kaplan MS, Hinds JW (1977) Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092-1094.

Kappeler C, Saillour Y, Baudoin JP, Tuy FPD, Alvarez C, Houbron C, Gaspar P, Hamard G, Chelly J, Metin C, Francis F (2006) Branching and nucleokinesis defects in migrating interneurons derived from doublecortin knockout mice. Human Molecular Genetics 15:1387-1400.

Karatsoreos IN, Yan L, LeSauter J, Silver R (2004) Phenotype matters: Identification of light-responsive cells in the mouse suprachiasmatic nucleus. Journal of Neuroscience 24:68-75.

References

Kee N, Teixeira CM, Wang AH, Frankland PW (2007) Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. Nat Neurosci 10:355-362.

Kempermann G (2012) New neurons for 'survival of the fittest'. Nat Rev Neurosci 13:727-736.

Kempermann G (2008) The neurogenic reserve hypothesis: what is adult hippocampal neurogenesis good for? Trends Neurosci ..

Kempermann G, Brandon EP, Gage FH (1998a) Environmental stimulation of 129/SvJ mice causes increased cell proliferation and neurogenesis in the adult dentate gyrus. Curr Biol 8:939-942.

Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH (2003) Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. Development 130:391-399.

Kempermann G, Kuhn HG, Gage FH (1997b) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493-495.

Kempermann G, Kuhn HG, Gage FH (1997a) Genetic influence on neurogenesis in the dentate gyrus of adult mice. Proc Natl Acad Sci U S A 94:10409-10414.

Kempermann G, Kuhn HG, Gage FH (1998b) Experience-induced neurogenesis in the senescent dentate gyrus. Journal of Neuroscience 18:3206-3212.

Kempermann G, Song H, Gage FH (2008) Neurogenesis in the Adult Hippocampus. In: Adult Neurogenesis (Gage FH, Kempermann G, Song H, eds), pp 159-174. New York: Cold Spring Harbor Laboratory Press.

Kheirbek MA, Drew LJ, Burghardt NS, Costantini DO, Tannenholz L, Ahmari SE, Zeng H, Fenton AA, Hen R (2013) Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus. Neuron 77:955-968.

Kheirbek MA, Klemenhagen KC, Sahay A, Hen R (2012) Neurogenesis and generalization: a new approach to stratify and treat anxiety disorders. Nat Neurosci 15:1613-1620.

Kikuchi M, Nagata H, Watanabe N, Watanabe H, Tatemichi M, Hibi T (2010) Altered expression of a putative progenitor cell marker DCAMKL1 in the rat gastric mucosa in regeneration, metaplasia and dysplasia. BMC Gastroenterol 10:65.

Kim MH, Cierpicki T, Derewenda U, Krowarsch D, Feng YY, Devedjiev Y, Dauter Z, Walsh CA, Otlewski J, Bushweller JH, Derewenda ZS (2003) The DCX-domain tandems of doublecortin and doublecortin-like kinase. Nature Structural Biology 10:324-333.

Kohwi M, Osumi N, Rubenstein JL, varez-Buylla A (2005) Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. J Neurosci 25:6997-7003.

Kohwi M, Petryniak MA, Long JE, Ekker M, Obata K, Yanagawa Y, Rubenstein JL, varez-Buylla A (2007) A subpopulation of olfactory bulb GABAergic interneurons is derived from Emx1- and Dlx5/6-expressing progenitors. J Neurosci 27:6878-6891.

Koizumi H, Tanaka T, Gleeson JG (2006) Doublecortin-like kinase functions with doublecortin to mediate fiber tract decussation and neuronal migration. Neuroscience Research 55:S238.

Kokoeva MV, Yin HL, Flier JS (2007) Evidence for constitutive neural cell proliferation in the adult murine hypothalamus. Journal of Comparative Neurology 505:209-220.
Kokoeva MV, Yin HL, Flier JS (2005) Neurogenesis in the hypothalamus of adult mice: Potential role in energy balance. Science 310:679-683.

Konefal S, Elliot M, Crespi B (2013) The adaptive significance of adult neurogenesis: an integrative approach. Front Neuroanat 7:21.

Koopmans G, Blokland A, van NP, Prickaerts J (2003) Assessment of spatial learning abilities of mice in a new circular maze. Physiol Behav 79:683-693.

Kosaka K, Aika Y, Toida K, Heizmann CW, Hunziker W, Jacobowitz DM, Nagatsu I, Streit P, Visser TJ, Kosaka T (1995) Chemically defined neuron groups and their subpopulations in the glomerular layer of the rat main olfactory bulb. Neurosci Res 23:73-88.

Kriegstein A, Alvarez-Buylla A (2009) The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32:149-84.:149-184.

Kriegstein AR, Noctor SC (2004) Patterns of neuronal migration in the embryonic cortex. Trends Neurosci 27:392-399.

Kronenberg G, Reuter K, Steiner B, Brandt MD, Jessberger S, Yamaguchi M, Kempermann G (2003) Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. J Comp Neurol 467:455-463.

Kruidering M, Schouten T, Evan GI, Vreugdenhil E (2001) Caspase-mediated cleavage of the Ca2+/calmodulin-dependent protein kinase-like kinase facilitates neuronal apoptosis. Journal of Biological Chemistry 276:38417-38425.

Kuhn HG, Biebl M, Wilhelm D, Li M, Friedlander RM, Winkler J (2005) Increased generation of granule cells in adult Bcl-2-overexpressing mice: a role for cell death during continued hippocampal neurogenesis. Eur J Neurosci 22:1907-1915.

Kuhn HG, DickinsonAnson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. Journal of Neuroscience 16:2027-2033.

Lagace DC, Benavides DR, Kansy JW, Mapelli M, Greengard P, Bibb JA, Eisch AJ (2008) Cdk5 is essential for adult hippocampal neurogenesis. Proc Natl Acad Sci U S A 105:18567-18571.

Lancaster MA, Knoblich JA (2012) Spindle orientation in mammalian cerebral cortical development. Curr Opin Neurobiol 22:737-746.

Laplagne DA, Esposito MS, Piatti VC, Morgenstern NA, Zhao C, van PH, Gage FH, Schinder AF (2006) Functional convergence of neurons generated in the developing and adult hippocampus. PLoS Biol 4:e409.

Latchney SE, Hein AM, O'Banion MK, cicco-Bloom E, Opanashuk LA (2013) Deletion or activation of the aryl hydrocarbon receptor alters adult hippocampal neurogenesis and contextual fear memory. J Neurochem 125:430-445.

Lau BW, Yau SY, So KF (2011) Reproduction: a new venue for studying function of adult neurogenesis? Cell Transplant 20:21-35.

Lazarini F, Lledo PM (2010) Is adult neurogenesis essential for olfaction? Trends Neurosci.

Leak RK, Moore RY (2001) Topographic organization of suprachiasmatic nucleus projection neurons. Journal of Comparative Neurology 433:312-334.

Lee DA, Bedont JL, Pak T, Wang H, Song J, Miranda-Angulo A, Takiar V, Charubhumi V, Balordi F, Takebayashi H, Aja S, Ford E, Fishell G, Blackshaw S (2012) Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche. Nat Neurosci 15:700-702.

Lee DA, Blackshaw S (2012) Functional implications of hypothalamic neurogenesis in the adult mammalian brain. Int J Dev Neurosci 30:615-621.

Lee HS, Lim BV, Jang MH, Shin MC, Lee TH, Kim YP, Shin HS, Cho SY, Kim H, Shin MS, Kim EH, Kim CJ (2002) Hypothermia inhibits cell proliferation and nitric oxide synthase expression in rats. Neuroscience Letters 329:53-56.

Lehmann ML, Brachman RA, Martinowich K, Schloesser RJ, Herkenham M (2013) Glucocorticoids orchestrate divergent effects on mood through adult neurogenesis. J Neurosci 33:2961-2972.

Li J, Tang Y, Cai D (2012) IKKbeta/NF-kappaB disrupts adult hypothalamic neural stem cells to mediate a neurodegenerative mechanism of dietary obesity and pre-diabetes. Nat Cell Biol 14:999-1012.

Li Y, Luikart BW, Birnbaum S, Chen J, Kwon CH, Kernie SG, Bassel-Duby R, Parada LF (2008) TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. Neuron 59:399-412.

Li YF, Zhang YZ, Liu YQ, Wang HL, Yuan L, Luo ZP (2004) Moclobemide up-regulates proliferation of hippocampal progenitor cells in chronically stressed mice. Acta Pharmacologica Sinica 25:1408-1412.

Lightman SL, Wiles CC, Atkinson HC, Henley DE, Russell GM, Leendertz JA, McKenna MA, Spiga F, Wood SA, Conway-Campbell BL (2008) The significance of glucocorticoid pulsatility. European Journal of Pharmacology 583:255-262.

Lim DA, Huang YC, Alvarez-Buylla A (2008) Adult Subventricular Zone and Olfactory Bulb Neurogenesis. In: Adult Neurogenesis (Gage F, Kempermann G, Song H, eds), pp 175-206. New York: Cold Spring Harbor Laboratory Press.

Lin PT, Gleeson JG, Corbo JC, Flanagan L, Walsh CA (2000) DCAMKL1 encodes a protein kinase with homology to doublecortin that regulates microtubule polymerization. J Neurosci 20:9152-9161.

Lister RG (1990) Ethologically-based animal models of anxiety disorders. Pharmacol Ther 46:321-340.

Liu Y, Chirino AJ, Misulovin Z, Leteux C, Feizi T, Nussenzweig MC, Bjorkman PJ (2000) Crystal structure of the cysteine-rich domain of mannose receptor complexed with a sulfated carbohydrate ligand. J Exp Med 191:1105-1116.

Liu YW, Curtis MA, Gibbons HM, Mee EW, Bergin PS, Teoh HH, Connor B, Dragunow M, Faull RL (2008) Doublecortin expression in the normal and epileptic adult human brain. Eur J Neurosci 28:2254-2265.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.
Lledo PM, Alonso M, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nature Reviews Neuroscience 7:179-193.

Lledo PM, Saghatelyan A (2005) Integrating new neurons into the adult olfactory bulb: joining the network, life-death decisions, and the effects of sensory experience. Trends Neurosci 28:248-254.

Lledo P-M (2008) Adult neurogenesis in the olfactory bulb. In: Adult Neurogenesis (Gage FH, Kempermann G, Song H, eds), pp 425-444. New York: Cold Spring Harbor Laboratory Press.

Lucassen PJ, Meerlo P, Naylor AS, Van Dam AM, Dayer AG, Fuchs E, Oomen CA, Czeh B (2010a) Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action. European Neuropsychopharmacology 20:1-17.

Lucassen PJ, Stumpel MW, Wang Q, Aronica E (2010b) Decreased numbers of progenitor cells but no response to antidepressant drugs in the hippocampus of elderly depressed patients. Neuropharmacology 58:940-949.

Luzzati F, De MS, Fasolo A, Peretto P (2006) Neurogenesis in the caudate nucleus of the adult rabbit. J Neurosci 26:609-621.

Machado DG, Cunha MP, Neis VB, Balen GO, Colla AR, Grando J, Brocardo PS, Bettio LE, Dalmarco JB, Rial D, Prediger RD, Pizzolatti MG, Rodrigues AL (2012) Rosmarinus officinalis L. hydroalcoholic extract, similar to fluoxetine, reverses depressive-like behavior without altering learning deficit in olfactory bulbectomized mice. J Ethnopharmacol 143:158-169.

MacQueen GM, Campbell S, Mcewen BS, Macdonald K, Amano S, Joffe RT, Nahmias C, Young LT (2003) Course of illness, hippocampal function, and hippocampal volume in major depression. Proceedings of the National Academy of Sciences of the United States of America 100:1387-1392.

Mak GK, Enwere EK, Gregg C, Pakarainen T, Poutanen M, Huhtaniemi I, Weiss S (2007) Male pheromone-stimulated neurogenesis in the adult female brain: possible role in mating behavior. Nat Neurosci 10:1003-1011.

Mak GK, Weiss S (2010) Paternal recognition of adult offspring mediated by newly generated CNS neurons. Nat Neurosci 13:753-758.

Malatesta P, Appolloni I, Calzolari F (2008) Radial glia and neural stem cells. Cell Tissue Res 331:165-178.

Malatesta P, Hack MA, Hartfuss E, Kettenmann H, Klinkert W, Kirchhoff F, Gotz M (2003) Neuronal or glial progeny: Regional differences in radial glia fate. Neuron 37:751-764.

Malberg JE, Duman RS (2003) Cell proliferation in adult hippocampus is decreased by inescapable stress: Reversal by fluoxetine treatment. Neuropsychopharmacology 28:1562-1571.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. Journal of Neuroscience 20:9104-9110.

Marin O, Rubenstein JL (2003) Cell migration in the forebrain. Annu Rev Neurosci 26:441-483.

Marin-Burgin A, Schinder AF (2012) Requirement of adult-born neurons for hippocampus-dependent learning. Behav Brain Res 227:391-399.

Martinez-Canabal A, Akers KG, Josselyn SA, Frankland PW (2013) Age-dependent effects of hippocampal neurogenesis suppression on spatial learning. Hippocampus 23:66-74.

Matsuzaki K, Katakura M, Hara T, Li G, Hashimoto M, Shido O (2009) Proliferation of neuronal progenitor cells and neuronal differentiation in the hypothalamus are enhanced in heat-acclimated rats. Pflugers Arch 458:661-673.

Mcewen BS (2007) Physiology and neurobiology of stress and adaptation: Central role of the brain. Physiological Reviews 87:873-904.

Mcewen BS (1998) Stress, adaptation, and disease - Allostasis and allostatic load. Neuroimmunomodulation 840:33-44.

Mcewen BS, Gianaros PJ (2011) Stress- and Allostasis-Induced Brain Plasticity. Annual Review of Medicine, Vol 62, 2011 62:431-445.

Meijer JH, Michel S, vanderLeest HT, Rohling JHT (2010) Daily and seasonal adaptation of the circadian clock requires plasticity of the SCN neuronal network. European Journal of Neuroscience 32:2143-2151.

Merz K, Lie DC (2013) Evidence that Doublecortin is dispensable for the development of adult born neurons in mice. PLoS One 8:e62693.

Meshi D, Drew MR, Saxe M, Ansorge MS, David D, Santarelli L, Malapani C, Moore H, Hen R (2006) Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. Nature Neuroscience 9:729-731.

Meyer G, Gonzalezhernandez T, Carrillopadilla F, Ferrestorres R (1989) Aggregations of Granule Cells in the Basal Forebrain (Islands of Calleja) - Golgi and Cytoarchitectonic Study in Different Mammals, Including Man. Journal of Comparative Neurology 284:405-428.

Millan C, Martinez F, Cortes-Campos C, Lizama I, Yanez MJ, Llanos P, Reinicke K, Rodriguez F, Peruzzo B, Nualart F, Garcia MA (2010) Glial glucokinase expression in adult and post-natal development of the hypothalamic region. Asn Neuro 2:135-145.

Millhouse OE (1987) Granule Cells of the Olfactory Tubercle and the Question of the Islands of Calleja. Journal of Comparative Neurology 265:1-24.

Ming GL, Song HJ (2005) Adult neurogenesis in the mammalian central nervous system. Annual Review of Neuroscience 28:223-250.

Ming GL, Song HJ (2011) Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions. Neuron 70:687-702.

Mirescu C, Gould E (2006) Stress and adult neurogenesis. Hippocampus 16:233-238.
Mirescu C, Peters JD, Gould E (2004) Early life experience alters response of adult neurogenesis to stress. Nat Neurosci 7:841-846.

Mirescu C, Peters JD, Noiman L, Gould E (2006) Sleep deprivation inhibits adult neurogenesis in the hippocampus by elevating glucocorticoids. Proc Natl Acad Sci U S A 103:19170-19175.

Moores CA, Perderiset M, Francis F, Chelly J, Houdusse A, Milligan RA (2004) Mechanism of microtubule stabilization by doublecortin. Molecular Cell 14:833-839.

Moores CA, Perderiset M, Kappeler C, Kain S, Drummond D, Perkins SJ, Chelly J, Cross R, Houdusse A, Francis F (2006) Distinct roles of doublecortin modulating the microtubule cytoskeleton. EMBO J 25:4448-4457.

Morin LP (2007) SCN organization reconsidered. Journal of Biological Rhythms 22:3-13.

Mullier A, Bouret SG, Prevot V, Dehouck B (2010) Differential Distribution of Tight Junction Proteins Suggests a Role for Tanycytes in Blood-Hypothalamus Barrier Regulation in the Adult Mouse Brain. Journal of Comparative Neurology 518:943-962.

Muramatsu R, Ikegaya Y, Matsuki N, Koyama R (2007) Neonatally born granule cells numerically dominate adult mice dentate gyrus. Neuroscience 148:593-598.

Murata K, Imai M, Nakanishi S, Watanabe D, Pastan I, Kobayashi K, Nihira T, Mochizuki H, Yamada S, Mori K, Yamaguchi M (2011) Compensation of Depleted Neuronal Subsets by New Neurons in a Local Area of the Adult Olfactory Bulb. J Neurosci 20;31:10540-10557.

Murphy M, Ebling FJ (2011) The role of hypothalamic tri-iodothyronine availability in seasonal regulation of energy balance and body weight. J Thyroid Res 2011:387562.
Nacher J, Crespo C, Mcewen BS (2001) Doublecortin expression in the adult rat telencephalon. Eur J Neurosci 14:629-644.

Nadarajah B, Brunstrom JE, Grutzendler J, Wong RO, Pearlman AL (2001) Two modes of radial migration in early development of the cerebral cortex. Nat Neurosci 4:143-150.

Ng KL, Li JD, Cheng MY, Leslie FM, Lee AG, Zhou QY (2005) Dependence of olfactory bulb neurogenesis on prokineticin 2 signaling. Science 308:1923-1927.

Nissant A, Pallotto M (2011) Integration and maturation of newborn neurons in the adult olfactory bulb--from synapses to function. Eur J Neurosci 33:1069-1077.

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR (2001) Neurons derived from radial glial cells establish radial units in neocortex. Nature 409:714-720.

Noctor SC, Martinez-Cerdeno V, Kriegstein AR (2008) Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. J Comp Neurol 508:28-44.

Ohmae S, Takemoto-Kimura S, Okamura M, chi-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 281:20427-20439.

Oomen CA, Mayer JL, de Kloet ER, Joels M, Lucassen PJ (2007) Brief treatment with the glucocorticoid receptor antagonist mifepristone normalizes the reduction in neurogenesis after chronic stress. Eur J Neurosci 26:3395-3401.

Oomen CA, Soeters H, Audureau N, Vermunt L, van Hasselt FN, Manders EM, Joels M, Lucassen PJ, Krugers H (2010) Severe early life stress hampers spatial learning and neurogenesis, but improves hippocampal synaptic plasticity and emotional learning under high-stress conditions in adulthood. J Neurosci 30:6635-6645.

Out C, Hageman J, Bloks VW, Gerrits H, Sollewijn G, Bos T, Havinga R, Smit MJ, Kuipers F, Groen AK (2011) Liver receptor homolog-1 is critical for adequate up-regulation of Cyp7a1 gene transcription and bile salt synthesis during bile salt sequestration. Hepatology 53:2075-2085.

Pan YW, Chan GC, Kuo CT, Storm DR, Xia Z (2012) Inhibition of adult neurogenesis by inducible and targeted deletion of ERK5 mitogen-activated protein kinase specifically in adult neurogenic regions impairs contextual fear extinction and remote fear memory. J Neurosci 32:6444-6455.

Parrish-Aungst S, Shipley MT, Erdelyi F, Szabo G, Puche AC (2007) Quantitative analysis of neuronal diversity in the mouse olfactory bulb. Journal of Comparative Neurology 501:825-836.

Paxinos G, Franklin KBJ (2001) The Mouse Brain in stereotaxic coordinates. Academic press.

Pekcec A, Loscher W, Potschka H (2006) Neurogenesis in the adult rat piriform cortex. Neuroreport 17:571-574.

Pencea V, Bingaman KD, Wiegand SJ, Luskin MB (2001) Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J Neurosci 21:6706-6717.

Perez-Martin M, Cifuentes M, Grondona JM, Lopez-Avalos MD, Gomez-Pinedo U, Garcia-Verdugo JM, Fernandez-Llebrez P (2010) IGF-I stimulates neurogenesis in the hypothalamus of adult rats. Eur J Neurosci 31:1533-1548.

Petrik D, Lagace DC, Eisch AJ (2012) The neurogenesis hypothesis of affective and anxiety disorders: are we mistaking the scaffolding for the building? Neuropharmacology 62:21-34.

Pham K, Nacher J, Hof PR, Mcewen BS (2003) Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. European Journal of Neuroscience 17:879-886.

Phillips RG, Ledoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci 106:274-285.

Pickard B (2011) Progress in defining the biological causes of schizophrenia. Expert Reviews in Molecular Medicine 13.

References

Plumpe T, Ehninger D, Steiner B, Klempin F, Jessberger S, Brandt M, Romer B, Rodriguez GR, Kronenberg G, Kempermann G (2006) Variability of doublecortin-associated dendrite maturation in adult hippocampal neurogenesis is independent of the regulation of precursor cell proliferation. BMC Neurosci 7:77.

Pramparo T, Youn YH, Yingling J, Hirotsune S, Wynshaw-Boris A (2010) Novel embryonic neuronal migration and proliferation defects in Dcx mutant mice are exacerbated by Lis1 reduction. J Neurosci 30:3002-3012.

Prevot V (2002) Glial-neuronal-endothelial interactions are involved in the control of GnRH secretion. Journal of Neuroendocrinology 14:247-255.

Prosser RA, Rutishauser U, Ungers G, Fedorkova L, Glass JD (2003) Intrinsic role of polysialylated neural cell adhesion molecule in photic phase resetting of the mammalian circadian clock. Journal of Neuroscience 23:652-658.

Raber J, Rola R, LeFevour A, Morhardt D, Curley J, Mizumatsu S, VandenBerg SR, Fike JR (2004) Radiation-induced cognitive impairments are associated with changes in indicators of hippocampal neurogenesis. Radiat Res 162:39-47.

Rakic P (2002) Adult neurogenesis in mammals: An identity crisis. Journal of Neuroscience 22:614-618.

Rakic P (2006) No more cortical neurons for you. Science 313:928-929.

Rami A, Brehier A, Thomasset M, Rabie A (1987) Cholecalcin (28-Kda Calcium-Binding Protein) in the Rat Hippocampus - Development in Normal Animals and in Altered Thyroid States - An Immunocytochemical Study. Developmental Biology 124:228-238.

Rao MS, Shetty AK (2004) Efficacy of doublecortin as a marker to analyse the absolute number anddendritic growth of newly generated neurons in the adult dentate gyrus. European Journal of Neuroscience 19:234-246.

Reif A, Fritzen S, Finger M, Strobel A, Lauer M, Schmitt A, Lesch KP (2006) Neural stem cell proliferation is decreased in schizophrenia, but not in depression. Molecular Psychiatry 11:514-522.

Reiner O, Coquelle FM, Peter B, Levy T, Kaplan A, Sapir T, Orr I, Barkai N, Eichele G, Bergmann S (2006) The evolving doublecortin (DCX) superfamily. Bmc Genomics 7.

Reul JMHM, deKloet ER (1985) 2 Receptor Systems for Corticosterone in Rat-Brain - Microdistribution and Differential Occupation. Endocrinology 117:2505-2511.

Rhodes JS, van Praag H, Jeffrey S, Girard I, Mitchell GS, Garland T, Gage FH (2003) Exercise increases hippocampal neurogenesis to high levels but does not improve spatial learning in mice bred for increased voluntary wheel running. Behavioral Neuroscience 117:1006-1016.

Rodriguez EM, Blazquez JL, Guerra M (2010) The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: The former opens to the portal blood and the latter to the cerebrospinal fluid. Peptides 31:757-776.

Rodriguez EM, Blazquez JL, Pastor FE, Pelaez B, Pena P, Peruzzo B, Amat P (2005) Hypothalamic tanycytes: a key component of brain-endocrine interaction. Int Rev Cytol 247:89-164.:89-164.

Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37:e45.

Saaltink DJ, Havik B, Verissimo CS, Lucassen PJ, Vreugdenhil E (2012) Doublecortin and doublecortin-like are expressed in overlapping and non-overlapping neuronal cell population: Implications for neurogenesis. J Comp Neurol 520:2805-2823.

Saghatelyan A, de CA, Schachner M, Lledo PM (2004) Tenascin-R mediates activity-dependent recruitment of neuroblasts in the adult mouse forebrain. Nat Neurosci 7:347-356.

Sah A, Schmuckermair C, Sartori SB, Gaburro S, Kandasamy M, Irschick R, Klimaschewski L, Landgraf R, Aigner L, Singewald N (2012) Anxiety- rather than depression-like behavior is associated with adult neurogenesis in a female mouse model of higher trait anxiety- and comorbid depression-like behavior. Transl Psychiatry 2:e171.

Sahay A, Hen R (2007) Adult hippocampal neurogenesis in depression. Nat Neurosci 10:1110-1115.

Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, Fenton AA, Dranovsky A, Hen R (2011a) Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature 472:466-U539.

Sahay A, Wilson DA, Hen R (2011b) Pattern Separation: A Common Function for New Neurons in Hippocampus and Olfactory Bulb. Neuron 70:582-588.

Samuels BA, Hen R (2011) Neurogenesis and affective disorders. Eur J Neurosci 33:1152-1159.

Sanai N, Berger MS, Garcia-Verdugo JM, varez-Buylla A (2007) Comment on "Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension". Science %19;318:393.

Sanchez E, Vargas MA, Singru PS, Pascual I, Romero F, Fekete C, Charli JL, Lechan RM (2009) Tanycyte Pyroglutamyl Peptidase II Contributes to Regulation of the Hypothalamic-Pituitary-Thyroid Axis through Glial-Axonal Associations in the Median Eminence. Endocrinology 150:2283-2291.

Sandeman R, Sandeman D (2000) "Impoverished" and "enriched" living conditions influence the proliferation and survival of neurons in crayfish brain. Journal of Neurobiology 45:215-226.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805-809.

Sapolsky RM (2004) Is impaired neurogenesis relevant to the affective symptoms of depression? Biol Psychiatry 56:137-139.

Sapolsky RM (2000) Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Archives of General Psychiatry 57:925-935.

Sapolsky RM (2001) Depression, antidepressants, and the shrinking hippocampus. Proceedings of the National Academy of Sciences of the United States of America 98:12320-12322.

Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia ADR, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR (2006) Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. Proceedings of the National Academy of Sciences of the United States of America 103:17501-17506.

Schaar BT, Kinoshita K, McConnell SK (2004) Doublecortin microtubule affinity is regulated by a balance of kinase and phosphatase activity at the leading edge of migrating neurons. Neuron 41:203-213.

Schenk GJ, Engels B, Zhang YP, Fitzsimons CP, Schouten T, Kruidering M, de Kloet ER, 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 26:3411-3420.

Schoenfeld TJ, Gould E (2012) Stress, stress hormones, and adult neurogenesis. Exp Neurol 233:12-21.

Schwartz WJ, Reppert SM (1985) Neural regulation of the circadian vasopressin rhythm in cerebrospinal fluid: a pre-eminent role for the suprachiasmatic nuclei. J Neurosci 5:2771-2778.

Scott JP (1966) Agonistic behavior of mice and rats: a review. Am Zool 6:683-701.
Scotto LS, Strambi C, Strambi A, Charpin P, Augier R, Aouane A, Cayre M (2000) Influence of environmental stimulation on neurogenesis in the adult insect brain. J Neurobiol 45:162-171.

Seibler J, Kleinridders A, Kuter-Luks B, Niehaves S, Bruning JC, Schwenk F (2007) Reversible gene knockdown in mice using a tight, inducible shRNA expression system. Nucleic Acids Res 35:e54.

Seri B, Garcia-Verdugo JM, Collado-Morente L, Mcewen BS, varez-Buylla A (2004) Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. J Comp Neurol 478:359-378.

Seri B, Garcia-Verdugo JM, Mcewen BS, varez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153-7160.

Shapiro LA, Ng K, Zhou QY, Ribak CE (2009) Subventricular zone-derived, newly generated neurons populate several olfactory and limbic forebrain regions. Epilepsy & Behavior 14:74-80.

Sheline YI, Sanghavi M, Mintun MA, Gado MH (1999) Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. Journal of Neuroscience 19:5034-5043.

Sheline YI, Wang PW, Gado MH, Csernansky JG, Vannier MW (1996) Hippocampal atrophy in recurrent major depression. Proceedings of the National Academy of Sciences of the United States of America 93:3908-3913.

Shen HM, Glass JD, Seki T, Watanabe M (1999) Ultrastructural analysis of polysialylated neural cell adhesion molecule in the suprachiasmatic nuclei of the adult mouse. Anatomical Record 256:448-457.

Shen HM, Watanabe M, Tomasiewicz H, Rutishauser U, Magnuson T, Glass JD (1997) Role of neural cell adhesion molecule and polysialic acid in mouse circadian clock function. Journal of Neuroscience 17:5221-5229.

Shin E, Kashiwagi Y, Kuriu T, Iwasaki H, Tanaka T, Koizumi H, Gleeson JG, Okabe S (2013) Doublecortin-like kinase enhances dendritic remodelling and negatively regulates synapse maturation. Nat Commun 4:1440.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12:578-584.

Shu TZ, Tseng HC, Sapir T, Stern P, Zhou Y, Sanada K, Fischer A, Coquelle FM, Reiner O, Tsai LH (2006) Doublecortin-like kinase controls neurogenesis by regulating mitotic spindles and M phase progression. Neuron 49:25-39.

Sidibe A, Mullier A, Chen P, Baroncini M, Boutin JA, Delagrange P, Prevot V, Jockers R (2010) Expression of the orphan GPR50 protein in rodent and human dorsomedial hypothalamus, tanycytes and median eminence. J Pineal Res 48:263-269.

Silverman MA, 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 274:2631-2636.

Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. Nature 476:458-461.

Sossey-Alaoui K, Hartung AJ, Guerrini R, Manchester DK, Posar A, Puche-Mira A, Andermann E, Dobyns WB, Srivastava AK (1998) Human doublecortin (DCX) and the homologous gene in mouse encode a putative Ca2+-dependent signaling protein which is mutated in human X-linked neuronal migration defects. Hum Mol Genet 7:1327-1332.

Sossey-Alaoui K, Srivastava AK (1999) DCAMKL1, a brain-specific transmembrane protein on 13q12.3 that is similar to doublecortin (DCX). Genomics 56:121-126.

Sousa-Ferreira L, Alvaro AR, Aveleira C, Santana M, Brandao I, Kugler S, de Almeida LP, Cavadas C (2011) Proliferative hypothalamic neurospheres express NPY, AGRP, POMC, CART and Orexin-A and differentiate to functional neurons. PLoS One 6:e19745.

Steffens DC, Byrum CE, McQuoid DR, Greenberg DL, Payne ME, Blitchington TF, MacFall JR, Krishnan KRR (2000) Hippocampal volume in geriatric depression. Biological Psychiatry 48:301-309.

Steiner B, Klempin F, Wang L, Kott M, Kettenmann H, Kempermann G (2006) Type-2 cells as link between glial and neuronal lineage in adult hippocampal neurogenesis. Glia 54:805-814.

Steinsapir J, Bianco AC, Buettner C, Harney J, Larsen PR (2000) Substrate-induced down-regulation of human type 2 deiodinase (hD2) is mediated through proteasomal degradation and requires interaction with the enzyme's active center. Endocrinology 141:1127-1135.

Stockmeier CA, Mahajan GJ, Konick LC, Overholser JC, Jurjus GJ, Meltzer HY, Uylings HBM, Friedman L, Rajkowska G (2004) Cellular changes in the postmortem hippocampus in major depression. Biological Psychiatry 56:640-650.

Stranahan AM, Khalil D, Gould E (2006) Social isolation delays the positive effects of running on adult neurogenesis. Nature Neuroscience 9:526-533.

Surget A, Saxe M, Leman S, Ibarguen-Vargas Y, Chalon S, Griebel G, Hen R, Belzung C (2008) Drug-dependent requirement of hippocampal neurogenesis in a model of depression and of antidepressant reversal. Biological Psychiatry 64:293-301.

Tanaka T, Koizumi H, Gleeson JG (2006) The Doublecortin and Doublecortin-like kinase 1 genes cooperate in murine hippocampal development. Cerebral Cortex 16:I69-I73.

Tanapat P, Hastings NB, Rydel TA, Galea LAM, Gould E (2001) Exposure to fox odor inhibits cell proliferation in the hippocampus of adult rats via an adrenal hormone-dependent mechanism. Journal of Comparative Neurology 437:496-504.

Taupin P (2007) BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev 53:198-214.

Taupin P, Gage FH (2002) Adult neurogenesis and neural stem cells of the central nervous system in mammals. J Neurosci Res 69:745-749.

Toni N, Laplagne DA, Zhao C, Lombardi G, Ribak CE, Gage FH, Schinder AF (2008) Neurons born in the adult dentate gyrus form functional synapses with target cells. Nat Neurosci 11:901-907.

Toni N, Sultan S (2011) Synapse formation on adult-born hippocampal neurons. Eur J Neurosci 33:1062-1068.

Toni N, Teng EM, Bushong EA, Aimone JB, Zhao C, Consiglio A, van PH, Martone ME, Ellisman MH, Gage FH (2007) Synapse formation on neurons born in the adult hippocampus. Nat Neurosci 10:727-734.

Treves A, Tashiro A, Witter MP, Moser EI (2008) What is the mammalian dentate gyrus good for? Neuroscience 154:1155-1172.

Tronel S, Belnoue L, Grosjean N, Revest JM, Piazza PV, Koehl M, Abrous DN (2012) Adult-born neurons are necessary for extended contextual discrimination. Hippocampus 22:292-298.

Tu HM, Kim SW, Salvatore D, Bartha T, Legradi G, Larsen PR, Lechan RM (1997) Regional distribution of type 2 thyroxine deiodinase messenger ribonucleic acid in rat hypothalamus and pituitary and its regulation by thyroid hormone. Endocrinology 138:3359-3368.

Tuy FP, Saillour Y, Kappeler C, Chelly J, Francis F (2008) Alternative transcripts of Dclk1 and Dclk2 and their expression in doublecortin knockout mice. Dev Neurosci 30:171-186.

Ubeda-Banon I, Novejarque A, Mohedano-Moriano A, Pro-Sistiaga P, Insausti R, Martinez-Garcia F, Lanuza E, Martinez-Marcos A (2008) Vomeronasal inputs to the rodent ventral striatum. Brain Research Bulletin 75:467-473.

Urbach A, Robakiewicz I, Baum E, Kaczmarek L, Witte OW, Filipkowski RK (2013) Cyclin D2 knockout mice with depleted adult neurogenesis learn Barnes maze task. Behav Neurosci 127:1-8.

van Hooijdonk LW, Ichwan M, Dijkmans TF, Schouten TG, de Backer MW, Adan RA, Verbeek FJ, Vreugdenhil E, Fitzsimons CP (2009) Lentivirus-mediated transgene delivery to the hippocampus reveals sub-field specific differences in expression. BMC Neurosci 10:2.

van Praag H, Christie BR, Sejnowski TJ, Gage FH (1999a) Running enhances neurogenesis, learning, and long-term potentiation in mice. Proceedings of the National Academy of Sciences of the United States of America 96:13427-13431.

van Praag H, Kempermann G, Gage FH (1999b) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nature Neuroscience 2:266-270.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030-1034.

van Rijzingen I, Gispen WH, Spruijt BM (1995) Olfactory bulbectomy temporarily impairs Morris maze performance: an ACTH(4-9) analog accellerates return of function. Physiol Behav 58:147-152.

Varea E, Castillo-Gomez E, Gomez-Climent MA, Guirado R, Blasco-Ibanez JM, Crespo C, Martinez-Guijarro FJ, Nacher J (2009) Differential evolution of PSA-NCAM expression during aging of the rat telencephalon. Neurobiology of Aging 30:808-818.

Veenema AH, de Kloet ER, de Wilde MC, Roelofs AJ, Kawata M, Buwalda B, Neumann ID, Koolhaas JM, Lucassen PJ (2007) Differential effects of stress on adult hippocampal cell proliferation in low and high aggressive mice. J Neuroendocrinol 19:489-498.

Verissimo CS, Elands R, Cheng S, Saaltink DJ, Ter Horst JP, Alme MN, Pont C, van de WB, Havik B, Fitzsimons CP, Vreugdenhil E (2013) Silencing of Doublecortin-Like (DCL) Results in Decreased Mitochondrial Activity and Delayed Neuroblastoma Tumor Growth. PLoS One 8:e75752.

Verissimo CS, Molenaar JJ, Meerman J, Puigvert JC, Lamers F, Koster J, Danen EHJ, van de Water B, Versteeg R, Fitzsimons CP, Vreugdenhil E (2010a) Silencing of the microtubule-associated proteins doublecortin-like and doublecortin-like kinase-long induces apoptosis in neuroblastoma cells. Endocrine-Related Cancer 17:399-414.

Verissimo CS, Molenaar JJ, Meerman J, Puigvert JC, Lamers F, van Kuik-Romeijn P, Danen EHJ, van de Water B, Versteeg R, Fitzsimons CP, Vreugdenhil E (2010b) Exploring A New Therapy for Neuroblastoma: Silencing of Doublecortin-Like Kinase Using Rna-Interference. Neuro-Oncology 12:64.

Vollmayr B, Simonis C, Weber S, Gass P, Henn F (2003) Reduced cell proliferation in the dentate gyrus is not correlated with the development of learned helplessness. Biological Psychiatry 54:1035-1040.

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

Vreugdenhil E, Engels B, Middelburg R, van Koningsbruggen S, Knol J, Veldhuisen B, de Kloet ER (2001) Multiple transcripts generated by the DCAMKL gene are expressed in the rat hippocampus. Molecular Brain Research 94:67-74.

Vreugdenhil E, Kolk SM, Boekhoorn K, Fitzsimons CP, Schaaf M, Schouten T, Sarabdjitsingh A, Sibug R, Lucassen PJ (2007) Doublecortin-like, a microtubule-associated protein expressed in radial glia, is crucial for neuronal precursor division and radial process stability. European Journal of Neuroscience 25:635-648.

Waclaw RR, Allen ZJ, Bell SM, Erdelyi F, Szabo G, Potter SS, Campbell K (2006) The zinc finger transcription factor Sp8 regulates the generation and diversity of olfactory bulb interneurons. Neuron 49:503-516.

Walker TL, Yasuda T, Adams DJ, Bartlett PF (2007) The doublecortin-expressing population in the developing and adult brain contains multipotential precursors in addition to neuronal-lineage cells. Journal of Neuroscience 27:3734-3742.

Wang C, Liu F, Liu YY, Zhao CH, You Y, Wang L, Zhang J, Wei B, Ma T, Zhang Q, Zhang Y, Chen R, Song H, Yang Z (2011) Identification and characterization of neuroblasts in the subventricular zone and rostral migratory stream of the adult human brain. Cell Res 21:1534-1550.

Wang JW, David DJ, Monckton JE, Battaglia F, Hen R (2008) Chronic fluoxetine stimulates maturation and synaptic plasticity of adult-born hippocampal granule cells. J Neurosci 28:1374-1384.

Wang LP, Kempermann G, Kettenmann H (2005) A subpopulation of precursor cells in the mouse dentate gyrus receives synaptic GABAergic input. Mol Cell Neurosci 29:181-189.

Warner-Schmidt JL, Duman RS (2006) Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment. Hippocampus 16:239-249.

Watanabe Y, Gould E, Mcewen BS (1992) Stress Induces Atrophy of Apical Dendrites of Hippocampal Ca3 Pyramidal Neurons. Brain Research 588:341-345.

Weimer JM, Anton ES (2006) Doubling up on microtubule stabilizers: Synergistic functions of doublecortin-like kinase and doublecortin in the developing cerebral cortex. Neuron 49:3-4.

Welsh DK, Takahashi JS, Kay SA (2010) Suprachiasmatic Nucleus: Cell Autonomy and Network Properties. Annual Review of Physiology 72:551-577.

Werner L, Muller-Fielitz H, Ritzal M, Werner T, Rossner M, Schwaninger M (2012) Involvement of doublecortin-expressing cells in the arcuate nucleus in body weight regulation. Endocrinology 153:2655-2664.

Wibrand K, Messaoudi E, Havik B, Steenslid V, Lovlie R, Steen VM, Bramham CR (2006) Identification of genes co-upregulated with Arc during BDNF-induced long-term potentiation in adult rat dentate gyrus in vivo. Eur J Neurosci 23:1501-1511.

Wichterle H, Garcia-Verdugo JM, varez-Buylla A (1997) Direct evidence for homotypic, glia-independent neuronal migration. Neuron 18:779-791.

Wiersinga WM, Chopra IJ (1982) Radioimmunoassay of thyroxine (T4), 3,5,3'-triiodothyronine (T3), 3,3',5'-triiodothyronine (reverse T3, rT3), and 3,3'-diiodothyronine (T2). Methods Enzymol 84:272-303.

Wilson DA, Best AR, Sullivan RM (2004) Plasticity in the olfactory system: Lessons for the neurobiology of memory. Neuroscientist 10:513-524.

Wiskott L, Rasch MJ, Kempermann G (2006) A functional hypothesis for adult hippocampal neurogenesis: avoidance of catastrophic interference in the dentate gyrus. Hippocampus 16:329-343.

Wojtowicz JM, Kee N (2006) BrdU assay for neurogenesis in rodents. Nat Protoc 1:1399-1405.

Wong EY, Herbert J (2004) The corticoid environment: a determining factor for neural progenitors' survival in the adult hippocampus. Eur J Neurosci 20:2491-2498.

Xu HY, Chen Z, He J, Haimanot S, Li XK, Dyck L, Li XM (2006) Synergetic effects of quetiapine and venlafaxine in preventing the chronic restraint stress-induced decrease in cell proliferation and BDNF expression in rat hippocampus. Hippocampus 16:551-559.

Xu Y, Tamamaki N, Noda T, Kimura K, Itokazu Y, Matsumoto N, Dezawa M, Ide C (2005) Neurogenesis in the ependymal layer of the adult rat 3rd ventricle. Experimental Neurology 192:251-264.

Young EA, Haskett RF, MurphyWeinberg V, Watson SJ, Akil H (1991) Loss of Glucocorticoid Fast Feedback in Depression. Archives of General Psychiatry 48:693-699.

Zhang CL, Zou Y, He W, Gage FH, Evans RM (2008) A role for adult TLX-positive neural stem cells in learning and behaviour. Nature 451:1004-1007.

Zhang J, Giesert F, Kloos K, Vogt Weisenhorn DM, Aigner L, Wurst W, Couillard-Despres S (2010) A powerful transgenic tool for fate mapping and functional analysis of newly generated neurons. BMC Neurosci 11:158.

Zhang XM, Cai Y, Chu Y, Chen EY, Feng JC, Luo XG, Xiong K, Struble RG, Clough RW, Patrylo PR, Kordower JH, Yan XX (2009) Doublecortin-expressing cells persist in the associative cerebral cortex and amygdala in aged nonhuman primates. Front Neuroanat 3:17.

Zhao C, Teng EM, Summers RG, Jr., Ming GL, Gage FH (2006) Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci 26:3-11.

Zhao M, Janson Lang AM (2009) Bromodeoxyuridine infused into the cerebral ventricle of adult mice labels nigral neurons under physiological conditions--a method to detect newborn nerve cells in regions with a low rate of neurogenesis. J Neurosci Methods 184:327-331.

Zhao M, Momma S, Delfani K, Carlen M, Cassidy RM, Johansson CB, Brismar H, Shupliakov O, Frisen J, Janson AM (2003) Evidence for neurogenesis in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A 100:7925-7930.