Doublecortin-like knockdown in the adult mouse brain: implications for neurogenesis, neuroplasticity and behaviour

Saaltink, D.J.

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.

Table of contents

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).

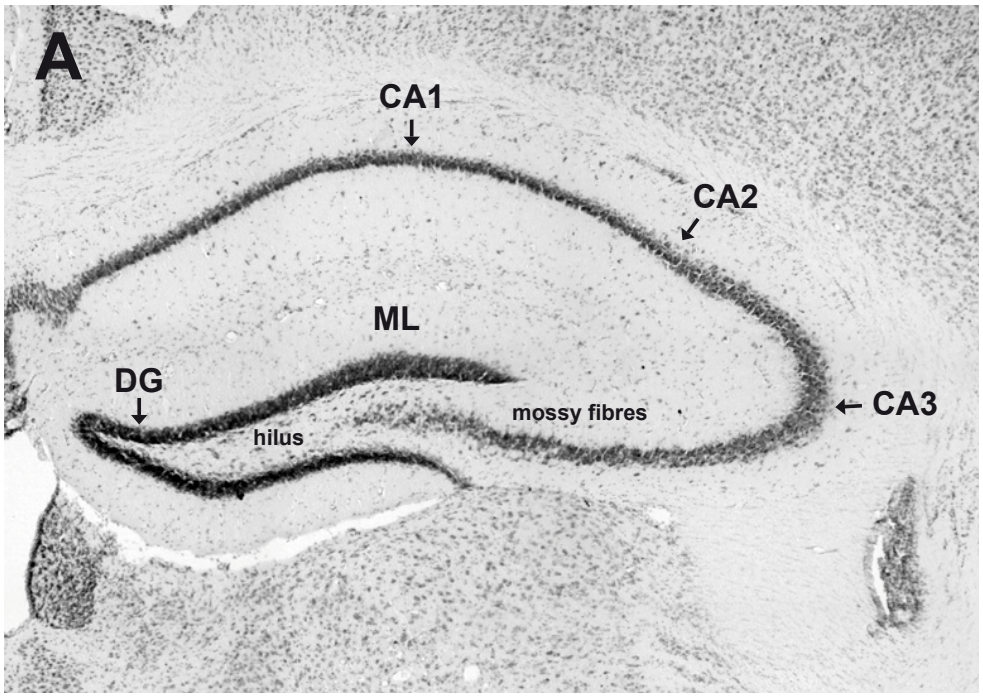
How the specification into several different interneurons is defined, has been intensively studied. Some crucial factors in the development of different PGC types are Paired box 6 (Pax6), a homeobox transcription factor, Olig2 (Hack et al., 2005;Kohwi et al., 2005), and the transcription factor Sp8 (Waclaw 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.

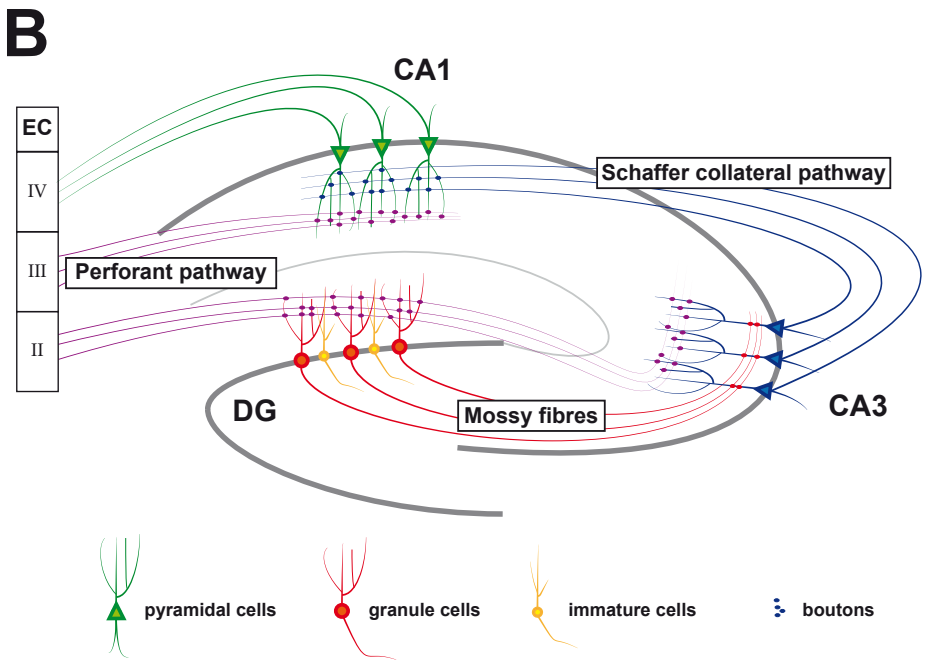
2.2 Hippocampal neurogenesis

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

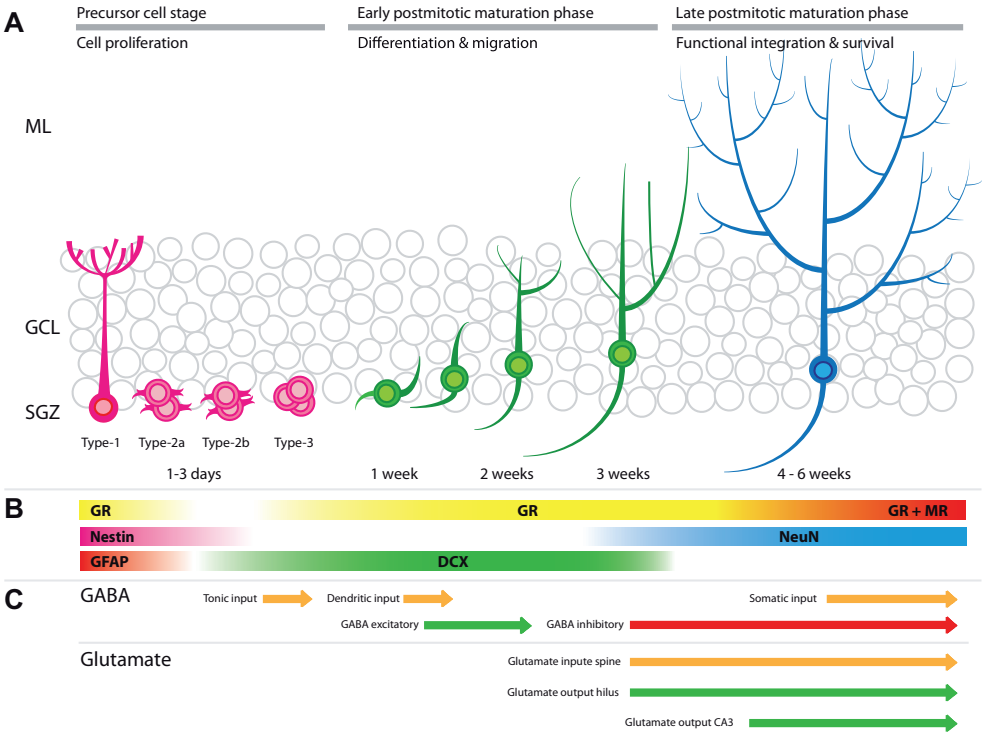


Figure 2: Overview of the adult neurogenesis process in the hippocampal dentate gyrus. A: The process can be divided into 3 different program sections. In the precursor cell stage, stem cells (Type-1) residing in the subgranular zone (SGZ) give birth to neuronal precursor cells (NPCs). Three different 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

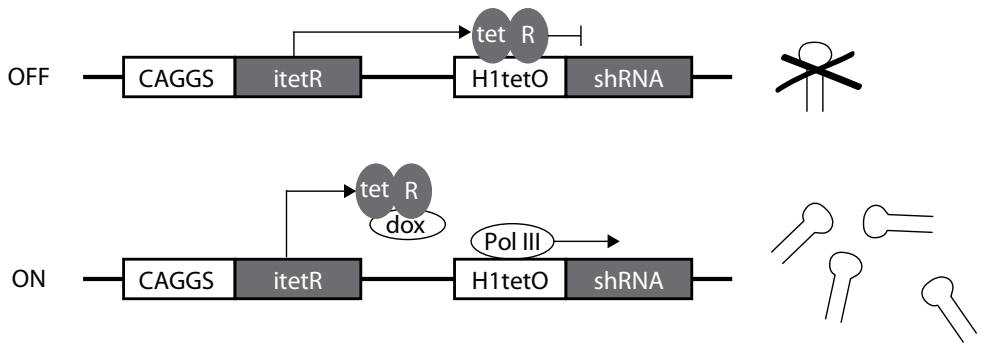


Figure 3: Inducible and reversible shRNA transcribing construct. In a normal condition (OFF), the CAGGS promoter transcribes the tet repressor (tetR) which blocks the H1tetO promoter. No shRNA is transcribed. When dox is applied (ON), dox binds to the tetR which can no longer bind to the H1tetO promoter. 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.