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Functional Analysis of Desmoplakin During Postnatal Development of the Hippocampus

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To My Family, my stalwarts

CONTENTS

ABBREVIATIONS ..... **vii**

1.INTRODUCTION **1**
1.1 Hippocampus **1**
1.2 Morphology and Connectivity of the hippocampus **1**
1.3 Development of the dentate gyrus **4**
1.4 Hippocampus in learning and memory **9**
1.5 Transcription factor Bcl11b **10**
1.6 Desmoplakin **11**
1.7 Aim **13**

2. Materials and Methods **15**
2.1 Materials **15**
2.1.1 Chemical and Enzymes 15
2.1.2 Solutions and Reagents 15
2.1.3 Plasmid vectors 17
2.1.4 Oligonucleotides 18
2.1.5 Antibodies 18
2.1.6 Culture media 19
2.1.7 Cell culture media 19
2.1.8 Slice culture media 19
2.2 Methods **19**
2.2.1 Preparation of plasmid DNA and DNA fragments 19
2.2.2 Isolation of genomic DNA from tail and ear tissue 20
2.2.3 Restriction digestion, ligation of DNA fragments and transformation of competent bacteria 20
2.2.4 Polymerase Chain Reaction (PCR) 20
2.2.5 RT-PCR 21
2.2.6 In-vitro transcription reaction and DIG labeling of RNA probe 21
2.2.7 RNA isolation and purification 22
2.2.8 First strand cDNA synthesis 22
2.2.9 Preparation of mouse tissue 23
2.2.10 Preparation of methacrylate sections 23
2.2.11 Cresyl violet staining of methacrylate sections 24
2.2.12 Preparation of frozen sections 24
2.2.13 Immunohistology 24
2.2.14 Detection of cell proliferation and apoptosis 25
2.2.15 Golgi staining 26
2.2.16 Timm staining 26
2.2.17 In-situ hybridization 27
2.2.18 Behavioral experiments 27
2.2.18.1 Study of emotional behavior – open field test 27
2.2.18.2 Study of cognitive abilities – radial maze 28
2.2.19 Neuronal cell culture 29
2.2.20 Ex-utero electroporation and slice culture 30
2.2.21 Statistical analysis 31
2.2.22 Approval for animal experiments 31

2.2.23 Funding 31

3. Results 33
3.1 Analysis of hippocampal desmoplakin expression 33
3.2 Analysis of mice with a forebrain specific mutation of desmoplakin 34
3.2.1 Characterization of desmoplakin mutants 35
3.2.2 Morphological analysis of the dentate gyrus of desmoplakin mutants . 36
3.2.3 Analysis of apoptosis in the dentate gyrus of desmoplakin mutants 39
3.2.4 Analysis of proliferation of dentate gyrus progenitor cells 41
3.2.5 Analysis of granule cell differentiation in desmoplakin mutants..... 43
3.2.6 Analysis of hippocampal circuitry of desmoplakin mutants 45
3.2.6.1 Analysis of dendritic spines in desmoplakin mutants 46
3.2.6.2 Mossy fiber distribution and thorny excrescences 47
3.2.7 Analysis of learning and memory behavior of desmoplakin mutants ... 48
3.2.7.1 Open field (exploration) 48
3.2.7.2 Spatial learning 48
3.3 Slice culture analysis of desmoplakin over-expression 50
3.4 Rescue of the Bcl11b mutants phenotype by desmoplakin 53

4. Discussion 56
4.1 Desmoplakin is essential for postnatal neurogenesis in the dentate gyrus 57
4.2 Impaired progenitor cell proliferation of desmoplakin mutants 58
4.3 Reduced granule cells apoptosis of desmoplakin mutants 62
4.4 Desmoplakin is essential for differentiation of granule cells 63
4.5 Desmoplakin is essential for dendritic spine development 65
4.6 Emotional and cognitive learning behavior in desmoplakin mutants 66
4.7 Phenotype comparison of desmoplakin and Bcl11b mutants 68
4.8 Future directions 70

5. Summary 73

6. Bibliography 75

A. Acknowledgments 93

B. Curriculum Vitae 95

ABBREVIATIONS

BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine
°C	Degree Celsius
cDNA	Complementary DNA
CA	Cornu Ammonis
cm	Centimeter
DEPC	Diethylpyrocarbonate
DG	Dentate gyrus
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
G	Grams
H	Hour
HBSS	Hank's Balanced Salt Solution
l	Liter
m	Milli
M	Molar
MEM	Minimum Essential Media
Min	Minutes
µ	Micro
mRNA	Messenger Ribonucleic acid
n	Nano
NGF	Nerve growth factor
P	Postnatal day
PBS	Phosphate buffered saline
PBT-X	Triton X-100 in phosphate buffered saline
PCR	Polymerase Chain Reaction
Pen/strep	Penicillin Streptomycin
PFA	Paraformaldehyde
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SSC	Saline-sodium citrate
TE	Tris-EDTA buffer
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
tRNA	Transfer ribonucleic acid

1 Introduction

1.1 Hippocampus

The hippocampus belongs to the phylogenetically one of the oldest part of the cerebral cortex, the archicortex. The hippocampus is located in the medial aspect of the temporal lobe, forming the medial wall of the lateral ventricle. As the central part of the limbic system, the hippocampus receives most of the highly pre-processed information from all sensory modalities via the entorhinal cortex as well as from the cingulate gyrus and the hypothalamus. In addition to the connections to the cognitive area of the neocortex, the hippocampus plays a significant role in higher brain functions like memory consolidation (Squire LR. 1986). The hippocampal formation includes the dentate gyrus, the hippocampal cortex consisting of the regions of the Ammon's horn, subiculum, presubiculum, parasubiculum and entorhinal cortex. Following the nomenclature of Lorente de Nó (1934), the hippocampal cortex neurons due to their density and neuronal connections can be further subdivided into sectors CA1, CA2 and CA3 (Cornu Ammonis). In the dentate gyrus, one can distinguish three histological layers: a relatively cell-free layer, the molecular layer or Stratum moleculare (predominantly occupied by apical dendrites of the dentate granule cells), the granule cell layer or stratum granulosum, consisting largely of densely packed granule cells and the third layer, the polymorphic layer of the hilus, which contains a high proportion of mossy cells and various interneuron types.

1.2 Morphology and Connectivity in the hippocampus

The cytoarchitectonic structure of the hippocampus is relatively simple and well conserved among mammals (Insausti et al, 1993). It is characterized by a tight U-shaped structure; one end of it is embedded in the backward facing V or U shaped granule cell band of the dentate gyrus and the other end forms the pyramidal cell band (stratum pyramidale) of the cornu ammonis of the hippocampus. The dendrites from these two major cell types of the hippocampus are spread across the cell-free

layers: the apical dendrites of the pyramidal cells spread across the stratum radiatum and the stratum lacunosum moleculare while their basal dendrites spanning to the stratum oriens. The dendrites of the granule cells form the stratum moleculare of the dentate gyrus, which is further subdivided into an inner and outer layer.

The hippocampus is linked to other brain regions by extrinsic connections through several fiber tracts. Through intrinsic synaptic connections the hippocampus is connected to its own region (Amaral et al, 1989). The dentate gyrus acts as a primary gateway regulating the cortical input to the hippocampus. The major afferents to the hippocampus are the cortical, glutamatergic afferents of the entorhinal cortex, the so-called perforant pathway. These afferents project through angular bundles, traverse the pyramidal cells of the subiculum to reach the outer molecular layer of the dentate gyrus. These fibers then terminate at the distal dendrites of the granule cells. The proximal dendrites of the granule cells of the inner molecular layer are however innervated by contralateral or ipsilateral, glutamatergic commissural and association fibers of the hilus (Blackstad, 1956, Blackstad, 1958, Swanson et al, 1981), thus generating the layer specific innervation pattern of the molecular layer of the dentate gyrus (Fig.1 B). In addition some of the afferents of the entorhinal cortex also terminate in the stratum lacunosum moleculare of the CA1 and CA3 regions of the hippocampus.

The axons of the granule cells form the mossy fiber bundles that are projecting via the hilus to the CA3 region of the hippocampal cortex and terminate mostly on the apical dendrites of the pyramidal cells. In addition they also terminate partly at the mossy cells residing in the hilus. The CA3 pyramidal cell axons, the Schaffer collaterals, in turn traverse the CA2 region and innervate the CA1 region, whose axons in turn project back to the entorhinal cortex. This allows to establish a simple, mostly uni-directional loop known as tri-synaptic loop, which is considered to be the principal circuitry of the hippocampal formation: Entorhinal cortex afferents terminating on the granule cells of the dentate gyrus via the perforant pathway (synapse 1), dentate granule cell axons projecting via the mossy fiber tract to CA3 pyramidal cells (synapse 2), Schaffer collaterals of CA3 pyramidal neurons innervating the CA1 cells (synapse 3) (Fig.1 A).

The propagation of action potential within the hippocampus by excitatory glutamatergic neurons is regulated by a variety of inhibitory interneurons. The

somata of various interneuron types, unlike the principal cells in the hippocampus, are not arranged in specific layers, although their axons terminate specifically in various compartments of the granule cells, for example, the basket cell axons terminate at the somata of the granule cells, while the axo-axonic cells terminate at the initial segment of the mossy fibers (Soriano et al, 1989, Ribak et al, 1983, Freund et al, 1996, Houser CR, 2007, McBain et al, 2001). The interneurons are classified into different subtypes based on specific criteria, e.g. their morphology, distribution, physiology and expression of various neuro-peptides or calcium-binding proteins (Houser CR 2007). Despite such diversity, a unifying feature of the dentate gyrus interneurons is their use of GABA (γ-aminobutyric acid) as their major neurotransmitter.

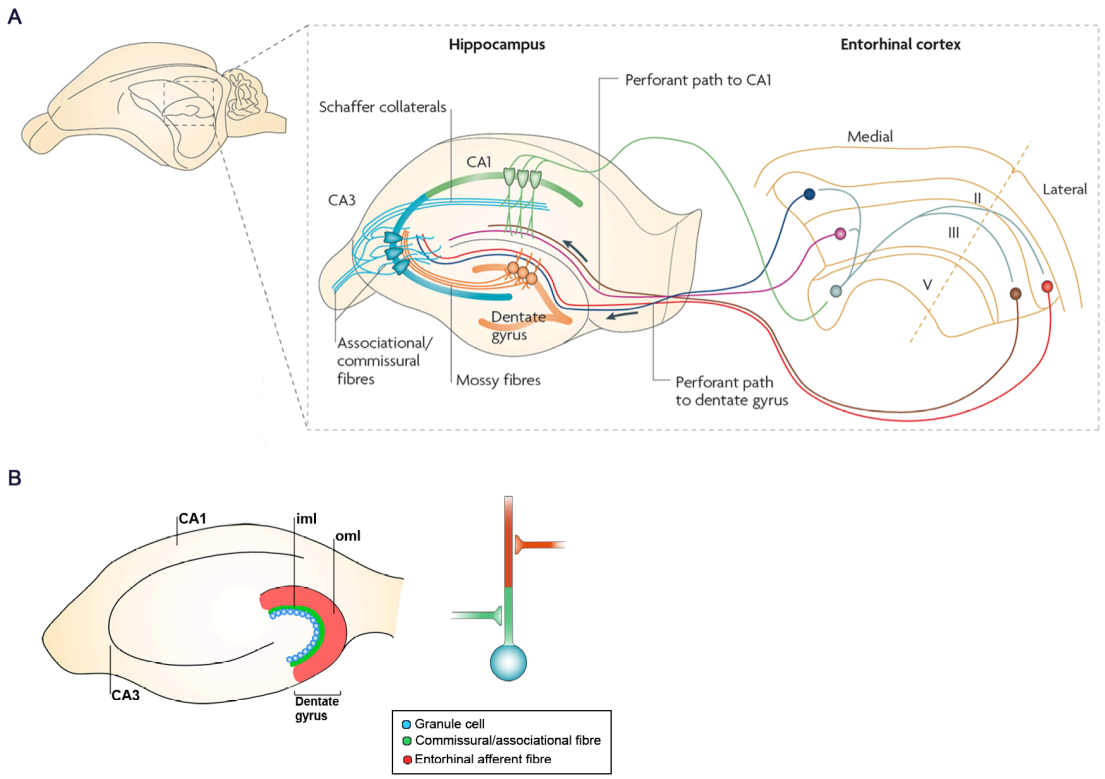

Fig 1.1 Schematic representation of the major uni-directional pathway of the hippocampus, the tri-synaptic pathway.
A. The intrinsic connection is characterized by: 1. Glutamatergic afferents from the lateral and medial entorhinal cortex innervate the outer and middle third of the dendritic tree of the granule cells of the dentate gyrus (blue and orange line). 2. The axons of granule cells project via the mossy fiber tract to the proximal apical dendrites of the CA3 pyramidal cells of the hippocampus (brown line). 3. CA3 pyramidal cell axons project ipsilateral to the CA1 pyramidal cells through Schaffer collaterals (Modified from Neves et al, 2008, page 65-75)(Reprinted by the permission from Macmillan Publishers Ltd: Nature Review Neuroscience, Synaptic plasticity, memory and the hippocampus: a neural network approach to causality, copyright 2008 January).
B. Specific layer structure and innervation of the dentate gyrus. Afferent fibers from the lateral and medial entorhinal cortex innervate the granule cells and terminate at the distal part of the dendritic tree (oml - outer molecular layer). The commissural/ associational fibers originating from the mossy cells in the contralateral and ipsilateral hilar region terminate in the proximal dendritic tree of the granule cells (iml- inner molecular layer) (Modified from, Förster et al, 2006, page 259-67) (Reprinted by the permission from Macmillan Publishers Ltd: Nature Review Neuroscience, Laminating the hippocampus, copyright 2006 April).

1.3 Development of dentate gyrus

The first granule neurons are born at E15 in the subventricular zone of the primary dentate neuroepithelium in the vicinity of the cortical hem, which migrate to the

dentate anlage and expand to generate the dentate precursor pool. This becomes apparently adjacent to the dentate notch at mid-gestation termed the secondary matrix (Altman and Bayer, 1990, Altman et al, 1965). Immediately adjacent to the dentate anlage resides the pool of immature neurons, the so-called Cajal-Retzius cells and radial glial cells (Barry et al, 2008). Both these cell types form a scaffold on which the precursor cells migrate to generate the future dentate gyrus (Rickmann et al 1987, Alcantara et al, 1998, Del Rio et al, 1997). These dividing precursor cells are destined to settle in the hilus and subgranular zone, migrating from the dentate notch radially on the primary radial glial scaffolding in a subpial route towards the dentate anlage (first dentate migration). The radial nature of this migration, along the horseshoe-shaped scaffolding of the radial fibers (Rickmann et al 1987), leads to a neurogenic gradient, such that the cells migrating first position themselves in the dorsal (supra-pyramidal layer) blade and the cells migrating later are positioned in the ventral (infra-pyramidal layer) blade (Eckenhoff et al, 1984, Nowakowski et al, 1981, Altman et al, 1990, Bayer SA, 1980) where they begin to differentiate into mature dentate gyrus cells (Fig 2). Postnatally, migrating progenitor cells increasingly settle in the hilar region of the dentate gyrus (second dentate migration). In contrast to the first dentate migrating cells, the second dentate migrating cells retain the ability to form neuronal progenitor cells when they reach the dentate gyrus. This newly formed neurogenic niche of the hilus beneath the condensing dentate granule cell layer was termed the tertiary matrix by Altman and Bayer (1990a, b). More than 80% of granule cells are born in the first four weeks of postnatal dentate gyrus development from this tertiary matrix, which develops its own radial glial matrix. Newborn cells migrate along these radially oriented fibers from the hilus and differentiate into mature granule cells. By postnatal day 10, the tertiary matrix and the radial glia cells are reorganized (Fig 2 C), such that the position of the progenitor cells is shifted from the hilus to the subgranular zone, located at the innermost border between the condensing granule cell layer and the hilus. In adulthood, granule cells continue to be born in this specialized niche, migrate a short distance into the granular zone and functionally integrate by forming neuronal circuits.

This unique development of the dentate gyrus, i.e. the establishment of a secondary neurogenic niche in the hilus and later in the subgranular zone requires the expression of a set of specific developmentally regulated genes. The secondary

neurogenic niche also is necessary for maintaining the proliferation of the progenitor cell population as well as to control the migration and differentiation of the newborn neurons.

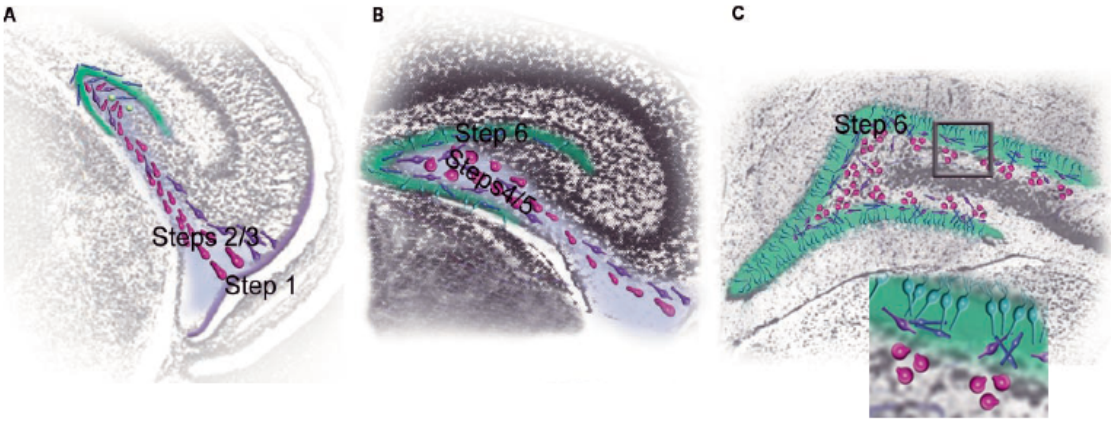

Fig 1.2. **Schematic representation of the morphogenesis of the dentate gyrus**.
The morphogenesis of the dentate gyrus includes the following steps: A. Formation of a pool of proliferating progenitor cells (secondary matrix) from the neuroepithelium. (step 1). Formation of the matrix provided by the radial glia and Cajal-Retzius, on this matrix the proliferating progenitor cells migrate towards the dentate anlage (step 2/3). B. Postnatal migration of mitotic progenitor cells from the neuroepithelium, which increasingly settle in the hilus region (second dentate migration). These cells remain in the mitotic stage and form the secondary neurogenic niche (tertiary matrix) within the dentate gyrus. C. At postnatal day 10, the tertiary matrix undergoes reorganization and the position of the mitotic progenitor cells shifts from the hilar region to the inner subgranular layer, where neurogenesis continues throughout adulthood (Modified from Li et al, 2005, page 93-99, with the permission from Karger AG, Basel).

The cortical hem is situated in the most caudomedial zone of the neuroepithelium and acts as a signaling center by expressing a gradient of transcription factors. These factors regulate the development, including the formation of the neurogenic niche of proliferating progenitor cells in the neuroepithelium (secondary matrix) and their subsequent migration to the dentate anlage. These factors include the members of the Wnt signaling pathway, such as LEF1 (Galceran et al, 2000, Lee et al, 2000, Zhou et al, 2004), Sonic Hedgehog (Machold et al, 2003, Ahn et al, 2005), the BMP (Bone morphogenetic protein) family (Furuta et al, 1997, Caronia et al, 2010) and various transcription factors, such as the homeobox genes Emx2 (Pellegrini et al, 1996, Yoshida et al, 1997), Lhx2 (Bulchand et al, 2001, Porter et al, 1997) and Lhx5 (Zhao et al, 1999). The Wnt and sonic hedgehog pathways have also essential functions during

adult neurogenesis in the dentate gyrus (Pozniak et al, 2006, Lie et al, 2005, Ahn et al, 2005, Lai et al, 2002).

In addition, most of the Cajal-Retzius cells of the cortical hem (Bielle et al, 2005, Zhao et al, 2006) are known to secret reelin, an extracellular matrix glycoprotein, which is essential for the differentiation of progenitor cells into radial glia and the orientation of its fibers during matrix formation (Hartfuss et al, 2003, Solecki DJ, 2012). This matrix is necessary for the migration and correct positioning of the immature neurons in the dentate gyrus. During early postnatal development of the dentate gyrus, reelin is essential for reorganizing the radial glial fibers after the formation of the tertiary matrix in the hilus. In mutants lacking reelin or having defects in the reelin signaling cascade, granule cells of the dentate gyrus fail to form a regular densely packed layer of cells, instead the cells are loosely distributed in the entire dentate gyrus (Standfield et al, 1979, Zhao et al, 2004, Förster et al, 2002)

The terminal differentiation of immature neurons into functional granule cells, mostly during postnatal development of the dentate gyrus, includes outgrowth of the axons (mossy fibers) of the granule cells to the CA3 region. During this time dendritogenesis takes place in the molecular layer of the dentate gyrus, followed by the formation of synaptic interconnections. The mature granule cells are functionally integrated into the hippocampal circuitry as early as the end of the first postnatal week (Jones SP et al, 2003).

The transcription factor NeuroD, a basic helix-loop-helix protein and homologue of the Drosophila atonal family, is essential for the specification and further maturation of the newborn progenitor cells. Mutation of *NeuroD* causes a defect in the formation of the granule cell layer of the dentate gyrus (Liu et al, 2000, Schwab et al, 2000, Gage 2010). While the formation of the early born precursor cell population from the neuroepithelium and the migration of the mutant cells to the putative dentate gyrus is largely unaffected, the mutation of *NeuroD* affects the terminal differentiation of these immature neurons. This affects the earliest born granule cells, so that cells migrating afterwards to the dentate gyrus form a loosely packed group of cells instead of a compact suprapyramidal layer. This defect leads to the disorganization of the tertiary matrix and also the impairment of the proliferation of mitotic migrating progenitor cells, which results in an increase of cell death causing a dysplastic dentate gyrus. Since only a small subpopulation of the mitotically active

precursor cells express NeuroD, it is believed that the impairment in proliferation of the progenitor cells is non-cell autonomous. The absence of an intact granule cell layer is thought be the secondary effect of the NeuroD mutation (Schwab et al, 2000, Miyata et al, 1999, Liu et al, 2000).

The armadillo protein β-catenin is essential for the dendritic development of immature granule cells (Goa et al, 2007) and this leads to the integration of the granule cells in the hippocampal circuitry. This signal molecule controls the transcription of downstream target genes of the Wnt signaling pathway in mitotic progenitor cells, which in turn influence the mitotic progenitor cell behavior in the postnatal dentate gyrus (Lie et al, 2005). In addition β-catenin has adhesive functions in the formation of cell contacts and is also involved in the stabilization of cytoskeletal elements, such as actin filaments. The conditional deletion of β-catenin in postmitotic, immature granule cells impairs their dendrite development and prevents the further maturation of these immature granule cells into functional granule cells. It is believed that this function might not be due to β-catenin controlling the expression of the target genes of the Wnt signaling cascade, but rather depending on its cytoplasmic adhesive function and its impact on the cytoskeletal elements (Gao et al, 2007).

Active neurogenesis occurs in only two regions of the adult mammalian brain, in the subgranular layer (SGL) of the dentate gyrus and in the subventricular zone (SVZ) of the lateral ventricles (Reynolds et al, 1992, Kuhn et al, 1996, Kempermann et al, 1997, Kempermann et al, 2004, Cameron et al, 1998, Gage FH, 2002). Interestingly, it was found that the neurogenic progenitor cells of SGL and SVZ have molecular and structural characteristics of astrocytes (Seri et al, 2001). The behavior of neurogenic progenitor cells is not only controlled by intrinsic genetic signals, but also depends on the extrinsic signals from the environment (e.g. neurotransmitters, hormones, growth factors, electrical activity). Pathological conditions such as brain injury, epilepsy or neurodegenerative diseases alter the behavior of progenitor cells and affect adult neurogenesis (Ming et al, 2005, Lledo et al, 2006). The functional significance of adult neurogenesis is not well understood, but an increasing number of behavioral studies of rodents show a direct relationship between the continuing neurogenesis in the adult dentate gyrus and hippocampal functions in learning and memory processes. A better understanding of the molecular control of the development of the

hippocampus and neurogenesis is essential to gain knowledge about the physiology and development of pathological changes in the hippocampus (Jun et al, 2012).

1.4 Hippocampus in learning and memory

The evidence for hippocampal functions in learning and memory processes was established after the resection of the medial temporal lobe in a human subject often cited as H.M. (Scoville et al, 1957). Subsequent studies showed that lesions that were exclusively limited to regions of the hippocampus causes deficits in the formation of the declarative memory (Zola-Morgan et al, 1986, Vargha-Khadem et al, 1997). The declarative (explicit or conscious) memory is made up of facts and events, whereas the non-declarative memory is a collection of skills and habits (e.g. tying shoes, riding bicycle). Both of these memories are stored as long-term memory (reference memory). The declarative memory contents are stored in the neocortex (Milner et al, 1998, Squire 1995) and the non-declarative memories are stored in subcortical areas (amygdala cerebellum) (Squire et al, 2004). Through neuropsychological and electrophysiological studies of amnesic patients with hippocampal lesions as well as in animal models, theories were developed with respect to the exact function of the hippocampus in memory processes. Behavioral experiments in rodents with hippocampal lesions showed a more significant function of the hippocampus in spatial learning and memory processes (Morris et al, 1982, Aggleton et al, 1986). In this regard, O'Keefe J and Nadel L proposed a theory ''The hippocampus as a cognitive map'' (1978). According to this theory, the primary function of the hippocampus is to store the structural (internal) cognitive map of the area, in which the individual resides, more precisely the positions of the objects of the environment and the orientation of the objects in space. This theory is based on the electrophysiological observation of the activity of hippocampal neurons (pyramidal cells of CA1 and CA3 region) (Fox et al, 1975, O'Keefe et al, 1971). Overall, the hippocampus has an essential function in memory consolidation and thus is involved in learning processes, i.e. conversion of new information from the short-term memory (working memory) into the declarative (long-term) memory (Nadel et al, 1997, Squire LR, 1986).

1.5 Transcription factor Bcl11b

Our group has demonstrated that the transcription factor Bcl11b plays an important role in the development of the hippocampus, in particular the dentate gyrus (Simon et al, 2012). Bcl11b, a zinc finger protein, encodes six highly conserved C_2H_2 (Cystein2 – Histidin2) DNA binding zinc finger domains. Bcl11b was initially identified as a protein interacting with the members of the COUP – TF family (chicken ovalbumin upstream promoter transcription factor), also known as orphan nuclear receptors which are involved in transcriptional repression (Cherrier et al, 2013, Topark-Ngarm et al, 2006). Therefore Bcl11b is also denoted as Ctip2 (COUP-TF interaction protein 2) (Avram et. al. 2000). Members of the COUP-TF family have a pivotal function in the regulation of cell migration of the basal telencephalon as well as the formation of commissural projections by controlling axonal growth (Tripodi et al, 2004, Armentano et al, 2006).

In addition Bcl11b also regulates transcription in a COUP-TF independent way (Avram et al, 2002). *Bcl11b* exhibits a high degree of sequence similarity (95% similarity with centrally located zinc finger domains) with another zinc finger transcription factor, Bcl11a (Ctip1). In transient transfection experiments it was shown that both proteins bind to the same consensus DNA sequence thereby inhibiting the transcription of reporter genes (Avram et al, 2002).

Bcl11b exhibits specific spatial and temporal patterns of expression during embryogenesis and becomes more restricted within the CNS postnatally. Outside the nervous system *Bcl11b* is expressed in the thymus (Leid et al, 2004), hematopoietic system, specifically in T cells (Kastner et al, 2010), and the skin (Golonzhka et al, 2007, Ganguli-Indra et al, 2009). Expression of *Bcl11b* occurs in different regions of the CNS including the hippocampus (Arlotta et al, 2005), deep layers of the cortex (Chen B et al 2008), striatum (Arlotta et al, 2008), olfactory bulbs, basal ganglia, cerebellum and amygdala as well as the dorsal spinal cord.

Bcl11b null mutants die shortly after birth due to unknown causes (Wakabayashi et al, 2003, Arlotta et al, 2005). Relatively little is known about specific functions of the transcription factor Bcl11b during embryonic and postnatal stages in different regions of the CNS. In the neocortex *Bcl11b* is essential for the differentiation of pyramidal cells of the deep layers of the cortex (lamina V) and controls the

development of subcortical axonal projections of neurons (corticospinal motor neurons) that extend via the corticospinal tract to the spinal cord (Arlotta et al, 2005). *Bcl11b* expression in the neocortex is confined to the deep layers. In upper cortical layers (layers 2 through 4), which makes corticocortical connections (e.g. corpus callosum to the contralateral hemisphere), *Bcl11b* is repressed by the transcription factor Satb2 (Alcamo et al, 2008, Britanova et al, 2008). In Satb2 mutants, ectopic expression of *Bcl11b* in neurons of layer II/III alters the trajectory of corticocortical projections towards subcortical targets (subcortical targets include the thalamus, midbrain, hindbrain and spinal cord), instead of ipsilateral and contralateral targets (including the corpus callosum) (Alcamo et al, 2008, Britanova et al, 2008). Furthermore, in the striatum *Bcl11b* is essential for the cellular architecture and necessary for the differentiation of a specific type of neurons, the medium spiny neurons, (Arlotta et al, 2008). It was shown that loss of *Bcl11b* in the striatum is likely to contribute to the pathology of Huntingtion's disease, possibly by regulating the transcription of striatum specific genes (Desplats et al, 2008).

Bcl11b is also essential for the proliferation and differentiation of the dentate granule cells. Ablation of Bcl11b in the hippocampus leads to impairment of learning and memory due to the aberrant distribution of mossy fibers in CA3 region (Simon et al, 2012).

Moreover Bcl11b also plays an essential role in lymphopoiesis. Phenotype analysis of Bcl11b knockout mice showed that Bcl11b is essential for differentiation and survival for certain subpopulations of T-lymphocytes (Wakabayashi et al, 2003). In addition to its role in the immune system, Bcl11b also functions as a tumor suppressor in lymphatic cancer cells (Wakabayashi et al, 2003, Kamimura et al, 2007).

1.6 Desmoplakin

Carrying out a comparative transcriptome analysis to elucidate the putative target genes of *Bcl11b* during development of the dentate gyrus our group determined desmoplakin as a direct target gene of Bcl11b.

Desomplakin, a member of the plakin family, is one of the components forming desmosomes, which link the cytoplasmic intermediate filaments to desmosomal

plaques (Jones et. al, 1985, Stappenbeck et. al, 1992, Green KJ 1990, Mueller et al, 1983, O'Keefe et al, 1989). The protein structure of desmoplakin consists of a globular amino- and carboxy-terminus, which are connected by a central alpha helical coiled-coil rod domain (Green et al, 1992, Virata et al, 1992). The amino terminal head interacts with plakophilins and plakoglobins (Bornslaeger et. al, 1996, Delya et al, 2009, Smith et al, 1998, Vallron et al, 1996), while the carboxy terminal tail contains three plakin repeat domains (PRDs), which bind to intermediate filaments (Kazerounian et al, 2002, Stappenbeck et al, 1994). The glycine- serine- arginine rich domains within the carboxy terminus are thought to regulate desmoplakin binding to intermediate filaments (Choi et. al, 2002, Steppenbeck et. al, 1994, Kouklis et. al, 1994, Getsios et al, 2004). Two major isoforms of desmoplakin, *DSPI* and *DSPII* and one minor isoform *DSPIa* (Cabral et. al, 2010) were reported and all three isoforms are produced by alternative splicing. The resultant proteins from these isoforms only differ in the length of the central rod domain. Although all isoforms of desmoplakin are widely expressed in numerous tissues, *DSPII* is absent in heart tissue while *DSPIa* is expressed only in the aorta (Cabral et. al, 2010, Ruhrberg et al, 1997). Relatively little is known about specific functions of desmoplakin in brain development.

The importance of desmoplakin was highlighted by *in vivo* experiments using desmoplakin-null mice. These mice exhibit embryonic lethality at day 6.5 due to the loss of the integrity of the extraembryonic tissue (Gallicano et. al, 1998). Rescue of the desmoplakin null embryos by expressing desmoplakin in extraembryonic tissue prolonged the survival rate of the mutants to embryonic day 10, but these mice displayed pronounced defects in heart, epidermis, neuroepithelium and vasculature development. Detailed analysis of extra embryonic tissue at day 6.5 in desmoplakin null mutants, showed a reduction in the proliferation of cells in embryonic as well as extra embryonic tissues, but was not attributed to concomitant increases of apoptosis (Gallicano et. al, 2001).

In addition to being a cytoplasmic linker between keratin intermediate filaments and desmosomes, desmoplakin also plays an important role in the re-organization of microtubules during epidermal differentiation, by recruiting a subset of microtubule binding proteins like, Ninein (NIN), Ndel1, CLIP170 and Lis1, which in turn stabilize desmosomes (Lechler et. al, 2007, Sumigray et. al, 2011). In addition conditional

knockout mice lacking desmoplakin in the skin show a differentiation defect (Vasioukhin et. al, 2001, Wan et al, 2007).

Suppression of desmoplakin expression in cultured atrial myocytes and in mouse hearts leads to the release of plakoglobin, also known as γ-catenin, from the desmosomes. The released plakoglobin translocates to the nucleus and suppresses the canonical Wnt/β-catenin-Tcf/Lef1 signaling (Gracia-Gras et al, 2006).

1.7 Aim

Desmoplakin is an imperative desmosomal component, which are highly specialized complexes that ensure cell-cell adhesions and serve as anchorage sites for the keratin network. These desmosomes are found in abundance in the tissues such as epithelium, cardiac muscle and other tissues (Boyer et al, 2010, Sonnenberg et al, 2007). Previous studies have demonstrated that desmoplakin is a direct downstream target for Bcl11b, a C_2H_2 zinc finger transcription factor (Simon et al, 2012) expressed in the skin, the hematopoietic system and the central nervous system (CNS), especially in the brain. There is very little known about the functional role of desmoplakin in the hippocampus during postnatal development.

The aim of the present study is to analyze the function of desmoplakin during postnatal development of the hippocampus using mice with a conditional forebrain specific deletion of desmoplakin. The phenotype of these conditional desmoplakin mutants is analyzed by morphological and immunohistological methods during postnatal development. Several aspects of the development of the hippocampal neurons of desmoplakin mutants and control animals are comparatively analyzed, such as the proliferation of progenitor cells, the differentiation, survival of the neuronal cells and synaptic connectivity of the granule cells in the dentate gyrus.

I expect that my research will contribute to a better understanding of the molecular mechanisms underlying the postnatal development of the hippocampus. I hope that the improved knowledge of the precise functions of the hippocampus leads to a greater understanding of the diseases associated with the structural and/or functional pathologies of the hippocampus.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and Enzymes

All chemicals, enzymes, oligonucleotides and antibodies were, unless stated specifically, from the following companies: Amersham-Pharmacia (Freiburg), Gibco/ BRL (Karlsruhe), Heraeus Kulzer (Wehrheim), Invitek (Berlin), MBI Fermentas (St. Leon-Rot), Merck (Darmstadt, Germany), MWG-Biotech (Ebersberg), New England Biolabs (Frankfurt), Promega (Mannheim), Qiagen (Hilden), Roche (Mannheim), Roth (Karlsruhe), Serva (Heidelberg), Shandon (Frankfurt), Sigma (Deisenhofen), Santa cruz, Chemicon, Abcam.

2.1.2 Solutions and Reagents

All solutions and reagents were prepared with water that had been purified by a treatment system (Milli-Q Biocel Water System, Millipore) to quality grade. The solutions were autoclaved or sterile-filtered.

Table 1 Solution and Reagents

Name	Composition
Acetic anhydride buffer	296 ml MilliQ-H_2O 4 ml Triethanolamine 0.5 ml HCl 0.75 ml Acetic anhydride
B1 buffer	0.1 M Tris-HCl, pH 7.5 0.15 M NaCl
Borate buffer	1.546 g of borate 250 ml of H_2O, adjust the pH 8.5
EDTA	0.5 M EDTA pH 8.0 with NaOH
H_2O_{DEPC}	0.1% DEPC in MilliQ-H_2O

Hybridization buffer (in-situ)	50 ml Formamide 5XSSC, pH 7.0 5X Denhardt's solution 1:1000 tRNA (denatured at 95 °C for 10 min) 1:100 salmon sperm DNA (denatured at 95 °C for 10 min) 13.75 ml H_2O_{DEPC}
Complete Hank's Balanced Salt Solution (Complete HBSS)	50ml 10XHBSS, 1.25ml 1M HEPES (pH 7.4), 15ml 1M D-glucose, 5ml 100mM $CaCl_2$ 5ml 100mM $MgSO_4$ 2ml 1M $NaHCO_3$
Lysis buffer (Ear and tail tissue)	100 mM Tris-HCl pH 8.5 200 mM NaCl 5 mM EDTA 0.2% SDS 100 µg/ml Proteinase K
NTMT	100 mM NaCl 100 mM Tris-HCl pH 9.5 50 mM MgCl2 0.1% (v/v) Tween 20
PBS (Phosphate Buffered Saline)	8 g NaCl 0.2 g KCl 1.15 g Na_2HPO_4 0.2 g KH_2PO_4 1000 ml MilliQ-H_2O, pH 7.4
PBSDEPC	0.1% DEPC in PBS
PBT-X	0.1% Triton-X100 in PBS
Phosphate Buffer	0.2 M NaH_2PO_4/Na_2HPO_4
Permeabilization solution	3% BSA, 0.3% Triton X-100 in 1XPBS
Sodium acetate	3 M Sodium acetate pH 5.2 with acetic acid

Sorensen buffer	19.6 ml of 1.5 M Potassium di hydrogen phosphate 80.4 ml of 1.5 M Di sodium hydrogen phosphate, pH 7.4
Tris-HCl	1 M Tris base pH7.4, 7.5, 8.0, 9.5 with water
20X SSC	3 M NaCl 300 mM Sodium citrate di-hydrate, pH 7.0
Walpole buffer	0.2 M Acetic acid 0.2 M Sodium acetate, pH 4.45

Table 2 Bacterial strains

Name	Source
Escherichia coli XL1-Blue	Stratagene

2.1.3 Plasmid vectors

Table 3 Plasmid vectors used.

Name	Source
pBluescript-SK II (+/-)	Stratagene
pCAGGS	from Stefan Schumacher's lab
pRCCMVDSP	from Elaine Fuchs's lab
pRCCMV	In house
pCIG2-GFP	from Franck Polleux's lab
pCIG2-Cre-GFP	In house
pEGFP-N1	Addgene

2.1.4 Oligonucleotides

All oligonucleotides used in this study were obtained from MWG-Biotech (Ebersberg).

Table 4 Oligonucleotides used.

Name	Sequence (5' to 3')
DSP FP	TGTCTGTTGCCATGTGATGCC
DSP RP	GGTTGGGCCTCTCGAATCATGAGTGTCTAGCG
Dsp 5'	CGGACATTCATGCGAGATAC
Dsp 3'	GCCTTGAACTGGGAACACTC
Nex-For	GAGTCCTGGAATCAGTCTTTTTC
Nex-Rev-Cre	CCGCATAACCAGTGAAACAG
Bcl11b-flox UP	TGAGTCAATAAACCTGGGCGAC
Bcl11b-flox LP	GGAATCCTTGGAGTCACTTGTGC
EmxCre1	GTATTTGGTTTAGAGTTTGGC
EmxCre2	GGGGGACATGAGAGGATGTCAC

2.1.5 Antibodies

The primary antibodies used are listed, which bind to these antigens of mouse.

Table: list of primary antibodies used for staining frozen and free floating sections.

Name	Dilution	Source
Rat-BrdU	1:1000, 1:800	Ab-direct
Dsp	1:200	Progen
Chicken-GFP	1:2500	Abcam
NeuN	1:1000	Chemicon
NeuroD	1:1000	Santa cruz
Tubulin Tyrosin	1:2000	Sigma

2.1.6 Culture media

Media and plated for the propagation and cultivation of *Escherichia coli* were used according to standard protocols (Sambrook and Russell, 2001). The concentration of ampicillin and kanamycin in the media was 100 μg/ ml and 25 μg/ ml respectively.

2.1.7 Cell culture media

Plating media containing 20% glucose, 10% fetal bovine serum, 1X Pen/Strep in Minimal Essential Medium (MEM) were used. The growth media is prepared in MEM containing 2% B27, 0.25% Glutamine, 1X Pen/Strep, 1% N2 supplement and 0.05% NGF.

2.1.8 Slice culture media

The hippocampus slices were kept in slice culture medium, which is prepared as described in Hand et al, 2005 and Polleux et al, 2002.

2.2 METHODS

DNA isolation and purification

2.2.1 Preparation of plasmid DNA and DNA fragments

The mini-preparation of plasmid DNA was done by alkaline lysis (Birnboim and Doly, 1979). The resultant plasmid DNA was dissolved in 30-40 μl of TE buffer and stored at -20°C. The preparation plasmid DNA in large scale was carried out using the plasmid midi or maxi kit (Qiagen, Hilden, Germany) or the Nucleobond PC-500 Kit (Macherey-Nagel, Duren) or Invitrogen Purelink according to manufacturer's instructions. The DNA was then dissolved in 50-100 μl TE buffer and stored at -20 ° C.

The preparative isolation of DNA fragments was performed by using the QIAquick (Qiagen, Hilden, Vogelstein and Gillespie, 1979) or Nucleospin Extract II (Macherey-Nagel, Duren) gel Extraction Kit. The DNA was dissolved in 25-30 µl TE and stored at -20 ° C.

2.2.2 Isolation of genomic DNA from tail and ear tissue

The tail and ear tissue from young and adult mice were incubated in ear/ tail digesting buffer for 2h or overnight at 55°C. This was followed by inactivation of proteinase K for 10 min at 95°C. After inactivation, 200 µl H_2O was added, from this diluted solution 1-2 µl were used for genotyping in a PCR reaction.

2.2.3 Restriction digestion, ligation of DNA fragments and transformation of competent bacteria

Restriction digest of plasmid DNA with one or more restriction enzymes was carried out according to standard methods (Sambrook and Russel, 2001). The ligation of DNA fragments usually with 50 ng of vector DNA and approximately three to five fold molar excess of DNA fragment was ligated in a 10 µl reaction. The product was transformed into competent bacteria cells thereafter.

Amplification of DNA fragment

2.2.4 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (Saiki et al., 1985) was used for genotyping of young animals as well as for cloning of various plasmid constructs. The PCR was performed according to standard methods (Sambrook and Russell, 2001) using a thermocycler from Biometra (Göttingen). The standard PCR mixture contained 1µl dNTPs (2.5 mM each nucleotide; Invitrogen, Karlsruhe, Germany), 2 µl 10x buffer (Invitrogen,

Karlsruhe, Germany), 1µl Primer1 (0.5 mM), 1µl Primer2 (0.5 mM), 0.2µl Taq DNA polymerase (Invitrogen, Karlsruhe, Germany) and MilliQ H_2O to make up to 20µl. As the template for genotyping was either, 50-100 ng of Plasmid DNA or 1-2µl of genomic DNA (heat-inactivated lysate) used. The PCR program consisted of the following steps: 5 min 94 ° C, followed by 35 cycles of 95°C for 30 seconds, 30 seconds at 63°C and 1 min at 72°C, with a final incubation at 4°C. For preparative PCR and subsequent cloning, the amplified product was separated by electrophoresis and then gel extracted or directly used for cloning with the pGEM-T Easy kit (Promega, Mannheim, Germany).

2.2.5 RT-PCR

The cDNA fragments were generated by RT-PCR in a one-step reverse transcriptase kit (Invitrogen, Karlsruhe). The purified total RNA was used as template in reverse transcriptase reaction and an oligo(dT) primer to make a cDNA.

2.2.6 In-vitro transcription reaction and DIG labeling the RNA probe

To prepare RNA probes for in-situ hybridization, plasmid DNA initially was linearized and subsequently in vitro transcribed and DIG-labeled. The linearization was done with 30 micrograms of plasmid DNA in a 150µl reaction with approximately 50 units of the corresponding enzyme at 37°C overnight.

Then the linearized DNA was extracted with phenol / chloroform, precipitated with ethanol / sodium acetate (1/10 volume of 3M sodium acetate) and washed with 70% ethanol. The DNA pellet was dried and dissolved in 25µl H_2O and stored at -20°C. For the probe preparation, in-vitro transcription and DIG-labeling of RNA, unless otherwise specified, reagents and enzymes were from Roche (Mannheim).

The transcription reaction mixture containing 1 microgram of linearized DNA template, 2µl 10X transcription buffer, 2µl DIG labeling mix, 0.5µl RNase inhibitor (40 units/ µl, Invitrogen), 1µl of the appropriate polymerase (T7, T3, SP6; 20 units/ µl) and DEPC treated water was incubated for 2 h at 37°C and the reaction was stopped

on ice. This was followed by purification of DIG labeled RNA with the RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DIG labeled RNA probe was eluted with 50 µl H_2O_{DEPC} in a tube containing 50µl of formamide. The RNA probe was stored at -80°C.

2.2.7 RNA isolation and purification

To identify differentially expressed genes quantitative real time PCR was performed on RNA isolated from the hippocampus at postnatal day 14 and 30. The hippocampus of control and mutants animals were dissected in cold PBS and immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation. The total RNA was isolated and purified using a RNA cleanup kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of the total RNA was determined photometrically and the total RNA was used for cDNA synthesis.

2.2.8 First strand cDNA synthesis

The RNA was in-vitro transcribed to first strand cDNA by SuperScript II RT kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The reaction mixture for the first strand synthesis contained 1 µl of oligo$(dT)_{12\text{-}18}$ (500µg/ml), 2 micrograms of total RNA column purified, 1 µl of dNTP (10 mM of each nucleotide) and made up to 12 µl with sterile, distilled water. The mixture was heated up to 65°C for 5 minutes and quickly chilled on ice. Then 4 µl of 5X first strand buffer, 2 µl of 0.5 M DTT was added and incubated at 42°C for 2 minutes, 1 µl of SuperScript II RT (200 units, Invitrogen, Karlsruhe, Germany) was added. The first strand synthesis was carried out for 2 h at 42°C. The product was cooled on ice and used for quantitative real time PCR.

2.2.9 Preparation of mouse tissue

The tissue was obtained from postnatal stages and the tissue was prefixed by perfusing the animals with ice-cold PFA solution (4% PFA in 0.1 M phosphate buffer). Animals were euthanized by CO_2, by placing the animal in a chamber followed by filling it with CO_2. Soon after the animal was taken out on to a sterile platform and the heart was exposed by cutting open the ribcage. A butterfly needle was immediately inserted into the left ventricle of the heart and the right atrium was punctured with tweezers. Using a pump, 15-20 ml of ice-cold PBS solution was passed thought the needle followed by 15-20 ml of PFA solution. The animals were then decapitated and the brain was dissected out of the skull.

Histological methods

2.2.10 Preparation of methacrylate sections

For histological analysis, the prepared tissue was embedded in hydroxyethylmethacrylate (Technovit 7100, cold-curing plastic, Heraeus Kulzer, Wehrheim). The tissue was prepared by soaking it for 2 days in 4% PFA in PBS and then dehydrated gradually in an ascending series of alcohol (1 day each in 25%, 50%, 75%, 95%, 99% and 2 days in 100%) at 4°C. This was followed by pre-infiltration of the tissues with equal parts of Technovit 7100 and 100% ethanol for at least 2 days at room temperature, followed by preparative solution (Technovit 7100 with 1g of hardener I) for 5-7 days at room temperature. The tissue was embedded in preparative solution containing hardener II (15:1 ratio) in a mould. After complete polymerization of the plastic overnight or up to a day, the embedded tissue is fixed to the block holder with Technovit 3040 dissolved in Technovit universal liquid and stored at room temperature. The block was sectioned into semi thin sections (5 microns) with a rotary microtome (Microm, HM355S). The sections were placed on warm water and collected on slides (Roth, Karlsruhe, Germany) and dried over a heating block.

2.2.11 Cresyl violet staining of methacrylate sections

For cresyl violet staining for semi thin methacrylate section, the slides were incubated for 10-15 min in cresyl violet staining solution (0.02% cresyl violet in 0.2 M Walpole buffer). Subsequently, the sections were washed for 30 min with several changes of water, followed by a brief wash with 70% ethanol for 10 seconds. Then the sections were washed for 10-20 min with several changes of deionized water and dried overnight. The sections were covered with Entellan (Merck, Darmstadt) and cover slips.

2.2.12 Preparation of frozen sections

Immunohistochemistry and In-situ hybridization were performed on frozen tissue sections. Freshly dissected tissue was postfixed for 2 h with 4% PFA in phosphate buffer at 4°C. Subsequently, the tissue was washed thrice for 15 minutes each with PBS before incubating overnight with 20% D (+) sucrose in phosphate buffer at 4°C, for cryoprotection. Then the tissue was rinsed in OCT compound Polyfreeze Tissue Freezing medium (Polysciences, Warrington) and transferred to Peel-A-Way mould (Thermo) with OCT compound. The tissue was aligned before freezing the mould on a metal block cooled by liquid nitrogen and stored at -80°C.

The block was sectioned in a cryostat (Microm HM 560, Microm Walldorf) with the block temperature of -15 to -20°C and knife temperature –2 to -3°C above the block temperature. The sections of a varying thickness of 7 – 18 microns were collected on an adhesive slide (Superfrost plus, Thermo), dried at 37°C and then stored at -80°C in an airtight box.

2.2.13 Immunohistology

For immunohistology on frozen sections, the sections were thawed for 30 – 60 minutes at room temperature, and marked on the edges with a PAP pen (Liquid Blocker, Japan). The slides were washed 3 times for 5 minutes each in glass coupling

jars with PBT-X (PBS containing 0.1% Triton X-100) at room temperature. Thereafter, the slides were incubated in a humid chamber with blocking solution I (10% horse serum in PBT-X) for 1 h at room temperature, followed by incubation with primary antibody in blocking solution II (5% horse serum in PBT-X) at 4°C overnight. Next day the slides were washed thrice for 10 minutes each with PBT-X in a glass coplin jar and incubated with secondary antibody in blocking solution II for 1 h at room temperature. Then the slides were washed thrice for 10 minutes each with PBT-X in a glass coplin jar. The slides were washed once more in PBS before covering them with mounting media (Immu-mount, Thermo) and cover slips.

For immunohistology on "free-floating" tissues sections (40 microns vibratome sections), the sections were incubated for 2 h in blocking solution I (10% horse serum in PBT-X) and then with primary antibody for 48 h at 4°C. Followed by incubation with a secondary antibody for 2 – 4 h at room temperature. The sections were transferred on to slide and covered with mounting media (Immu-mount, Thermo) and cover slips.

2.2.14 Detection of cell proliferation and apoptosis

The quantification of mitotically active and apoptotic nuclei were performed on frozen tissue sections. To detect mitotically active and proliferating cells, the mouse was injected with BrdU (50 - 75 micrograms BrdU/g of body weight in 0.09% NaCl) intraperitoneally and the brain was dissected 2 h post injecting. BrdU being an analog of thymidine is incorporated into the DNA during the synthetic phase of the cell cycle of dividing cells. The incorporated BrdU can be detected immunohistochemically in the nuclei.

The frozen brain tissue sections were initially washed three times, for 5 min with PBS followed by postfixation of the section for 5 min in 4% PFA in 0.1 M phosphate buffer at RT. Subsequently, the sections were washed three times for 5 min with PBS and incubated for 30 min at 37°C in 2.4 M HCl, then washed again three times, for 5 min with PBS followed by blocking for 1 h in blocking solution (10% goat serum in PBT-X) at RT. The brain tissues were incubated with anti-BrdU antibody in 5% goat serum in PBT-X at 4°C. The next day the sections were washed three times with PBS before

incubating with the secondary antibodies for 2 h at RT. Finally, the sections were washed again with PBS three times for 5 min and the slides were covered with mounting media (Immu-mount, Thermo) and cover slips. The analysis of apoptosis was performed by detection of DNA fragmentation (Gavrieli et al, 1992) using the "Apop Taq Plus Fluorescein in situ Apoptosis Detection Kit" (Intergen, Gaithersburg, MD 20877, USA).

2.2.15 Golgi staining

To analyze the morphology of neurons, Golgi staining was performed with the FD Rapid GolgiStain Kit (FD Neurotechnologies) (Ellicott City, MD 21041, USA) according to the manufacturer's instructions. The brains of adult animals were prepared by briefly washing with H_2O and incubated for 2 weeks in an impregnation solution (solution A+B in 1:1 ratio) at RT in the dark. Then the brain tissue was incubated with solution C for at least 48 h at 4°C, before sectioning 150 microns thick sections with a vibratome. The sections were rinsed twice with H_2O for 10 min at RT and incubated in staining solution. Thereafter, the sections were washed twice with H_2O for 4 min, dehydrated in an ascending alcohol series (each 4 min 50%, 75%, 95% ethanol) and incubated for 4 min three times in xylene substitute. Finally, the tissue sections were carefully transferred on to a glass slide and mounted with Entellan and cover slip.

2.2.16 Timm staining

To analyze the mossy fiber distribution in CA3 region, Timm staining was performed (Schwegler and Lipp, 1983). The mouse was transcardially perfused with Sorensen buffer containing 1.75% sodium sulfide and 1.2% sodium di-hydrogen phosphate followed by 2.5% glutaraldehyde in Sorensen buffer. After the perfusion the brain tissue was dissected and stored overnight in 30% D (+) sucrose for cryoprotection. The tissue was embedded in OCT compound and the tissue block was sectioned to make thin slices of 40μM in a cryostat and the slides were stored at -80°C.

The slides were thawed and developed in Timm's solution containing 50% Arabic gum, 5% hydroquinone, 0.5 ml of 17% silver nitrate in 0.1M citrate buffer, incubated at 30°C until the sections developed.

2.2.17 In-situ Hybridization

On the first day of in situ hybridization on frozen sections, the sections were thawed for 1-2 h at RT, edges were marked with a PAP pen, then the sections were postfixed with 4% PFA in PBS_{DEPC} for 15 min at 4°C and washed three times for 5 min with PBS_{DEPC}. After that the sections were acetylated in acetylation buffer for 10 min at RT followed by washing three times for 5 min with PBS_{DEPC}. The sections were finally blocked for 2 h in 600 µl of pre-hybridization buffer in a humid chamber at RT. Meanwhile the cover slips were washed briefly with absolute ethanol and dried before covering the slides. Prior to hybridization, 3 µl of the DIG-labeled RNA probes were denatured for 5 min at 80°C in 150 µl of hybridization solution and then rapidly cooled on ice. The pre-hybridization buffer was eventually removed from the sections and replaced with the hybridization solution containing the RNA probe. The glass slides were subsequently covered with pre-washed cover slips and incubated in a moist chamber (with 5x SSC/50% formamide) at 70 ° C.

2.2.18 Behavioral experiment

2.2.18.1 Study of emotional behavior – open field test

The open field test is designed to detect motor activity and the exploration behavior of the mice with an emotional component. The open field consists of a 36 X 60 cm platform with a 28 cm high rim. On the floor of this platform lines are drawn into squares of 12 X 12 cm. The animals were placed individually in the center of the platform and allowed to move around freely. The exploratory behavior of the animals was observed and recorded such as leaning (upright on its hind paws, at least one front paw touches the wall) and rearing (rear on their hind legs in the open field

without touching the wall). Locomotor activity was recorded as number of lines crossed on the platform by the animal. Each animal was allowed to move around on the platform for a period of 10 min during which time the data was recorded. With this experiment one can obtain the behavior data in a fast and simple way, owning to the complex behavior component such as motivation, exploration, stress fear and spatial learning.

2.2.18.2 Study of cognitive abilities - radial maze

The objective of this experiment was to analyze the memory and spatial learning behavior of the animals. This was achieved by placing the animal in a radial maze, that consists of a platform with a central space from which 8 identical plexiglas arms extend radially, and the animal should find and eat the food pellet placed at the end of each arm hidden behind a small barrier.

The mice aged 12-13 weeks were used in the eight-arm radial maze experiment for a period of 7 days to analyze the spatial learning behavior of the animals. In the initial two days (day 1-2) of the experiment, the animals received a small amount of food to induce hunger, which was measured by the reduced body weight by 10-12%. This is necessary in order to motivate the animals to look for food and learn about the food source. During the entire behavior experiment the animals were housed individually and the body weight was maintained at 85-90% of the initial weight. In addition the animals were also habituated for 15 min/ day for the initial 2 days, during which the animals are allowed to move around the maze and eat the food pellet placed at the end of each arm. The maze was always oriented in the same way in the room and several extra-maze cues were provided close to the arms, to facilitate the orientation in the room.

The actual experiment was conducted for 5 days (day 3-7). The animals were placed in the center of the maze and were kept in the maze until all food pellets were eaten or up to a maximum of 10 min per day. An arm entrance was scored if an animal entered an arm with all four paws and an error was noted if an animal entered an arm previously visited or if it did not eat the food reward. During the test the following parameters were recorded for all the days (day 3-7): weight of the animal,

number and order of the radial arms entered, the number of novel arms entered and the numbers of arms revisited (errors) within the first 8 arms. The data from all 5 days (day 3-7) were used to calculate the learning ability of the animal, where as the significance was calculated for day 3-5 (day 5-7).

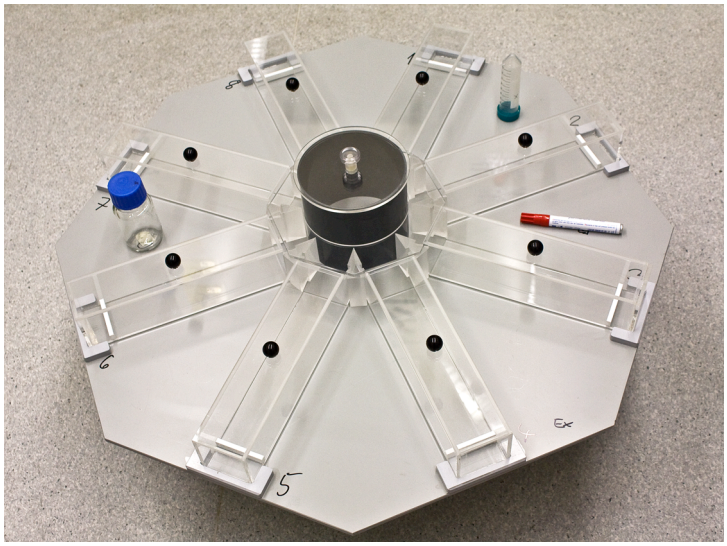

Fig 2.1 The 8 arm-radial maze to investigate the cognitive abilities of the mice.

2.2.19 Neuronal cell culture

Primary neuronal cell cultures were established according to a modified, previously published protocol (Banker et al, 1989, Bartlett et al, 1984, Goslin et al, 1990). Hippocampi from wild type, control and mutant animals were dissected at P1. After washing the hippocampi 3 times in 1XHBSS, they were incubated in 1XHBSS containing trypsin for 16-20 min at 37°C. The hippocampi were subjected to trituration and cells were plated on poly-L-lysine coated slides at a density of 7-15 x 10^4 cells/ml and cultured in serum-free neurobasal A medium. Fixation of the cells was done at RT for 20 min with 4% PFA and immunocytochemistry was performed according to the protocol (Poulter et al, 1999, Beaudoin et al, 2012).

2.2.20 Ex-utero electroporation and Slice culture

A pregnant female at E 15.5 was placed in a box, saturated with isoflurane for 1-2 min or until the mouse is unconscious. Then the mouse was euthanized by cervical dislocation and embryos were dissected and placed in a petri dish containing cold complete HBSS solution. Using a pair of scissors, single embryos were separated from the uterine horn. The uterine wall was cut open, the placenta severed and the embryo carefully released from the yolk sac. The head of the embryo was decapitated in a 60° angle, by using a pair of scissors. The head was transferred to a clean and dry petri plate. A needle containing either cDNA construct pRC CMV *Dsp* or the control vector pRC CMV along with GFP expressing vector pCAGGS (3:1 ratio to a final concentration of 4µg/ µl) was inserted into the middle of the hemisphere and 2-3µl of DNA solution was injected into one of the ventricles with help of a picospritzer. Before placing the electrodes, few drops of complete HBSS were applied on the head of the embryo. The electrodes were placed in such a way that the negative terminal was on the same side as the injected ventricle and the positive electrode on the opposite side, below the ear of the embryo's head and then 5 pulses of 50 volts were applied. After the electroporation, the brain was dissected by peeling off the skin and cutting open the skull with the help of fine forceps and scissors in complete HBSS. The brain was taken out of complete HBSS with a small scoop spatula and using fine tissue paper excess HBSS was drained before embedding into agarose. In a peel away mould, 4 % agarose was poured and the brain was embedded in it at 37 – 39 °C and kept on ice until the block was sectioned with a vibratome. The agarose block was trimmed and glued onto a stage followed by sectioning of 250 µm thick slices. Starting the sectioning from the hindbrain at slow speed, 5 – 7 sections were collected from the cerebrum. These slices were kept in cold complete HBSS until they were placed on the membrane. Before placing the sections on the membrane, 100 µl of complete HBSS was added in a cross fashion to facilitate the orientation of the sections on the membrane. The sections were transferred using a bent spatula onto the membrane and excess HBSS was taken off the membrane using a pipette. The membrane inserts were placed in a 6 well plate containing 1.8 ml of slice culture medium as well as 10 µM BrdU (Mancini JD et al, 2009) for the first 20 hours and thereafter media without BrdU for 11 days. Only half the medium was replaced with fresh medium every

second day. After culturing for 11 days, the membrane was cut with a scalpel blade and individual slice were fixed with 4 % PFA in PBS for 1 hour at room temperature. These fixed sections were washed thrice with PBS for 15 min each, followed by overnight incubation in permeabilization solution at 4 °C. After overnight incubation, immunohistochemistry was performed using appropriate primary antibodies diluted in permeabilization buffer (Venkataramanappa et al, 2014).

2.2.21 Statistical analysis

Histological analysis of the dentate gyrus, like area and granule cell number were determined by ImageJ software. All imaging for cell counting were done with a bright field or epifluorescence microscope and the representative image was obtained with a confocal microscope. The raw data were analyzed and expressed as the mean, standard deviation (± s.d.) and standard error of mean (± s.e.m.) calculated by using Microsoft Office Excel 2008. Calculating the significance of observed differences between groups were made by an unpaired two-tailed student's *t*-test and *P* values less than 0.05 were considered significant. Significance for quantitative real-time was calculated by $2^{-\Delta\Delta C_T}$ method as described previously by Livak and Schmittgen (2001).

2.2.22 Approval for animal experiments

All animal experiments were carried out in accordance with the German law and were approved by the respective government offices in Tübingen.

2.2.23 Funding

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to Prof. Dr. Stefan Britsch, SFB 497/A9.

3. Results

3.1 Analysis of hippocampal Desmoplakin expression.

To determine the expression pattern of desmoplakin in the hippocampus immunohistochemistry was performed on 7 µm frozen brain sections, using an antibody recognizing specifically desmoplakin in addition to antibodies recognizing Bcl11b [expressed only in the granular zone of the dentate gyrus and the CA1, CA2 region of the Cornu ammonis] (Fig. 3.1 A, C) and Sox2 [progenitor cell marker] (Fig. 3.1 B, D). Expression of desmoplakin in the hippocampus was only detected in the dentate gyrus (Fig. 3.1 A-D; Simon et. al 2012, Hagihara et. al 2009). Furthermore, immunohistochemistry on cultured primary neuronal cells derived from the hippocampus of P0 animals revealed the localization of the desmoplakin protein to the cytoplasm and along axons and dendrites (Fig. 3.2 A, B).

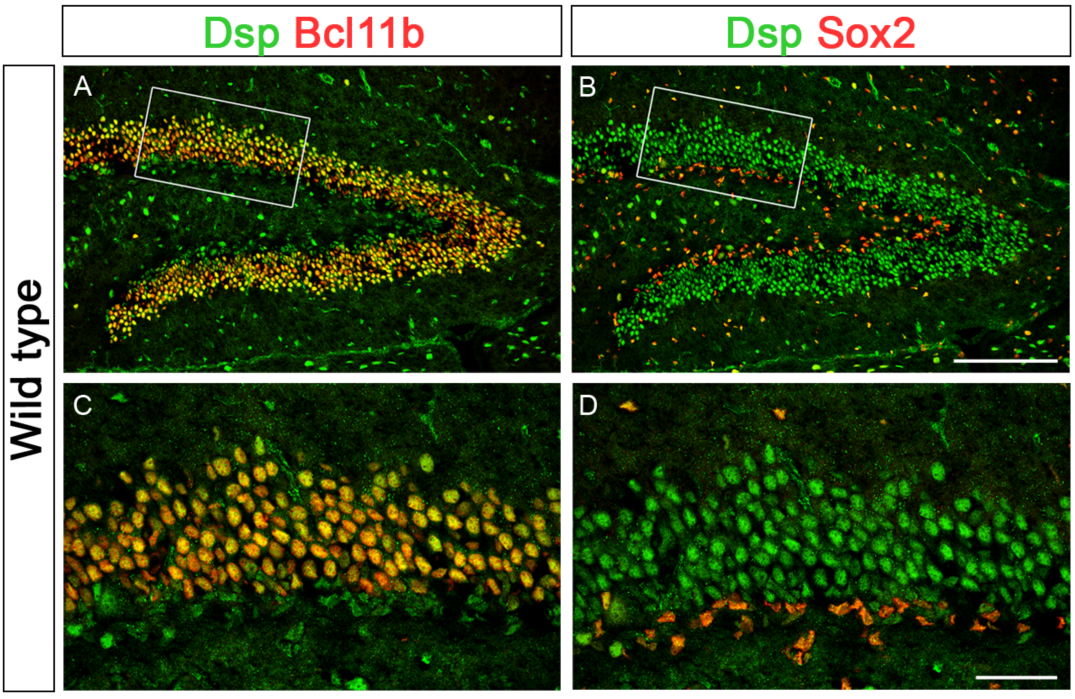

Fig. 3.1 Analysis of the desmoplakin expression pattern in the hippocampus at P14. Desmoplakin is expressed in the granule cells of the dentate gyrus (A-D; green) overlapping with Bcl11b (A, C; red) and a faint expression of desmoplakin co-localizes with Sox2 positive cells (B, D; red). Scale bar: 100µm (B) 50µm (D) (Simon et al, 2012, page 2922-36).

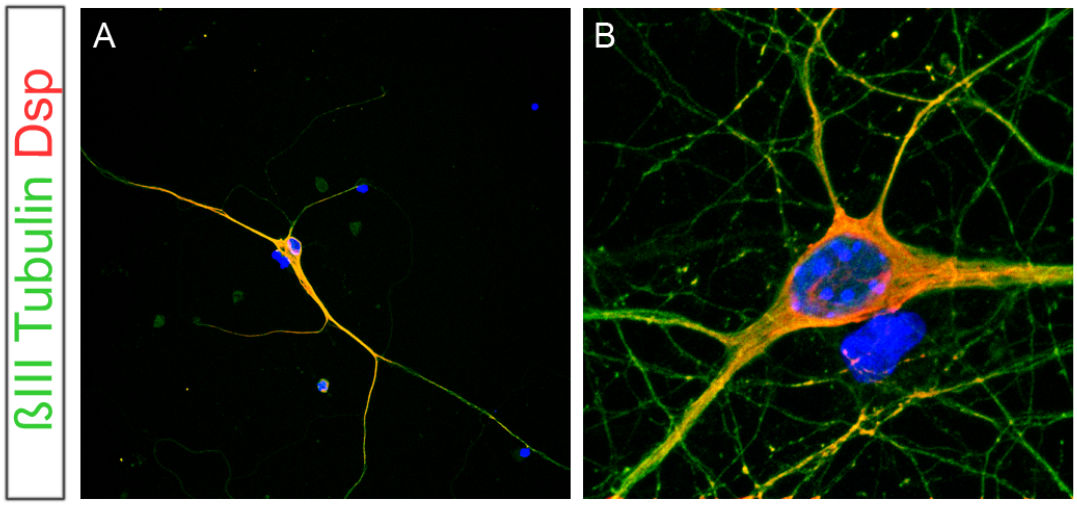

Fig. 3.2 Analysis of desmoplakin protein in cultured primary neuron cells.
Immunological analysis of the localization of the desmoplakin protein in hippocampal primary neuronal cells isolated from P0 wild type animals, and cultured in-vitro for 7 days. Cells were fixed and stained with tubulin (green, cytoskeleton marker) and Dsp (red) (A, B), 63X magnification; 3.5 zoom.

3.2 Analysis of mice with a forebrain specific mutation of desmoplakin

To analyze functions of desmoplakin in the dentate gyrus, a mouse line with a forebrain specific mutation of desmoplakin was generated. $Dsp^{flox/flox}$ mice (Vasioukhin et. al 2001) were mated with Emx1-*Cre* mice (Iwasato et. al 2000, Guo et al, 2000, Gorski et al, 2002) allowing forebrain specific *Cre* recombinase expression and ablation of desmoplakin.

The resultant progeny of heterozygous floxed desmoplakin animals were inter-mated to generate homozygous and control offspring. Genotypes of all animals were determined by genomic PCR using specific primers for desmoplakin and the respective *Cre* lines before experiments were conducted. Only those animals that were homozygous floxed desmoplakin, with Emx1-*Cre,* were used as mutants ($Dsp^{flox/flox}$;Emx1-*Cre*) for the following experiments along with control litter mates, which were heterozygous floxed desmoplakin ($Dsp^{flox/+}$;Emx1-*Cre*). The desmoplakin Nex-*Cre* (Goebbels et. al, 2006) line was also generated in the same way as described above.

Expression of desmoplakin mRNA in control and mutant animals was compared by in-situ hybridization and qPCR. In-situ hybridization was preformed on 12 – 15 µm

thin frozen brain sections of Dsp$^{flox/flox}$;Emx1-*Cre* (Fig. 3.3, B) and control animals (Fig. 3.3, A) at P14. While desmoplakin mRNA was clearly observed in the dentate gyrus of the control animals, minimal or no expression was reveled in the mutant hippocampus (Fig. 3.3, A, B). These results were also confirmed by qPCR analysis of hippocampal mRNA using specific primer pairs for desmoplakin (Fig. 3.3, C).

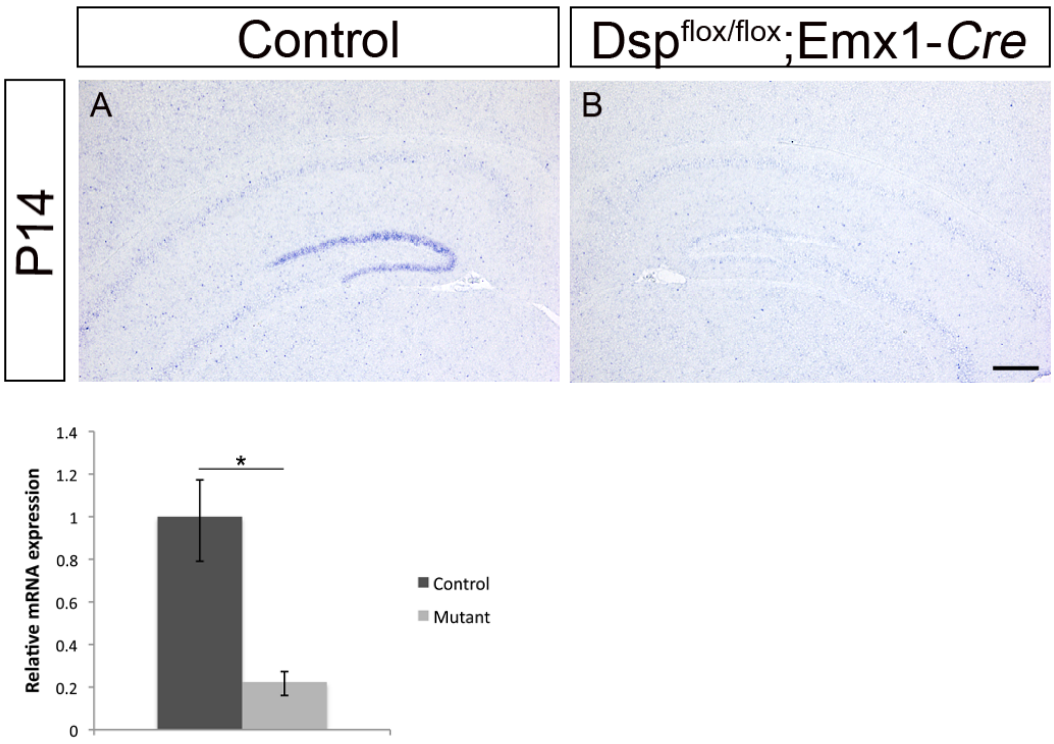

Fig. 3.3 Analysis of Desmoplakin expression in the hippocampus.
Desmoplakin RNA in-situ hybridization of hippocampal cryostat sections of control (A) and Dsp$^{flox/flox}$;Emx1-*Cre* (B) mice at age P14. Scale bar: 250μm. Determination of relative mRNA expression of desmoplakin control and mutant animals at P14, by qPCR (C). (*t*-test, *; *P*<0.0005; error bar, s.d,; n=3)

3.2.1 Characterization of desmoplakin mutants.

Desmoplakin mutant animals showed skin lesions on cheek, ears (Fig. 3.4 C), fore- and hindlimbs (Fig. 3.4 A, B, D) which were absent in control animals. In addition the mutants were also smaller at birth and early postnatal development when compared to controls, but regained weight and size during later development. The skin lesions were only found in Dsp$^{flox/flox}$;Emx1-*Cre* animals, most likely due to the expression of

Emx1-*Cre* in the limbs as well as forebrain (Xiao-Lu Jin et. al. 2000). No skin lesions were found with the desmoplakin Nex-*Cre* line.

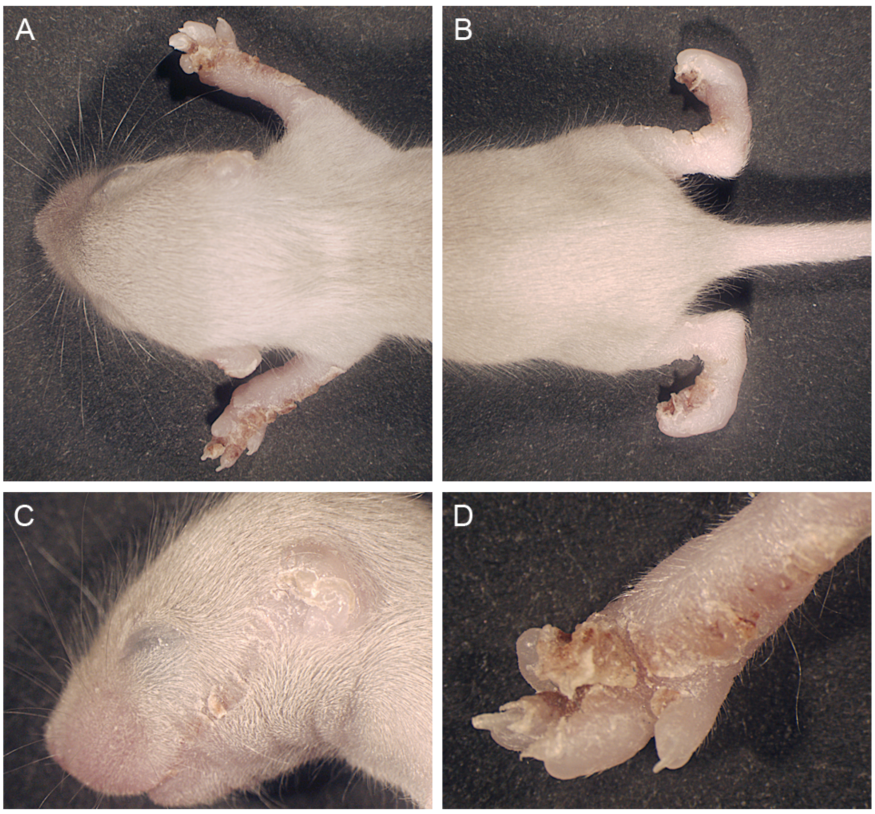

Fig. 3.4. Skin lesions observed on Dsp$^{flox/flox}$;Emx1-*Cre* animals.
Skin lesions occur on both, fore and hind limbs (A, B, D) as well as cheek and ears (C) due to the expression of Emx1-*Cre*.

3.2.2 Analysis of mice with forebrain specific mutation of desmoplakin.

To determine the phenotype of the desmoplakin mutation in the hippocampus, the dentate gyrus was examined histologically. Brain tissue was dissected, processed and embedded in hydroxyethylmethacrylate (Technovit 7100, Heraeus Kulzer, Wehrheim) and 5 μm sections were generated using a rotary microtome followed by cresyl violet staining. The desmoplakin phenotype was analyzed during postnatal development. Dentate gyrus granule cell numbers and area of control and desmoplakin mutant animals were determined using the ImageJ software.

In the incipient postnatal dentate gyrus approximately 25% reduction of granule cells was observed at P14 in Dsp$^{flox/flox}$;Emx1-*Cre* (Fig. 3.5 B, C, E. 1068.47 ± 87.37 s.d.) and at P10 in Dsp$^{flox/flox}$;Nex-*Cre* (Fig. 3.6 B, C, E. 953.2 ± 93.52 s.d.) animals, compared to control animals of the respective *Cre* line (Fig. 3.5 A, D, E. 1415.56 ± 157 s.d., P<0.02) (Fig. 3.6 A, D, E. 1303.04 ± 91.58 s.d., P<0.001). At P30, 17.6% and 24.8% reduction of granule cells was observed in Dsp$^{flox/flox}$;Emx1-*Cre* (Fig. 3.5 H, I, K. 1164.38 ± 139.22 s.d.) and Dsp$^{flox/flox}$;Nex-*Cre* (Fig. 3.6 H, I, K. 943.13 ± 97.30 s.d.) lines, respectively, compared to control animals of the respective *Cre* line (Fig. 3.5 G, I, J. 1414.55 ± 204.48 s.d., P<0.04) (Fig. 3.6 G, I, J. 1254.54 ± 126.8 s.d., P<0.003). A minor disarray of granule cells of the dentate gyrus was also observed in desmoplakin mutants.

To determine if the reduction in the cell number had an influence on the size of the dentate gyrus, we compared the areas of the dentate gyrus between control and mutant animals. The area of the control dentate gyrus was considered as 100% and the area of the mutant dentate gyrus was calculated accordingly. A reduction of approximately 20% in the area of the dentate gyrus was observed in P14 Dsp$^{flox/flox}$;Emx1-*Cre* (Fig. 3.5 B, E, F. 80.6 ± 5.54 s.d.) and P10 Dsp$^{flox/flox}$;Nex-*Cre* (Fig. 3.6 B, E, F. 81.72 ± 4.44 s.d.) animals, compared to control animals of both *Cre* lines (Fig. 3.5 A, D, F. 100 ± 14.11 s.d., P<0.02) (Fig. 3.6 A, D, F. 100 ± 7.82 s.d., P<0.003). At P30, approximately 40% and 20% reduction in the area of the dentate gyrus was observed in Dsp$^{flox/flox}$;Emx1-*Cre* (Fig. 3.5 H, K, L. 61.24 ± 16 s.d.) and Dsp$^{flox/flox}$;Nex-*Cre* (Fig. 3.6 H, K, L. 80.03 ± 6.59 s.d.) lines, respectively, compared to control animals of both *Cre* lines (Fig. 3.5 G, J, L. 100 ± 20.53 s.d., P<0.04) (Fig. 3.6 G, J, L. 100 ± 12.48 s.d., P<0.03).

Taken together, these results demonstrate that ablation of desmoplakin in the dentate gyrus results in the reduction of granule cells and the area of the dentate gyrus in desmoplakin mutants. This reduction in the cell number could be due to two reasons. Firstly, desmoplakin ablation may result in elevated apoptosis resulting in a lower granule cell count. Secondly desmoplakin may affect the progenitor cell proliferation. Hence removal of desmoplakin might result in a reduced proliferation of progenitor cells in the dentate gyrus.

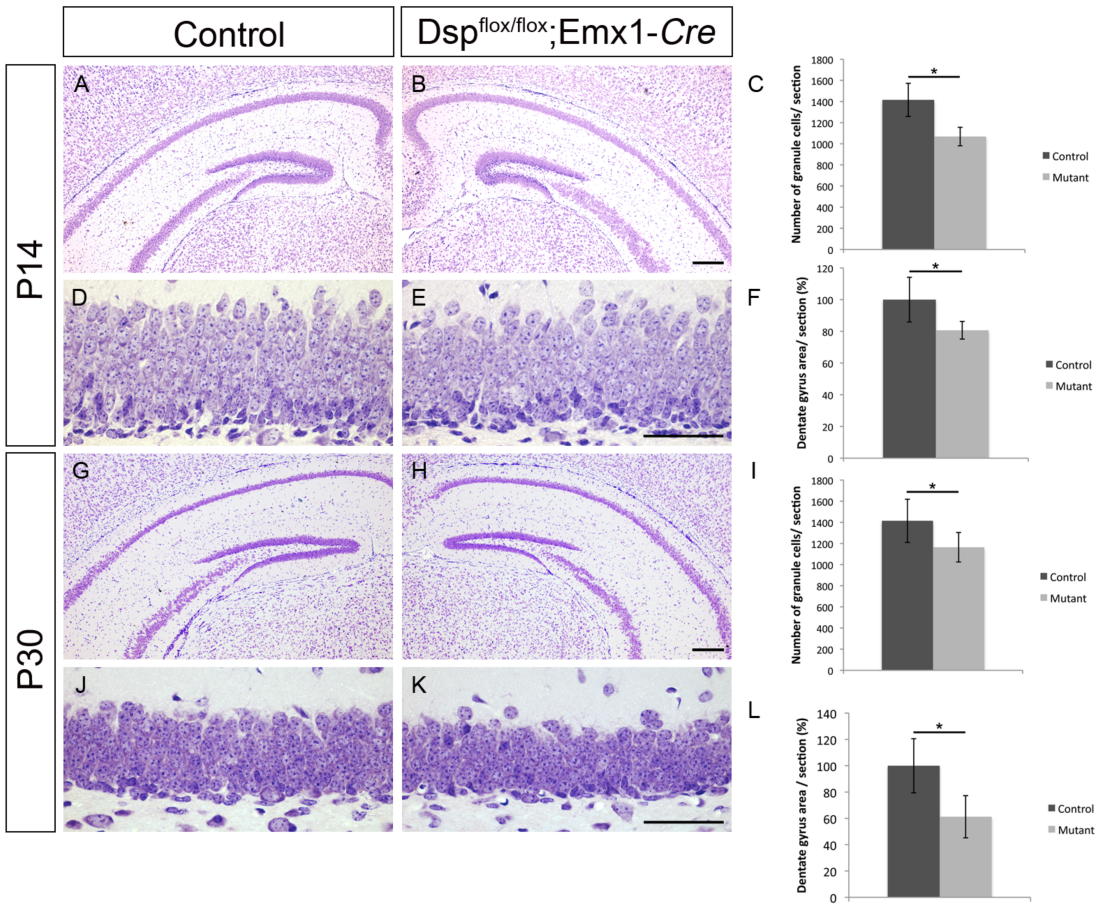

Fig.3.5. Morphological analysis of the hippocampus of Dsp$^{flox/flox}$;Emx1-Cre mice.
Cresyl-violet staining of hippocampus sections of control at P14 (A, D), P30 (G, J) and Dsp$^{flox/flox}$;Emx1-*Cre* at P14 (B, E), P30 (H, K). Quantitative analysis of the dentate gyrus cell number at P14(C), P30 (I) and area at P14 (F), P30 (L). (*t*-test, *;*P*<0.05; error bar, s.d,; n=4). Scale bar: 250μm (B), 50μm (E) (A-F published in Simon et al, 2012, page 2922-36).

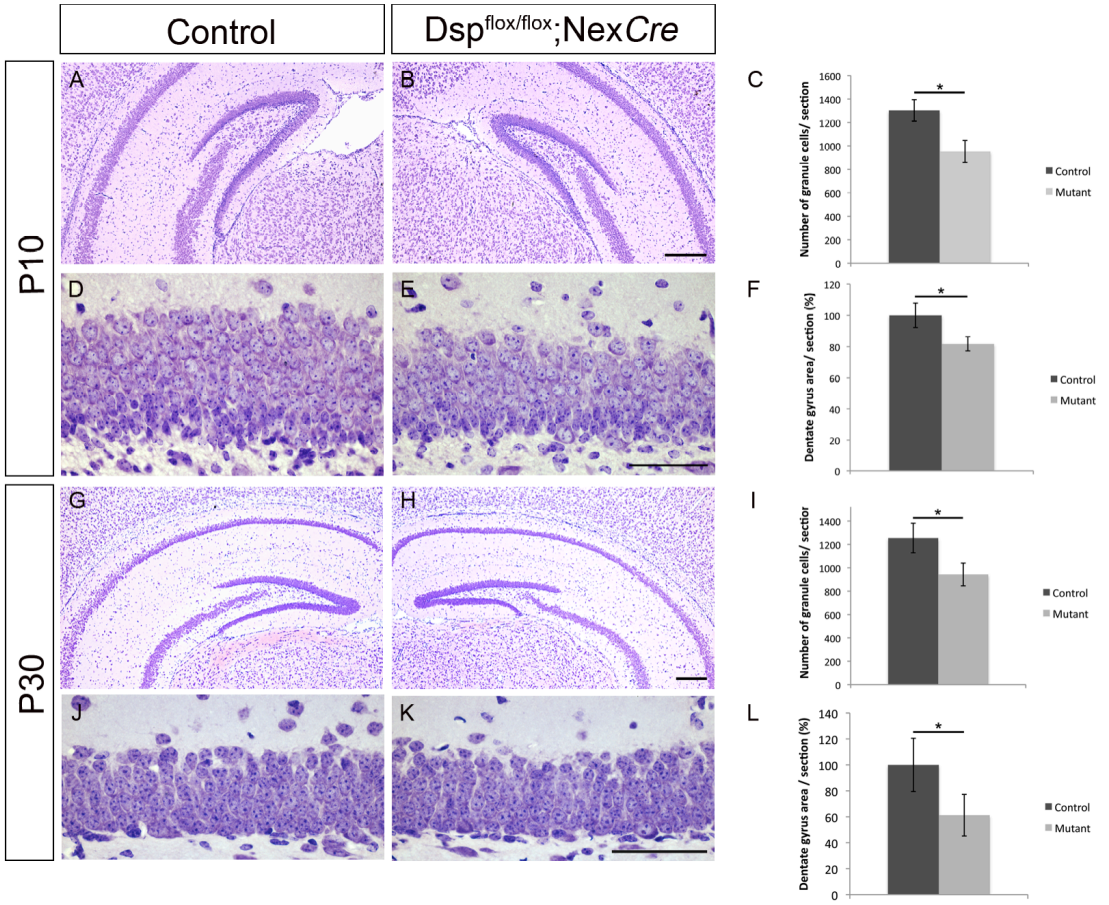

Fig.3.6. Morphological analysis of the hippocampus of Dsp$^{flox/flox}$;Nex-Cre mice.
Cresyl-violet staining of hippocampus sections of control at P10 (A, D), P30 (G, J) and Dsp$^{flox/flox}$;Nex-*Cre* at P10 (B, E), P30 (H, K). Quantitative analysis of the dentate gyrus cell number at P10(C), P30 (I) and area at P10 (F), P30 (L). (*t*-test, *;*P*<0.001; error bar, s.d,; n=4). Scale bar: 250μm (B, H), 50μm (E, K).

3.2.3 Analysis of apoptosis in the dentate gyrus of desmoplakin mutants.

To determine whether an elevated apoptotic cell death contributes to the reduced number of granule cells and the smaller dentate gyrus area of desmoplakin mutants, apoptotic nuclei were visualized by TUNEL assays and quantified by counting the number of TUNEL positive cells in the dentate gyrus.

In the incipient postnatal dentate gyrus, we observed approximately a 55% reduction of TUNEL positive cells in the dentate gyrus of P14 Dsp$^{flox/flox}$;Emx1-*Cre* (Fig. 3.7 B, C 1.55 ± 0.5 s.d.) and P10 Dsp$^{flox/flox}$;Nex-*Cre* animals (Fig. 3.8. B, C 1.16 ± 0.11 s.d.), compared to control animals of both *Cre* lines (Fig. 3.7 A, C 5.03 ± 1.68 s.d., P<0.03) (Fig. 3.8 A, 2.33 ± 0.32 s.d., P<0.01). At P30, the TUNEL positive cells were reduced by

40% in Dsp$^{flox/flox}$;Emx1-*Cre* (Fig. 3.7 E, F 3.77 ± 0.38 s.d.) and Dsp$^{flox/flox}$;Nex-*Cre* (Fig. 3.8 E, F 2.25 ± 0.60 s.d.) lines compared to there respective control animals of both *Cre* lines (Fig. 3.7 D, F 5.74 ± 0.73 s.d., P<0.05) (Fig. 3.8 D, F 6.05 ± 1.08 s.d., P<0.005). These results indicate that the reduction of the granule cell number in the dentate gyrus is not caused by apoptosis, but possibly by a changed rate of cell proliferation.

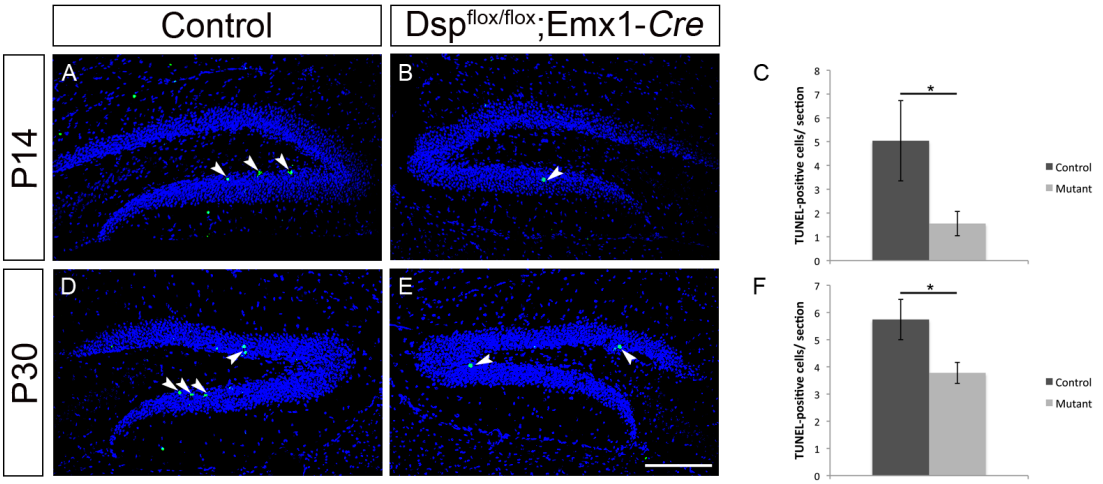

Fig. 3.7 Decreased cell death in Dsp$^{flox/flox}$;Emx1-*Cre* animals.
TUNEL staining of control P14 (A), P30 (D) and Dsp$^{flox/flox}$;Emx1-*Cre* P14 (B), P30 (E) hippocampi. Quantitative analysis of TUNEL positive cells at P14 (C) and P30 (F) show a reduction in apoptosis (*t*-test, *; *P*<0.05; error bar, s.d,; n=3). Scale bar: 100μm (E) (A, B and C published in Simon et al, 2012, page 2922-36).

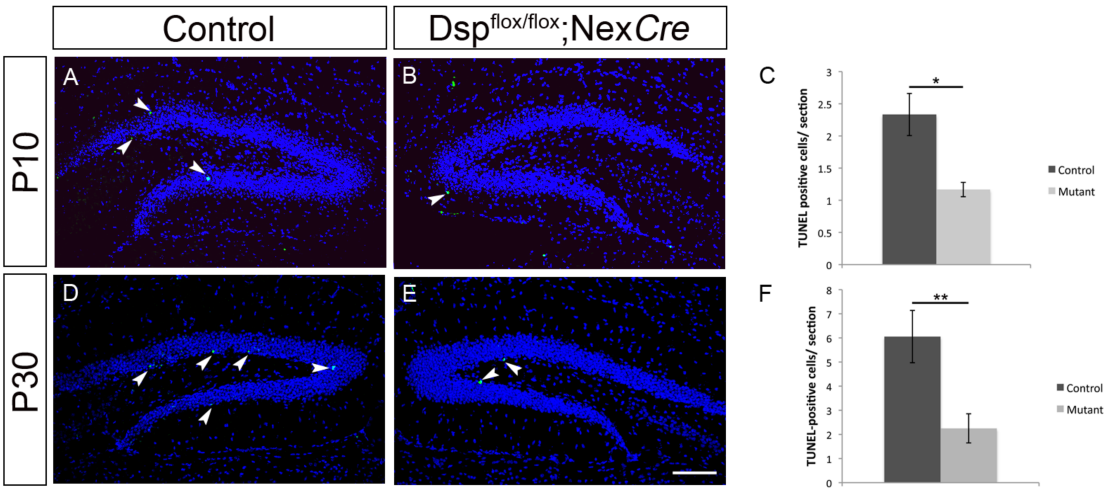

Fig. 3.8 Decreased cell death in Dsp$^{flox/flox}$;Nex-*Cre* animals.
TUNEL staining of control P10 (A), P30 (D) and Dsp$^{flox/flox}$;Nex-*Cre* P10 (B), P30 (E) hippocampi. Quantitative analysis of TUNEL positive cells at P10 (C) and P30 (F) show a reduction in apoptosis (*t*-test, *; *P*<0.05, **P<0.005; error bar, s.d,; n=3). Scale bar: 100μm (E).

3.2.4 Analysis of proliferation of dentate gyrus progenitor cells

To determine the proliferation activity of progenitor cells in the dentate gyrus at different postnatal developmental stages (P14, P30 in Emx1-*Cre,* P10, P30 in Nex-*Cre*), BrdU was injected into mice 2 h prior to the dissection of the brain. BrdU, a thymidine analog is incorporated into the DNA during the S phase of the cell cycle and was detected by immunohistochemistry using a specific BrdU antibody.

In the early postnatal dentate gyrus, we observed a approximately 25% and 35% reduction of BrdU positive cells in the dentate gyrus of P14 Dsp$^{flox/flox}$;Emx1-*Cre* (Fig. 3.9 B, C., 25.6 ± 1.45 s.d.) and P10 Dsp$^{flox/flox}$;Nex-*Cre* (Fig. 3.10 B, C. 37.6 ± 4.39 s.d.) lines, respectively, compared to control animals of both *Cre* lines (Fig. 3.9 A, C., 34.74 ± 1.57 s.d., P<0.006) (Fig. 3.10 A, C., 60 ± 8.15 s.d., P<0.004). This proliferation phenotype is further strengthened at P30 with a reduction of approximately 50% of BrdU positive cell in the dentate gyrus of Dsp$^{flox/flox}$;Emx1-*Cre* (Fig. 3.9 E, F, 6.85 ± 0.611 s.d.) and Dsp$^{flox/flox}$;Nex-*Cre* (Fig. 3.10 E, F, 8.16 ± 0.88 s.d.) lines, respectively, compared to control animals of both *Cre* lines (Fig. 3.9 D, F, 11.7 ± 1.31 s.d., P<0.006) (Fig. 3.10 D, F, 14.5 ± 2.35 s.d., P<0.005). Taken together, the data obtained from Emx1-*Cre* and Nex-*Cre* lines suggest that desmoplakin plays a pivotal role in the

proliferation of progenitor cells. This is probably due to a feedback mechanism that relays signals from postmitotic granule cells, where desmoplakin expression occurs, to progenitor cells located in the subgranular zone.

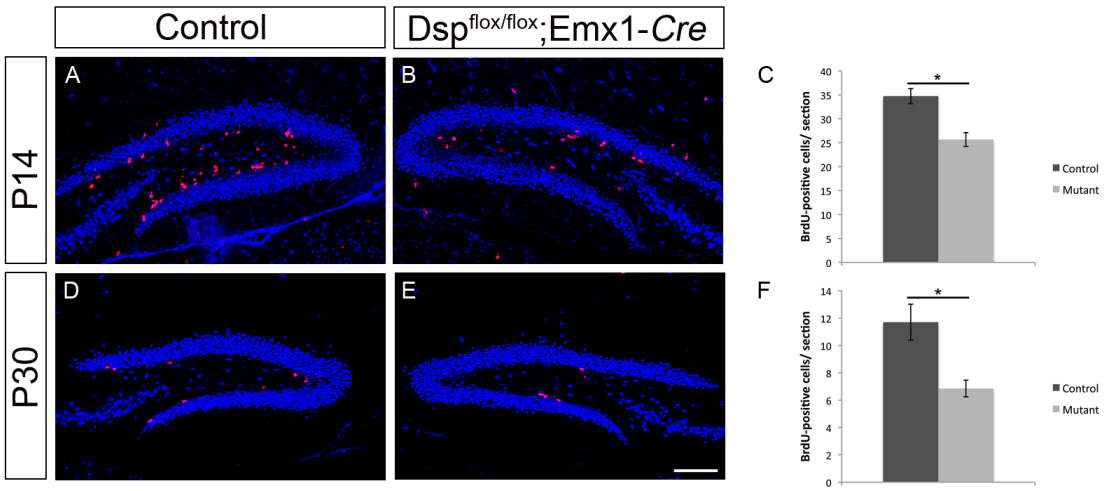

Fig. 3.9 Ablation of desmoplakin expression by Emx1-Cre affects progenitor cell proliferation of the postnatal dentate gyrus.
Immunohistochemical detection of BrdU positive cells (red) and *NeuN* (blue) in the dentate gyrus of control P14 (A), P30 (D) and Dsp$^{flox/flox}$;Emx1-*Cre* at P14 (B), P30 (E) animals. Quantitative analysis of BrdU positive cells at P14 (C) and P30 (F) *t*-test, *; $P<0.005$; error bar, s.d; n=3). Scale bar: 100μm (E) (A-C published in Simon et al, 2012, page 2922-36).

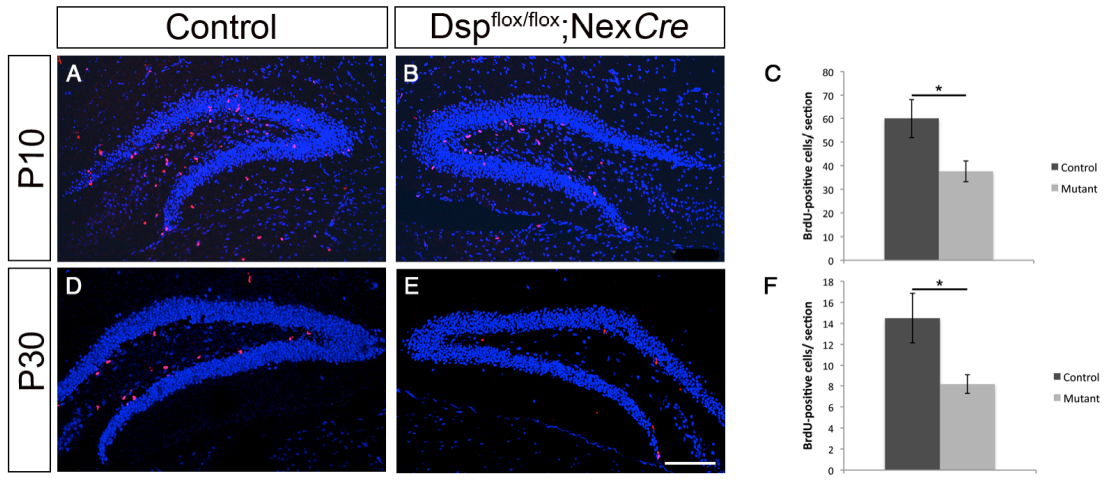

Fig. 3.10 Ablation of desmoplakin expression by Nex-Cre affects progenitor cell proliferation of the postnatal dentate gyrus.
Immunohistochemical detection of BrdU positive cells (red) and *NeuN* (blue) in the dentate gyrus of control P10 (A), P30 (D) and Dsp$^{flox/flox}$;Nex-*Cre* P10 (B), P30 (E) animals. Quantitative analysis of BrdU positive cells at P10 (C) and P30 (F) (*t*-test, *; $P<0.005$; error bar, s.d,; n=4). Scale bar: 100μm (E).

3.2.5 Analysis of granule cell differentiation in desmoplakin mutants.

To determine whether ablation of desmoplakin in the forebrain has an effect on the differentiation of granule cells, we analyzed the number of NeuroD positive cells in the dentate gyrus. Immunohistochemistry was performed on 12 - 15 μm thin frozen brain sections using a NeuroD specific antibody.

We observed a 35% and 30% increase of NeuroD-positive cells in the dentate gyrus of P14 $Dsp^{flox/flox}$;Emx1-*Cre* (Fig. 3.11 B, D, 821.2 ± 72.9 s.d.) and P10 $Dsp^{flox/flox}$;Nex-*Cre* (Fig. 3.12 B, D, 944.8 ± 170.6 s.d.) animals, respectively, when compared to control animals of the respective *Cre* line (Fig. 3.11 A, C, 591 ± 60.3 s.d., $P<0.006$) (Fig. 3.12 A, C, 727.72 ± 120.6 s.d., $P<0.009$). This differentiation is further strengthened at P30 with an increase to approximately 75% and 60 % of NeuroD positive cells in the dentate gyrus of $Dsp^{flox/flox}$;Emx1-*Cre* (Fig. 3.11 G, I, 277.5 ± 5.1 s.d.) and $Dsp^{flox/flox}$;Nex-*Cre* (Fig. 3.12 G, I, 180 ± 45.9 s.d.) animals, respectively, compared to control animals of both *Cre* lines (Fig. 3.11 F, H, 156.4 ± 3.99 s.d., $P<0.001$) (Fig. 3.12 F, H, 111.8 ± 24.5 s.d., $P<0.01$). Together these results suggest that ablation of desmoplakin in the dentate gyrus arrests the differentiation of immature granule cells.

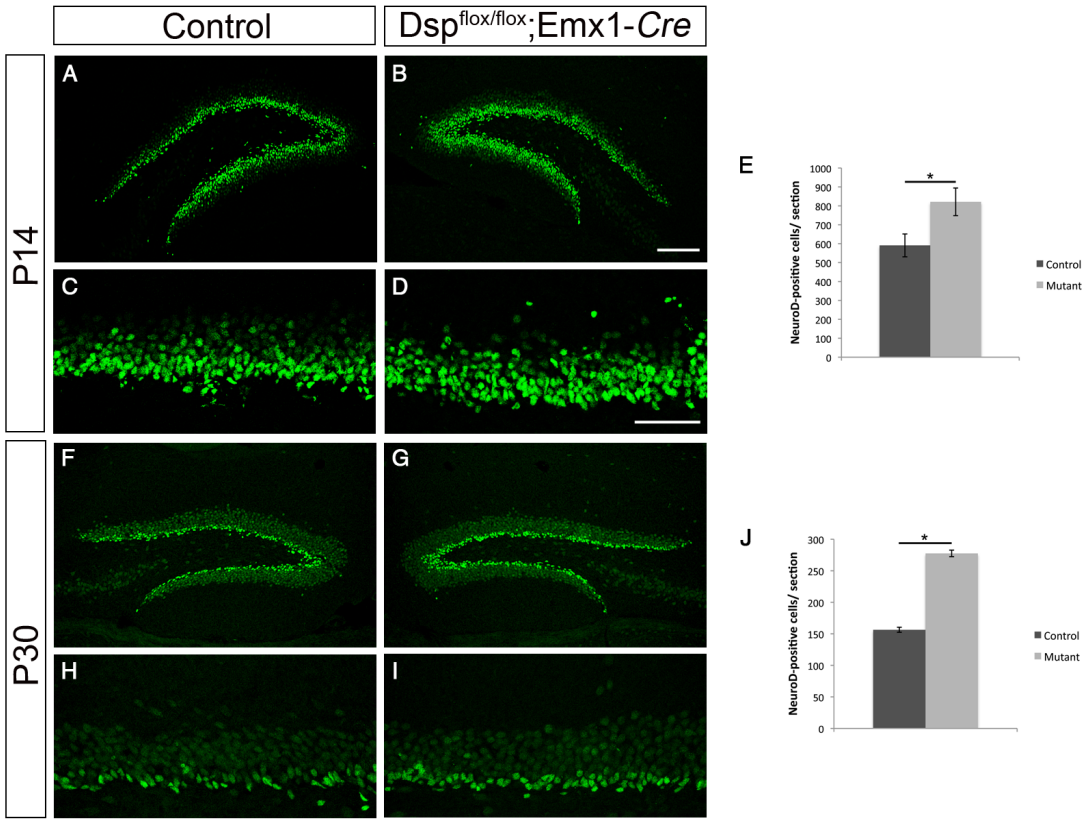

Fig. 3.11 Forebrain specific ablation of desmoplakin by Emx1-Cre affects granular cell differentiation in the dentate gyrus.
Immunohistological analysis of the differentiation marker NeuroD (green) in the hippocampi of control P14 (A, C), P30 (F, H) and Dsp$^{flox/flox}$;Emx1-*Cre* P14 (B, D), P30 (G, I) mice. Quantitative analysis of NeuroD positive cells at P14 (E) and P30 (J) (*t*-test, *; $P<0.005$; error bar, s.d,; n=3). Scale bar: 100μm (B) 50μm (D) (A-E published in Simon et al, 2012, page 2922-36).

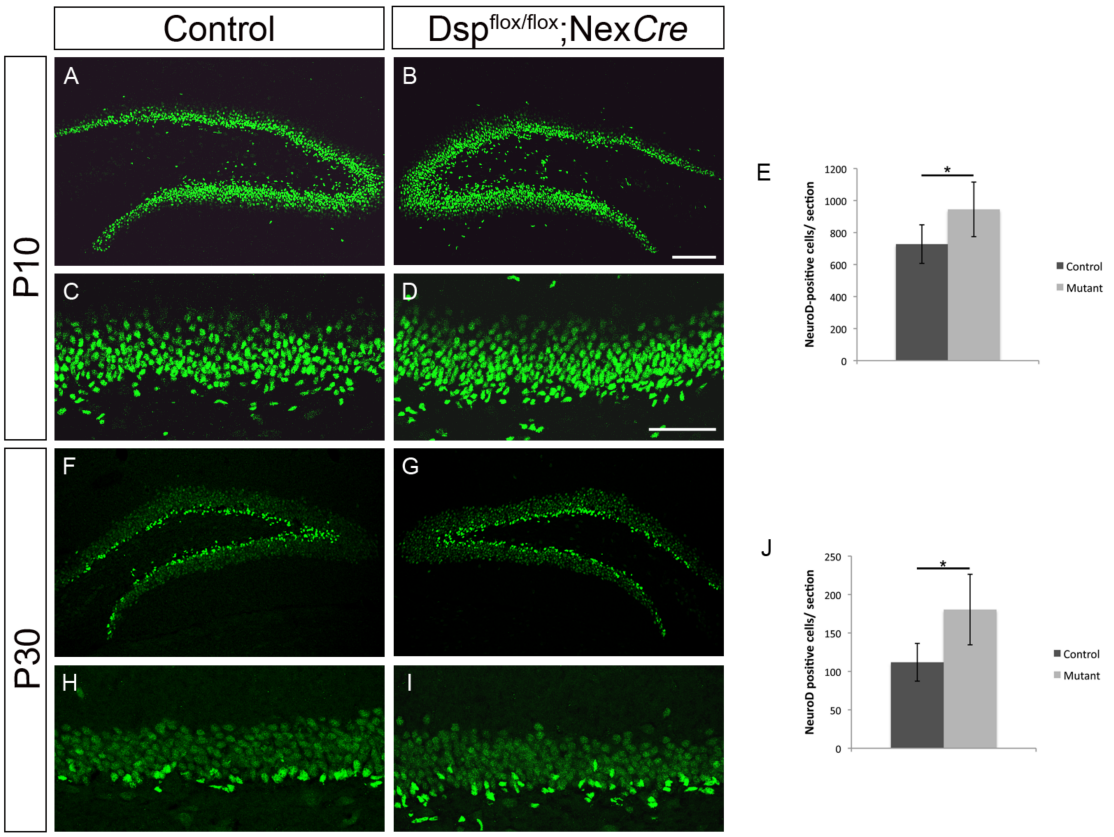

Fig. 3.12 Forebrain specific ablation of desmoplakin by Nex-Cre affects granular cell differentiation in the dentate gyrus.
Immunohistological analysis of the differentiation marker NeuroD (green) in the hippocampi of control P10 (A, C), P30 (F, H) and Dsp$^{flox/flox}$;Nex-*Cre* P10 (B, D), P30 (G, I) mice. Quantitative analysis of NeuroD positive cells at P10 (E) and P30 (J) (*t*-test, *; $P<0.001$, **$P<0.01$; error bar, s.d,; n=4 (E) n=3 (J)). Scale bar: 100μm (B), 50μm (D).

3.2.6 Analysis of hippocampal circuitry of desmoplakin mutants

To determine whether ablation of desmoplakin expression in the dentate gyrus has an impact on the formation of the hippocampal circuitry, we analyzed the dendritic spine number and distribution of the granule cell projections. To analyze the synaptic input to the dentate gyrus, we determined the spine numbers along granule cell dendrites as an indirect measure. Timm staining was used to analyze the distribution of mossy fiber terminals emanating from the dentate gyrus and terminating at thorny excrescences of pyramidal neurons in the CA3 region.

3.2.6.1 Analysis of dendritic spines in desmoplakin mutants.

To determine the dendritic spine number hippocampal brain sections were stained using the Golgi impregnation method (Ramon-Moliner E, 1970). Dendrite shafts were divided into 50μM segments starting at the cell soma to the end of the dendrites and only dendrites more than 150μM in length were chosen and equal numbers of dendrites were chosen from the dorsal and ventral blade of the dentate gyrus. We observed a significant reduction of dendritic spines in Dsp$^{flox/flox}$;Emx1-*Cre* mutants, in all three 50μM segments of the dendrites (Fig. 3.13 B, C, 0-50: 54.1 ± 8.51 s.d., 50-100: 70.5 ± 5.2 s.d., 100-150: 67.45 ± 5.3 s.d.) when compared to the controls (Fig. 3.13 A, C, 0-50: 75.8 ± 5.91 s.d, P<0.05, 50-100: 87.01 ± 4.4 s.d., P<0.05, 100-150: 83.08 ± 2.3 s.d., P<0.02).

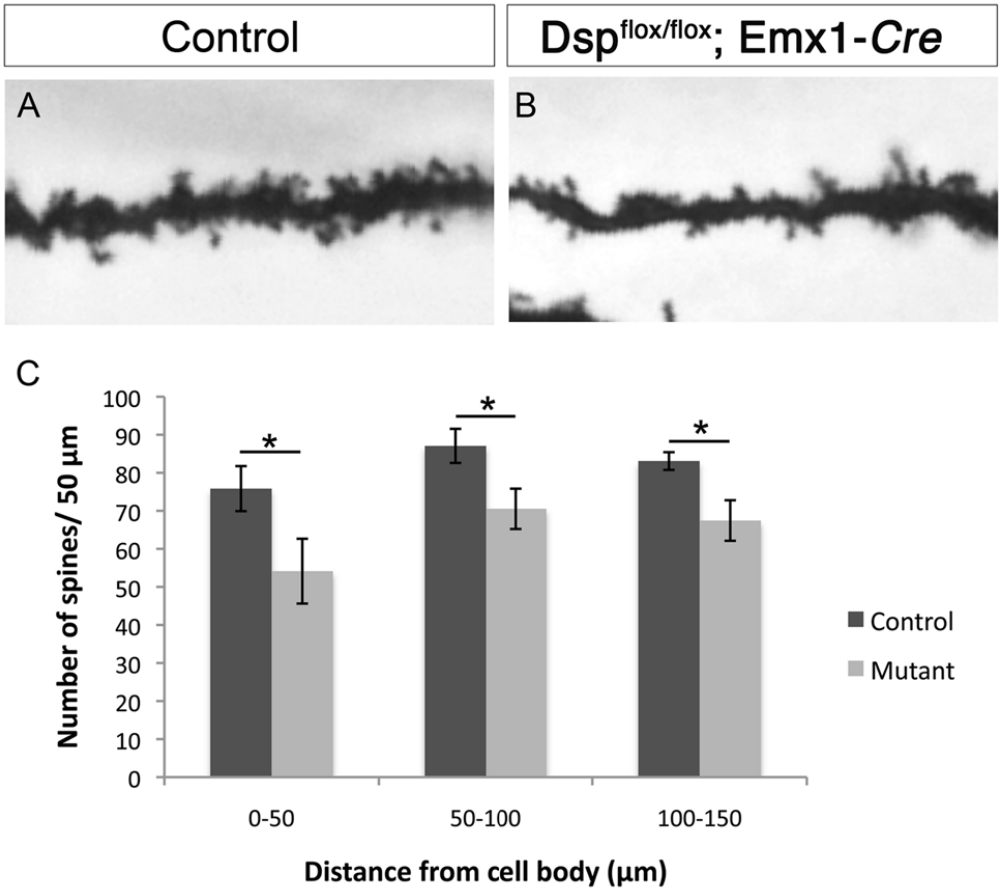

Fig. 3.13. Analysis of dendritic spines on granule cells of the dentate gyrus.
Golgi stained dendritic spines of control (A) and Dsp$^{flox/flox}$;Emx1-*Cre* (B) dentate granule cells. Quantitative analysis of dendritic spine numbers of control and Dsp$^{flox/flox}$;Emx1-*Cre* mutants (C). Dendrite shafts were subdivided in to 50μm sections starting at the cell soma (*t*-test, **; *P*<0.05; error bar, s.d; n=3).

3.2.6.2 Mossy fiber distribution and thorny excrescences.

To analyze the distribution of mossy fiber axons, which terminate on characteristic spines called thorny excrescences of the pyramidal cells in the CA3 region, Timm staining was performed (Zimmer et al, 1974). The Golgi impregnation method (Ramon-Moliner E, 1970) was used for analyzing thorny excrescences in CA3 region.
We observed no obvious difference in the distribution of mossy fibers in the CA3 region (Fig. 3.14 A, B) of the Dsp mutant, which is also corroborated by the unchanged number of thorny excrescences on proximal apical dendrites of CA3 neurons when compared to controls (Fig. 3.14 C control: 24.3 ± 1.08 s.d.; mutant 23.7 ± 0.66 s.d., P<0.4).

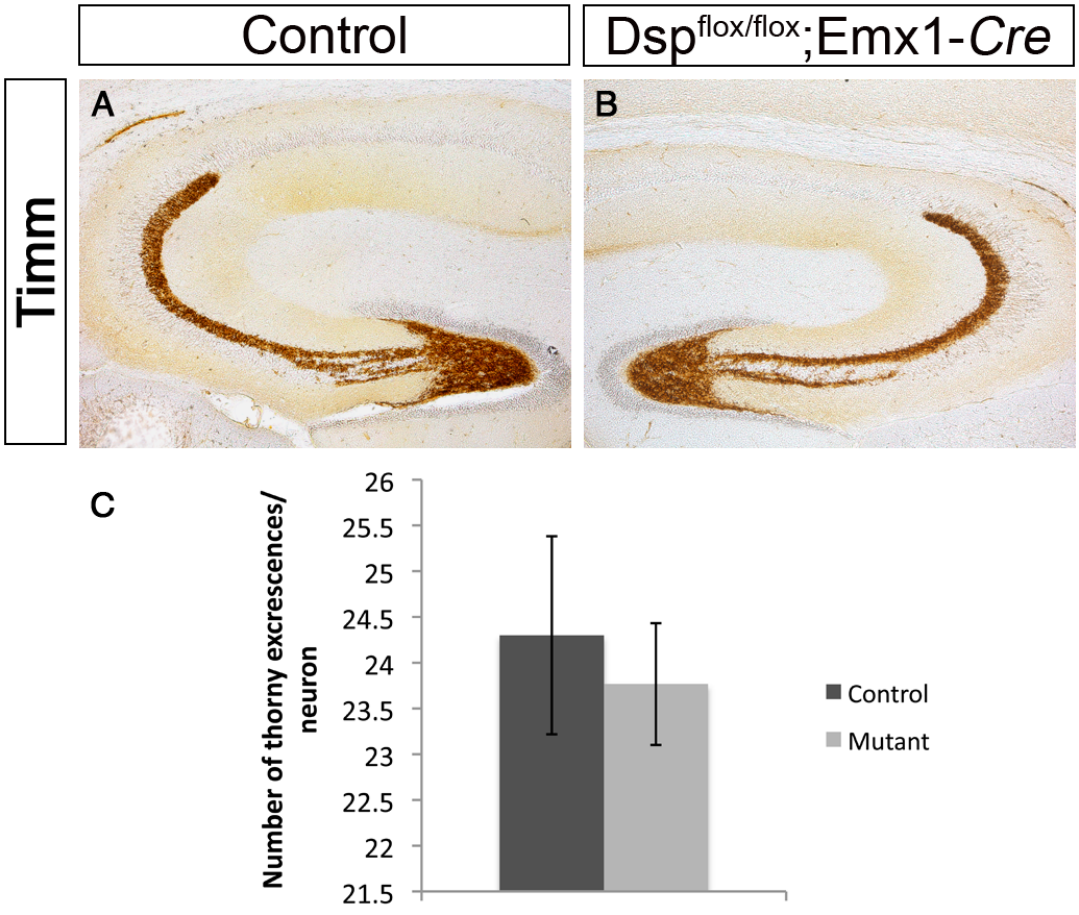

Fig. 3.14. Analysis of granule cell layer processes of Dsp$^{flox/flox}$;Emx1-*Cre* mice.
Timm stained mossy fibers of adult hippocampi of control (A) and Dsp$^{flox/flox}$;Emx1-*Cre* (B) animals. There is no detectable difference between control and mutant mossy fiber distribution. Quantitative analysis of thorny excrescences on apical dendrites of CA3 pyramidal neurons of Golgi impregnated control and desmoplakin mutant hippocampi (C) (s.d,; n=3) (A, B published in Simon et al, 2012, page 2922-36).

3.2.7 Analysis of learning and memory behavior of desmoplakin mutants

The hippocampus plays a pivotal role in learning and memory processing. To investigate whether the deranged development of the dentate gyrus in desmoplakin mutants has an influence on the emotional and cognitive learning behavior of the animals, desmoplakin mutants were examined in the open field (exploration) and radial arm maze (spatial learning) tests.

3.2.7.1 Open field (exploration)

In the open field test the behavior of the mice was observed in an area of 36 x 60 cm with a 28 cm high rim divided into 3 x 5 squares and the activity recorded: Locomotor activity (number of lines crossed), Leaning (mice standing erect and leaning with the front paws on the wall) and Rearing (mice in upright position on their hind legs). Activity of $Dsp^{flox/flox}$;Emx1-*Cre* were recorded in the open field test: locomotor activity (Fig. 3.15 A, 165 ± 28.2 s.e.m, n=10), leaning (Fig. 3.15 B, 41.1 ± 5.4 s.e.m, n=10), and rearing (Fig. 3.15 C, 27.2 ± 7.1 s.e.m, n=10), compared to controls, locomotor activity (Fig. 3.15 A, 233.4 ± 25.6 s.e.m, n=10, $P<0.07$), leaning (Fig. 3.15 B, 39.6 ± 4.9 s.e.m, n=10, $P<0.7$), and rearing (Fig. 3.15 C, 41.1 ± 6.2 s.e.m, n=10, $P<0.058$). While the desmoplakin mutants have a tendency towards explorative behavior, no significant differences were observed when compared to control animals.

3.2.7.2 Spatial learning

In the radial maze test (8-arm radial maze) various hippocampus dependent learning and memory processes can be analyzed, such as spatial learning, working memory or memory consolidation in mice (Bolhuis et.al, 1986; O'Keefe et al 1971; O'Keefe and Nadel 1985;Schwegler et al 1990). In contrast to the 'Morris water maze', where the animals are stressed because of swimming in water, radial maze is a dry version of

the maze experiment, which allows to largely exclude the stress factor. Food is placed at the end of the arms as an incentive to enter the arms of the radial maze.

Adult desmoplakin control and mutant animals aged about 12-13 weeks received training for 2 days in the radial maze for 10 min per day followed by the experiment on 5 successive days (Schwegler et at 1990). On the first day of the radial maze experiment, $Dsp^{flox/flox}$;Emx1-*Cre* animals displayed a higher number of errors when compared to controls. In the following days, $Dsp^{flox/flox}$;Emx1-*Cre* learned to navigate through the maze with a lesser number of working memory errors compared to the first day in the maze. When the data was statistically analyzed for working memory errors for five days, desmoplakin control and mutant animals showed no significant difference (Fig. 3.15 D). Data from day 3 – 5 of the radial maze experiment was analyzed for the number of novel arms entered (new entries) by desmoplakin control and mutant animals. We observed no significant difference between $Dsp^{flox/flox}$;Emx1-*Cre* (Fig. 3.15 E, 6.37± 0.17 s.e.m, n=10) and control animals (Fig. 3.15 E, 5.89 ± 0.13 s.e.m, n=10, $P<0.17$).

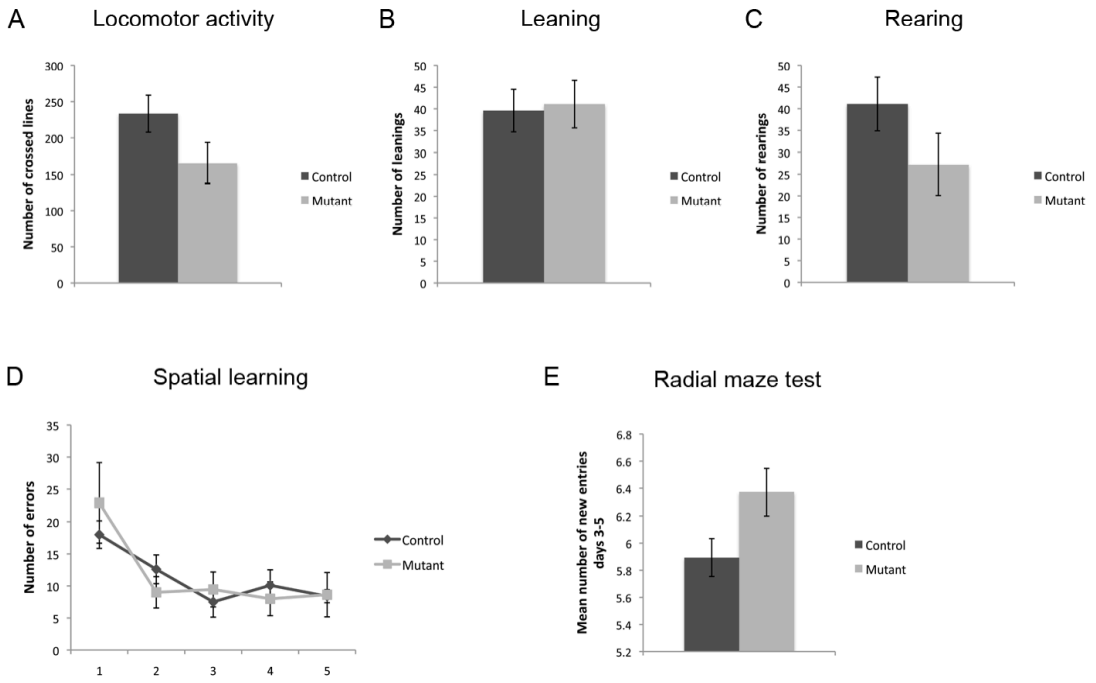

Fig. 3.17. Behavior analysis of Dsp$^{flox/flox}$;Emx1-*Cre*.
Dsp$^{flox/flox}$;Emx1-*Cre* animals were analyzed by subjecting the mice to locomotor activity (A), Leaning (B), Rearing (C), Spatial learning (D) and Radial maze (E) (A-E published in Simon et al, 2012, supplementary Figure S7).

3.3 Slice culture analysis of desmoplakin over-expression

Complex tissues such as the central nervous system with a composite order of architecture require temporally and spatially dynamic epigenetic cues that cannot be reproduced in dissociated cell culture. Organotypic cultures represent in-vitro models that keep the different cell types and retain the complex three-dimensional organization. A combination of electroporation-mediated gene transfer and organotypic slice culture was used to analyze the effects of over-expression of desmoplakin and a possible rescue of the desmoplakin phenotype.

Loss of desmoplakin expression leads to a decrease in progenitor cell proliferation. To examine whether over-expression of desmoplakin results in an increase of progenitor cell proliferation, vector pRC CMV *Dsp* was introduced into the dentate gyrus of control animals at E 15.5 by *ex-utero* electroporation. The embryos from wild type animals were dissected at E15.5 and prepared for *ex-utero* electroporation as described by Hand et al, 2005. Dissection of the brain and *ex-utero* electroporation

was performed as described in section 2.20. Immunohistochemistry was performed with appropriate primary antibodies (anti GFP antibody, anti desmoplakin antibody) along with anti-BrdU antibody.

Determining the BrdU incorporation in dentate gyrus slice cultures electroporated with pRC CMV *Dsp*, (Fig. 3.16 B, D) we observed an 61.8% increase in BrdU positive cells compared to dentate gyrus slice cultures electroporated with the control vector pRC CMV (Fig. 3.16 A, C control 21.9 ± 4.1 s.d.; over-expression 35.4 ± 6.4., P<2.8E-08). The electroporation of the spike vector pCAGGS GFP along with pRC CMV *Dsp* was used as a read out for the efficiency of the electroporation by quantifying the GFP positive cells (Fig. 3.16 E).

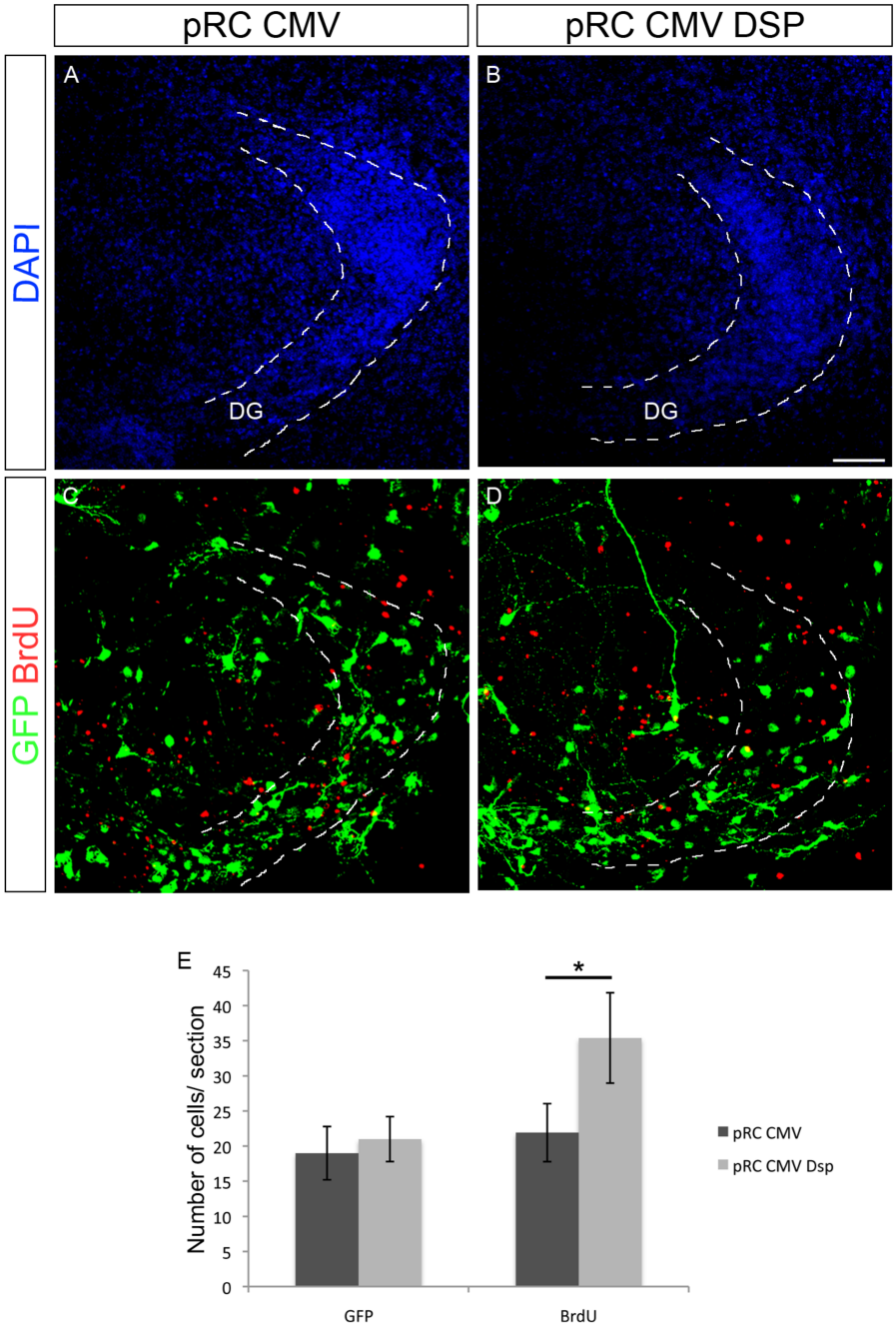

Fig. 3.16 Analysis of Desmoplakin over-expression by ex-utero electroporation. Immunohistological analysis of hippocampal slice cultures electroporated with vector pRC CVM (A, C) or vector pRC CVM Dsp (B, D), as well as vector pCAGGS expressing GFP, after 11 DIV and stained with DAPI (morphological marker). Quantitative analysis of GFP and BrdU-positive cells in pRC CMV and pRC CMV Dsp electroporated hippocampal slice cultures (E). *t*-test, *; $P<0.005$; error bar, s.d; n=7. Scale bar: 50μm (B), DG (Dentate gyrus).

3.4 Rescue of the Bcl11b mutant phenotype by desmoplakin

Previously desmoplakin was identified as a direct downstream target of Bcl11b (Simon R et al, 2012). Over expression of *Dsp* cDNA in the control animals increases the proliferation in the dentate (3.3.1). To evaluate the potential role of desmoplakin in Bcl11b mutants, *Dsp* cDNA was re-introduced by *ex-utero* electroporation followed by analysis of the proliferation phenotype (Fig. 3.17 A-F). Bcl11b mutant embryos were dissected at E15.5 and prepared for *ex-utero* electroporation as described by Hand et al, 2005. Immunohistochemistry was performed with appropriate primary antibodies (anti GFP antibody, anti NeuroD antibody, anti desmoplakin antibody) along with anti-BrdU antibody.

Analyzing the number of BrdU positive cells in the Bcl11b mutant dentate gyrus, electroporated with the empty vector pRC CMV showed a reduction of approximately 50% of BrdU incorporation (Fig.3.17 B, E) compared to Bcl11b expressing controls with or without the control vector pRC CMV (Fig.3.17 A, D) (control without electroporation: 22.2 ± 3.7 s.d.; control + pRC CMV: 21.6 ± 5.7 s.d; mutant + pRC CMV: 12.1 ± 4.7 s.d.; $P<0.01$). GFP positive cells indicate the electroporation efficiency. Re-introducing desmoplakin into the Bcl11b mutant dentate gyrus by pRC CMV *Dsp* (Fig.3.17 F insert) resulted in the complete recovery of the number of BrdU positive cells (Fig.3.17 C, F) (22.1 ± 5.3 s.d.) compared to the control (Fig.3.17 A, D). Furthermore most of the BrdU positive cells do not co-localize with GFP positive cells indicating an indirect regulation of progenitor cell proliferation by *Bcl11b/ Dsp* (Fig.3.17 H).

Analysis of the differentiation marker NeuroD, in the Bcl11b mutant dentate gyrus electroporated with the control vector pRC CMV and pRC CMV *Dsp* did not show any significant change in the number of GFP/ NeuroD positive cells.

Taken together the results from over-expression of desmoplakin in control animals (3.3.1) and rescue of proliferation phenotype in Bcl11b mutants by desmoplakin strongly indicate an indirect mechanism involved in proliferation of progenitor cells in dentate gyrus.

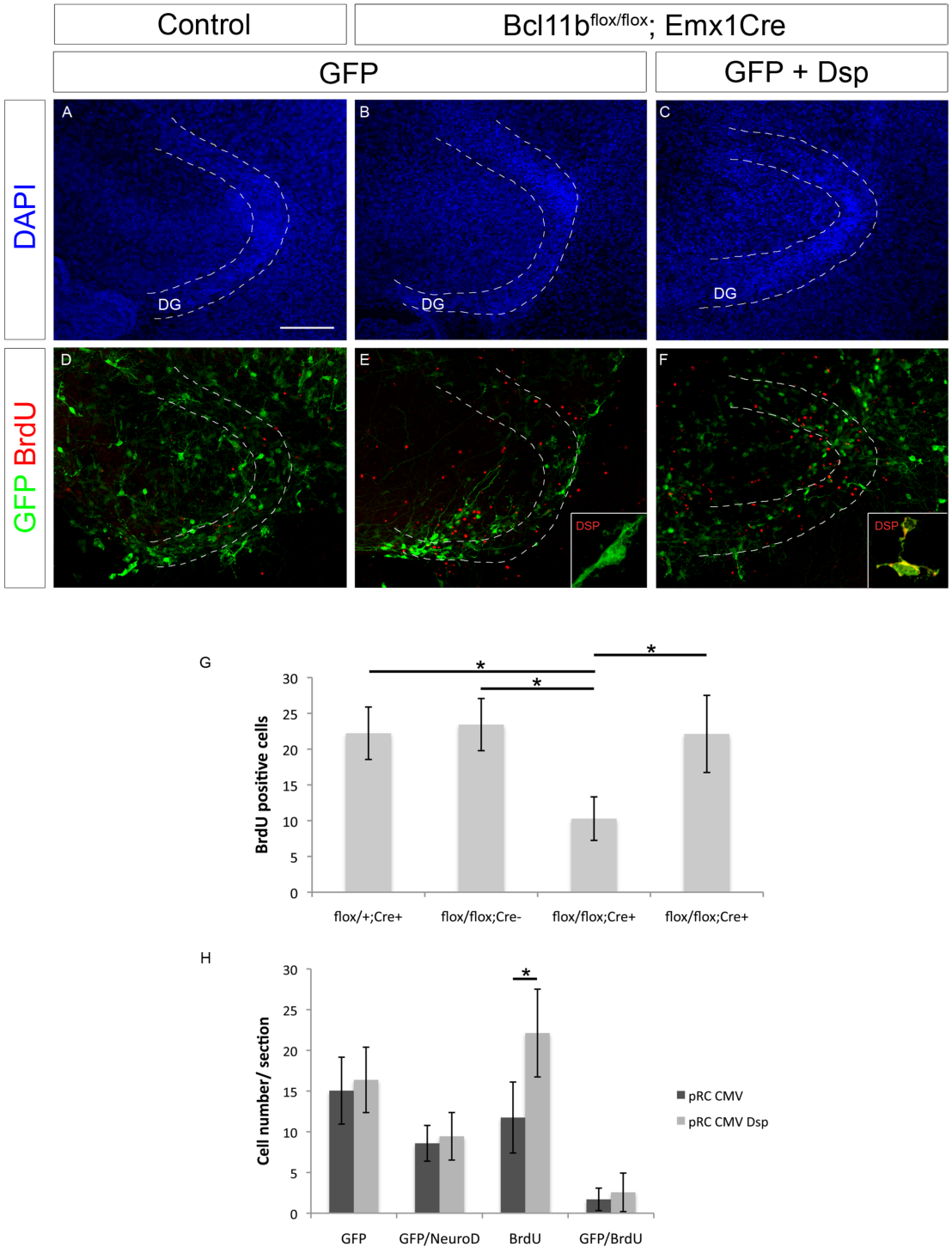

Fig. 3.15 Re-introduction of Desmoplakin expression rescues the Bcl11b phenotype. (A–F) Immunohistological analysis of control (A, D) and Bcl11b$^{flox/flox}$;Emx1-*Cre* (B, C, E, F) hippocampal slice cultures after 11 DIV electroporated with control pRC-CMV (A, B, D, E) and pRC CMV *Dsp* (C, F) expression vectors as well as vector pCAGGS expressing GFP. DAPI (blue) as morphological marker, GFP (green) as marker for electroporation efficiency and BrdU (red) as marker for cell proliferation. Inserts in (E) and (F) showing Dsp and GFP expression. Images taken at 20x magnification. (G) Statistical analysis of BrdU-positive cells in control and mutant hippocampal slice cultures. (H). Quantitative analysis of co-expression of GFP/NeuroD- and GFP/BrdU-positive cells (t-test, *; P<0.01; error bar, s.e.m.; n=4). Scale bar: 50µm (A), DG (Dentate gyrus). (A-H published in Simon et al, 2012, page 2922-36).

4. Discussion

In the work presented here, the functions of the desmoplakin protein were studied during the development of the hippocampus by analyzing the phenotype of mice with a forebrain specific mutation of the desmoplakin gene.

Neurogenesis during postnatal development of the hippocampus leads to proliferation of progenitor cells giving rise to postmitotic immature granule cells, which eventually undergo differentiation and integration into the hippocampal circuitry (Van Praag et al, 2002, Zhao et al, 2008, Yu DX et al, 2014, Luikart et al, 2012). This complex and dynamic process is controlled by the interaction of various factors that control proliferation, migration and differentiation as well as the survival of progenitor cells and other neuronal populations (Qu et al, 2013, Hodge et al, 2011, Machold et al, 2003, Pellegrini et al, 1996, Furuta et al, 1997). In my thesis I could demonstrate that the loss of desmoplakin expression causes the reduction of progenitor cell proliferation in the tertiary matrix of the dentate gyrus as well as a defect in the terminal differentiation of the postmitotic, immature granule cells. As a consequence of this impaired neurogenesis during the early postnatal development of the dentate gyrus, the number of granule cells and the dentate gyrus area are significantly reduced. In addition I observed a reduction in apoptotic granule cells in the dentate gyrus. Employing behavioral experiments I could show that there was no change in the emotional and cognitive learning behavior associated with the morphological changes in the dentate gyrus of the desmoplakin mutant mice.

Finally I could demonstrate that overexpression of desmoplakin cDNA in the dentate gyrus of wild type animals leads to an increase of the proliferation rate of granule cells in the dentate gyrus. Furthermore by reintroducing desmoplakin into the dentate gyrus of Bcl11b mutants, an upstream transcription factor of desmoplakin, the Bcl11b proliferation phenotype could be rescued.

These findings of desmoplakin's functional aspects during hippocampal development were unexpected and surprising. These properties are intriguing due to the fact that desmoplakin is established as a protein involved in maintaining cell and tissue integrity in keratinocytes and cardiomyocytes, but not in the development of neuronal tissue such as the dentate gyrus.

4.1 Desmoplakin is essential for postnatal neurogenesis in the dentate gyrus.

The morphogenesis of the dentate gyrus during development consists of two phases: the first phase takes place during prenatal development of the embryo, which includes the proliferation of the mitotic progenitor cells and postmitotic immature granule cells from the secondary matrix of the neuroepithelium. These cells migrate and settle in the dorsal (supra-pyramidal layer) blade of the newly formed granule cell layer in the dentate anlage. The second phase takes place postnatally and includes migration and formation of the secondary neurogenic niche consisting of mitotically active progenitor cells within the dentate gyrus known as tertiary matrix. This secondary neurogenic niche in the subgranular zone forms more than 80% of granule cells of the dentate gyrus during postnatal development. In addition, this neurogenic niche allows neurogenesis to occur throughout adulthood (Schlessinger et al, 1975, Altman et al, 1990).

The histological studies on desmoplakin mutants show a significant reduction of granule cells of the dentate gyrus in the postnatal hippocampus. This reduction was evident at postnatal day 10 in $Dsp^{flox/flox}$;Nex-*Cre* mice with 26 % reduction and at postnatal day 14 in $Dsp^{flox/flox}$;Emx1-*Cre* mice with 25 % reduction compared to control animals of the respective mouse line. This reduction continues even at postnatal day 30 with a 24 % reduction in granule cells in $Dsp^{flox/flox}$;Nex-Cre mice and 20 % in $Dsp^{flox/flox}$;Emx1-Cre mice, compared to the respective control animals. Although histological studies show no obvious morphological differences in the cellular architecture of the hippocampus in desmoplakin mutants, the size of the hippocampus or dentate gyrus was significantly reduced in the mutants of both *Cre* mouse lines. These observations indicate that the reduction of the granule cells in the dentate gyrus of desmoplakin mutants can be attributed to the reduction in the proliferation rate of progenitor cells of the tertiary matrix or an increase in apoptosis.

Analyzing the expression of desmoplakin during postnatal development of the dentate gyrus shows a differential expression pattern in the subgranular and granular zone. The postmitotic, differentiated granule cells highly express desmoplakin, which overlaps with the expression of *Bcl11b* (Simon et al, 2012). Previously, it was shown that desmoplakin is a direct target gene of Bcl11b, which is expressed only in postmitotic, differentiated cells and not in progenitor cells of the dentate gyrus

(Simon et al, 2012). In contrast to *Bcl11b*, faint expression of desmoplakin occurs also in progenitor cells of the dentate gyrus and overlaps with Sox2 expression, a progenitor cell marker. The differential expression of desmoplakin in postmitotic and progenitor cells might be regulated by different unknown factors. It was shown that Bcl11b regulates directly the expression of desmoplakin in postmitotic cells. It was also demonstrated that Bcl11b regulates progenitor cell proliferation in an indirect pathway, which might be also the case in the regulation of desmoplakin expression in progenitor cells (Simon et al, 2012).

4.2 Impaired progenitor cell proliferation of desmoplakin mutants.

During postnatal development thousands (50,000 cells/ day in rat, 700 cell/ day in adult humans)(Schlessinger et al, 1975, Spalding et al, 2013) of granule cells are born in the subgranular zone of the dentate gyrus due to the proliferation of progenitor cells, but only few cells survive (Biebl et al, 2000) and differentiate into mature and functional neurons. The process of postnatal neurogenesis requires a precise and finely tuned balance between proliferation and differentiation of progenitor cells, as well as elimination of non-functional neuronal cells by apoptotic cell death. This is essential for ensuring and maintaining the precise size, structure and functional integrity of the brain structure. I was able to show that loss of function of desmoplakin is associated with reduced proliferation of progenitor cells in the postnatal dentate gyrus, which decreases the ongoing neurogenesis.

A high level of desmoplakin expression is restricted to the postmitotic granule cells while mitotic cells of the dentate gyrus show low levels of expression. Progenitor cell proliferation in the dentate gyrus is significantly reduced in mutants of both Emx1-*Cre*, expressed in mitotic and postmitotic neurons, and Nex-*Cre*, expressed only in postmitotic neurons, mouse lines. Thus, the reduced number of mitotically active cells of the desmoplakin mutant might be caused by indirect, non cell-autonomous effects.

Desmoplakin, a structural protein involved in maintaining the cell and tissue integrity, interacts with many proteins like intermediate filaments, plakoglobin also known as γ-catenin, plakophilin as well as microtubule associated protein. The proliferation of progenitor cells in the dentate gyrus might be directly affected by the

desmoplakin mutation via plakoglobin. Examining atrial myocyte (HL-1) cell lines lacking desmoplakin expression as well as cardiac restricted Dsp-deficient mice shows the translocation of plakoglobin from the cytoplasm to the nucleus (Garcia-Gras et al, 2006). It is possible that the postmitotic cells expressing desmoplakin relay a feedback signal to the neurogenic niche, which influences the behavior of progenitor cells. Desmoplakin mutants also exhibit impaired differentiation of immature neurons to functional granule cells, which suggests that desmoplakin is involved in the differentiation process (Lechler et al, 2007). Additionally, this defective differentiation process of desmoplakin mutant cells could lead to retrograde signals that impair the proliferation of progenitor cells. For example, the signals could be mediated through cell-cell contact that control certain factors or signaling molecules in pathways whose 'Downstream' target genes in turn control the progenitor cells. Signaling molecules control various intracellular processes by acting as extracellular ligands by binding to corresponding receptors on the cell membrane and thereby transmitting the signals, which finally lead to gene modulation. The fate of progenitor cells in the postnatal dentate gyrus is regulated by a variety of signaling molecules and factors expressed by the progenitor cells themselves or provided by the cell environment. A highly specialized neurogenic niche, astrocytes, endothelial cells, microglia cells as well as soluble factors and extracellular matrix molecules, in close proximity are essential for the survival of the proliferation and differentiation of the progenitor cells (Alvarez-Buylla et al, 2004, Ming et al, 2005, Lledo et al, 2006, Gage et al, 1995).

The factors expressed within the niche, such as Sonic hedgehog (Shh), Notch signaling factors, bone morphogenic protein (BMP) or Wnt signaling factors regulate the proliferation and neuronal differentiation of progenitor cells in the postnatal dentate gyrus. These factors are expressed by stem cells or neuronal progenitor cells (Bonaguidi et al, 2008, Lai et al, 2003, Lie et al, 2005, Stump et al, 2002, Duan et al, 2008). Other factors such as VEGF (vascular endothelial grown factor), BDNF (brain derived neurotrophic factor) or TGF-β (transforming growth factor – beta), secreted by progenitor cells, endothelial cells or microglial cells, stimulate the proliferation of progenitor cells (Battista et al, 2006, Katoh-Semba et al, 2002, Jin et al, 2002, Sun et al, 2006).

In recent years, evidence obtained from human arrhythmogenic right ventricular dysplasia/ cardiomyopathy (ARVC) and non-small cell lung cancer studies demonstrated that desmoplakin can downregulate or inhibit the Wnt/β-catenin signaling pathway (Garcia-Gras et al, 2006, Yang et al, 2012). This modulation of the Wnt/β-catenin signaling pathway by desmoplakin could be regulated by plakoglobin. Plakoglobin, an integral part of desmosomes, interacts with the cytoplasmic tail of desmosomal cadherin and connects desmoplakin to the outer plaque on desmosomes. Suppression or deletion of the desmoplakin gene results in the release of plakoglobin from desmosomes and its translocation into the nucleus (Garcia-Gras et al, 2006, Chen et al, 2013). Plakoglobin, an armadillo protein is closely related to β-catenin. Plakoglobin competes with β-catenin to interact with the TCF/LEF enhancer factor and thereby modulates the downstream Wnt signaling pathway (Garcia-Gras et al, 2006, Miravet et al, 2002, Yang et al, 2012, Zhurinsky et al, 2000, Charpentier et al, 2000). An additional desmoplakin interacting protein, plakophilin, a p120-related arm-repeat protein, also modulates the Wnt/β-catenin signaling pathway (Chen et al, 2002). Thus, desmoplakin may play a role in regulation of cell differentiation through plakoglobin and plakophilin.

There is a substantial amount of data concerning the importance of cell adhesion and the stem cell niche (Knoblich JA, 2008). The concerted action of factors secreted by the stem cells and signals within the niche are required for a tight control of stem cell self-renewal. In addition to providing niche anchorage, adhesion mediated signaling events can help maintain stem cell self-renewal, proliferation and survival (Chen et al, 2013). The cadherin molecules participate in intercellular signaling via regulation of their interacting proteins, such as p120 (δ1-catenin), β-catenin as well as plakoglobin (Cavallaro et al, 2011). There is no clear evidence of involvement of adhesion molecules in the regulation of the stem cells in the dentate gyrus, but there are experimental data supporting involvement of adhesion molecules in the germ line and other types of stem cells. For example: E-cadherin-mediated cell adhesion is required for the anchorage of germline stem cells (GSCs) and follicular stem cells (FSC) in *Drosophila melanogaster* (Song et al, 2002, Song et al, 2002). E-cadherin is also required for polarizing GSCs towards the niche and properly orienting the GSC mitotic spindle (Inaba et al, 2010), allowing the self-renewing stem cells to remain in contact with the niche and the differentiating daughter cells to move out of the niche

(Cheng et al, 2011). In mouse, E-cadherin has an important function in pluripotency and reprogramming of fibroblast cells (Redmer et al, 2011).

The proliferation phenotype analysis of the $Dsp^{flox/flox}$: Nex-*Cre*, where *Cre*-recombinase is only active in postmitotic cells (Goebbels et al, 2006, Seuntjens et al, 2009) suggests a non-cell autonomous function of desmoplakin in dentate gyrus development. I could demonstrate this non-cell autonomous function of desmoplakin more precisely by overexpressing or reintroducing desmoplakin cDNA into wild type or Bcl11b mutant animals, respectively. Using Ex-*Utero* electroporation followed by slice culture (Venkataramanappa et al, 2014), the desmoplakin cDNA construct was introduced into E15.5 embryos. Overexpression of desmoplakin occurring only in a few cells of the dentate gyrus resulted in an increase of the proliferation rate of granule progenitor cells. The proliferation of neurons was analyzed by counting the BrdU positive cells in the prospective dentate gyrus. Overexpression of desmoplakin cDNA in wild type animals showed a significant increase of proliferation of cells compared to the animals electroporated with an empty vector. This approach was further utilized for the rescue of the proliferation phenotype in $Bcl11b^{flox/flox}$;Emx1-*Cre* animals by reintroducing the desmoplakin cDNA construct into the dentate gyrus by Ex-*Utero* electroporation. Desmoplakin, a direct downstream target gene of the zinc finger transcription factor Bcl11b is downregulated in the dentate gyrus of Bcl11b mutants. The phenotype associated with the ablation of Bcl11b in the dentate gyrus includes a reduced proliferation rate of progenitor cells (Simon R et al, 2012). Immunohistological analysis of the slice culture sections of desmoplakin over-expression as well as rescue of the proliferation phenotype revealed no significant difference in GFP-BrdU double positive cells (mitotically active cells that are marked by BrdU and transfected with a *Dsp* cDNA construct along with a GFP expressing vector), but a significant increase in the BrdU positive cells. This supports a non-cell autonomous function of desmoplakin in the regulation of progenitor cell proliferation of the dentate gyrus most likely executed through cell-cell contact.

A detailed expression analysis of various factors and signaling molecules present in the neurogenic niche of the desmoplakin mutant dentate gyrus is required to further define the mechanisms of this regulatory process. Furthermore, experimental investigations are required to analyze the molecular and cellular mechanisms of desmoplakin involved in the regulation of progenitor cell proliferation as well as non-

cell autonomous functions of desmoplakin in the overall development of the dentate gyrus.

4.3 Reduced granule cell apoptosis of desmoplakin mutants.

For maintaining structural and functional integrity of the dentate gyrus, it is critical to have a fine balance between the proliferation and apoptosis rate of granule cells. In desmoplakin mutants, cells undergoing apoptosis were significantly reduced compared to the control animals of both *Cre* lines (Emx1-*Cre* and Nex-*Cre*). The apoptotic cells were localized throughout the width of the granule cell layer of both dorsal and ventral blade and few cells were also found in the subgranular layer of the dentate gyrus. Usually, in the dentate gyrus type 2 cells undergo apoptosis (Garthe et al, 2012), but further investigation is necessary to identify the stage of the granule cells, which are undergoing apoptosis in this mutant. This reduction of apoptosis could be due to the reduced progenitor cell proliferation, which results in reduced neurogenesis. The cells undergoing apoptosis could be postmitotic differentiating granule cells, which by virtue of perturbed differentiation and lack of integration into the hippocampal circuitry, are eliminated by apoptotic cell death.

Since desmoplakin as well as the interacting partners of desmoplakin are expressed throughout the width of the granule cell layer, these proteins might have an anti-apoptotic effect on the granule cells of the dentate gyrus. For example knockdown by shRNA or deletion of desmoplakin leads to the translocation of the cytoplasmic protein plakoglobin into the nucleus (Garcia-Gras et al, 2006, Chen et al, 2013), which might regulate the genes necessary for cell survival (Aktary et al, 2012). Plakoglobin is known to negatively regulate apoptosis of SCC9 (Human squamous carcinoma cells) cells by modulating the expression of BCL-2, an anti-apoptotic protein, in cell culture (Hakimelahi et al, 2000, Aktary et al, 2012). Furthermore plakoglobin is closely related to β-catenin and can antagonize the Wnt/ β-catenin signaling pathway (Garcia-Gras et al, 2006, Hu et al, 2003) and might therefore cause the deregulation of genes involved in apoptosis.

Plakophilin-2, another cytoplasmic interacting partner of desmoplakin, also localizes to the nucleus in the presence or absence of desmosomes (Mertens et al, 1996). The

head domain of plakophilin-2 interacts with β-catenin and modulates Wnt/ β-catenin signaling activity and gene regulation (Chen et al, 2002). The amino terminal domain of plakophilin-2 is capable of interacting with single strand DNA (ssDNA) in the nucleus and may have cell survival effects on the cells or tissues (Sobolik-Delmaire et al, 2010). Taken together, one could hypothesize that the interacting partners of desmoplakin are not only involved in stabilizing the cytoskeletal architecture of the granule cells but also have signaling functions that might lead to the regulation of downstream genes. Further experimental investigations are required to analyze the function of desmoplakin and its interacting proteins in apoptosis.

4.4 Desmoplakin is essential for differentiation of granule cell.

A pronounced expression of NeuroD, a basic helix-loop-helix protein, by late neuronal progenitor cells and early postmitotic, immature neurons of the dentate gyrus is essential for the differentiation of granule cells during postnatal hippocampal development (Miyata et al, 1999). NeuroD is also required for the generation of granule cells of the dentate gyrus and olfactory neurons in the embryonic as well as adult brain (Gao et al 2009, Varela_nallar et al, 2013). In NeuroD mutants, a small cap of cells, which are abnormally differentiated, replaces the dentate gyrus. This abnormal differentiation is attributed to a defect in the transition from late precursor cells into differentiating granule cells (Liu et al, 2000, Miyata et al, 1999). Only a subpopulation of *NeuroD* positive cells also expresses desmoplakin (Simon et al, 2012). As the granule cell maturation advances, expression of *NeuroD* increases in differentiating granule cells from the inner most cells, the sub-granular zone to outer layers of the granule cells. On completion of the development of the dentate gyrus, the expression of *NeuroD* is limited to only a small number of immature cells in the inner layers of the granular cells. In desmoplakin mutants, a strong expression of *NeuroD* is maintained in the cells of the adjacent outer layer, which indicates that the terminal differentiation of immature neurons to functional mature granule cells is affected. Accordingly, in desmoplakin mutants, fewer cells express the mature neuronal cell marker, calbindin, in the entire width of the granule cell layer. Though only few cells

express calbindin, the level of expression of this marker does not seem to have changed in the cell soma as well as in the dendrites forming the molecular layer.

Doublecortin, another marker of late progenitor cells and differentiating immature neurons as well as an established marker for investigating dendritogenesis of immature neurons (Shapiro et al, 2005, Francis et al, 1999, Plümpe et al, 2006, Corbo et al, 2002, Deuel et al, 2006, Knoth et al, 2010), was used to analyze the dentate gyrus of mutant mice. In desmoplakin mutant mice at early postnatal stage, slightly more branching was observed in the molecular layer of dentate gyrus by doublecortin expressing granule cells, compared to the control animals (data not shown). Although the dendrites show increased branching, there is no change in the width of the molecular layer as well as the length of the granule cell dendrites compared to control animals. This increased branching of dendrites is more evident in early postnatal stages of desmoplakin mutants. These data suggest that the mutation of the desmoplakin gene in initial stages of cell proliferation may be the reason for the increased branching of granule cell dendrites. In addition the desmoplakin mutants exhibit little or no altered distribution of cell soma in the subgranular and granule cell layer (data not shown).

Desmoplakin null mutants exhibit aberrant terminal differentiation of cells in the epidermis (Vasioukhin et al, 2001). There is no clear evidence on the molecular mechanism to support the involvement of desmoplakin in differentiation up to date. But there is evidence that the interacting partners of desmoplakin may play a role in the terminal differentiation of cells. For example: Wnt signaling, which regulates neuronal differentiation, is suppressed by plakoglobin as well as plakophilin by competing with β-catenin signaling resulting in differentiation arrest in keratinocytes and COS cells (Hu et al, 2003, Chen et al, 2002, Munji et al, 2011). Additionally, overexpression of truncated or full-length plakoglobin can transactivate the promoters of genes like neuronal cell adhesion molecule, Cyclin D1 and rescue the differentiation phenotype in β-catenin-null mice (Teuliere et al, 2004).

4.5 Desmoplakin is essential for dendritic spine development.

The dendritic spines are sites of excitatory synaptic transmission. Changes in spine structure and/ or spine number are thought to represent a morphological correlation of altered brain function associated with hippocampal dependent learning and memory (Chakraborti et al, 2012). The morphology of the dendritic spines vary greatly in size and shape and are classified as thin, mushroom or stubby type spines (Peters et al, 1970). The spine head consists of the postsynaptic density (PSD), a membrane-associated disc of electron dense material, abundant with proteins such as signaling molecules (e.g. calcium/calmodulin dependent kinase II), receptors (e.g. glutamate receptors) cytoskeletal protein (e.g. actin), adhesion molecules (e.g. N-cadherin, α-catenin and β-catenin) (Tahiro et al, 2003, Bourne et al, 2008, Tanaka et al, 2012, Tashiro et al, 2003). The dendritic spines are highly dynamic, and their stabilization and morphology are influenced by many factors. Several molecular families, including receptors, scaffolding proteins and regulators of the cytoskeleton have been implicated in the regulation of spine number and morphology. As an example: upon activation of the glutamate receptor, the actin filaments are stabilized and decrease the motility of the spine (Fisher et al, 2000), as a result, the spine number increases upon inhibition of RhoA (Tashiro et al, 2000), but decreases as a result of inhibition of Rac1 (Nakayama et al, 2000). In humans, neurodegenerative diseases are associated with the reduction of the spine number, disruption of spine integrity and aberrant spine morphology as shown for Alzheimer's and Parkinson's disease (Bellot et al, 2014).

In desmoplakin mutants the dendritic spines were significantly reduced compared to the control animals. This reduction in dendritic spine density was not localized to a particular part of the dendrite but rather distributed throughout the length of the dendritic shaft, from cell soma to the tip of the dendrites (counted in three, 50μM segments from cell soma to the tip of dendrites). Although the dendritic spines were reduced, the desmoplakin mutants show no significant change in the length of the dendrites compared to control animals.

Recently there is increasing evidence that microtubules are involved in dendritic spine plasticity (Hoogenraad et al, 2010). Knockdown of microtubule binding protein or treating neurons with nocodazole, a microtubule polymerization inhibitor, reduces

dendritic spine numbers (Gu et al, 2008, Jaworski et al, 2009). Mass spectrometric analysis of the synaptosome revealed the co-localization of many proteins including desmoplakin (Tanaka et al, 2012) along with microtubule plus-end tracking protein, Lis1, as well as Ndel1 (Niethammer et al, 2000). In the epidermis, during differentiation, the desmosomes, specifically desmoplakin, recruit a group of microtubule-associated proteins as well as centrosomal proteins to the cell cortex (Sumigray et al, 2011). The microtubule-associated proteins include, Lis1 and CLIP170, which interact and stabilize microtubules. These factors are required for the generation of cortical microtubules and Lis1 is essential for stabilizing desmosomes (Sumigray et al, 2011). The model proposed by Tanaka H (2012), associates Neuronal specific cadherin (N-cadherin) with adhesion molecules of synaptic junctions, which are linked to the intermediate filaments through desmoplakin and plakoglobin. Taking this model into account, one can speculate that Lis1 is recruited by desmoplakin to the dendritic spine, thereby stabilizing the microtubules, which in turn may facilitate the polymerization of actin and increase dendritic spine stability (Merriam et al, 2013). Moreover, desmoplakin is directly involved in the regulation of microtubule organization (Lechler et al, 2007). Deleting desmoplakin expression might disturb the organization of microtubules and affect actin polymerization directly or indirectly, resulting in the reduction of dendritic spine numbers.

4.6 Emotional and cognitive learning behavior in desmoplakin mutants.

As discussed above, desmoplakin mutants exhibit reduced proliferation and defective differentiation of granule cells as well as reduced dendritic spine numbers in granule cells of the dentate gyrus. Through various behavioral experiments, I could demonstrate that these morphological changes in the dentate gyrus due to the desmoplakin mutation do not change the behavior of the mutant animals compared to controls. The emotional and cognitive learning behavior was analyzed by the open field, elevated plus maze (data not shown) and radial arm maze test. The open field and elevated plus maze tests are used to analyze locomotor activity, fear and anxiety. The $Dsp^{flox/flox}$;Emx1-*Cre* animals do not show any significant difference in their behavior when compared to control animals. The vertical activity in the form of

'Rearing' as well as the locomotor activity of Dsp$^{flox/flox}$;Emx1-*Cre* animals is reduced, but not significantly, which might be caused by the skin lesions in the fore- and hindlimbs of the Dsp$^{flox/flox}$;Emx1-*Cre* animals. The observation of reduced rearing activity can be interpreted as a delayed or absent habituation to the new environment and point to a limited spatial information acquisition and processing, as it is also observed in other animals with hippocampal lesions (Alvarez et al, 1995). Behavioral studies by W.E. Crusio (1989, a, b) show, that the rearing behavior of the mouse in the open field includes aspects that can be traced back to spatial learning behavior. These studies also show a direct relationship between this behavior and hippocampal structures. Furthermore the occurrence of this behavior in the open field correlates with the morphological variation (mossy fiber distribution and termination) in the hippocampal structure. Analyzing the hippocampus of Dsp$^{flox/flox}$;Emx1-*Cre* animals by TIMM staining did not reveal obvious changes in the distribution of the mossy fibers in CA3 region, compared to the control animals. The number of thorny excrescences, the characteristic spines of the CA3 pyramidal neurons on which the mossy fibers terminate, was also determined in Dsp$^{flox/flox}$;Emx1-*Cre* animal and no significant difference was found when compared to control animals.

In the radial maze, an established behavioral experiment to study hippocampus-dependent learning and memory processes in rodents (Schwegler et al, 1990, Nadel 1980), the spatial learning ability of Dsp$^{flox/flox}$;Emx1-*Cre* animals was comparable to the control animals and no significant difference was observed between these animals. The number of wrong entries to the arms of the radial maze is increased, but not significantly, in Dsp$^{flox/flox}$;Emx1-*Cre* animals compared to control animals. This tendency of a change in the learning behavior in the mutant could be a direct consequence of the reduced granule cell number and impaired neurogenesis in the postnatal dentate gyrus as well as a reduction in the excitation received due to the reduced dendritic spine number. The dentate gyrus is the primary gateway of the hippocampus via the connections of the afferents of the entorhinal cortex to the granule cells which project their axons, the mossy fibers, to the CA3 pyramidal cells. Due to the central position in the tri-synaptic excitation propagation within the hippocampus, the granule cells are not only involved in controlling the flow of information within the hippocampus, but probably are also directly involved in the processing and storage of spatial information (Winson et al, 1977, Jung et al, 1993).

This was confirmed by studies in which the selective ablation of granule cells in rodents by colchicine microinjection, significantly limited learning behavior of these animals in the radial maze and morris water maze experiments (Walsh et al, 1986, McNaughton et al, 1989, Xavier et al, 1999). It is also known that the continuous neurogenesis in the adult dentate gyrus is essential for specific learning and memory functions of the hippocampus, since the targeted destruction of neurogenic progenitor cells in mice is associated with learning deficits in morris water maze experiments (Snyder et al, 2005, Dupret et al, 2008).

Altogether these results demonstrate that desmoplakin does not have an essential role in axonal outgrowths and mossy fiber distribution. The results from the open field, elevated plus maze as well as radial maze does not show any significant difference in spatial learning and memory behavior of desmoplakin mutants compared to control animals. Further experiments are need to analyze the learning and memory behavior of desmoplakin mutants.

4.7 Phenotype comparison of desmoplakin and Bcl11b mutants.

The C_2H_2 zinc finger transcription factor *Bcl11b*, also known as *Ctip2* (Chicken ovalbumin upstream promoter transcription factor (COUP-TF) interacting protein 2), plays a crucial role during development of many tissues. Deletion of *Bcl11b* leads to abnormal development and postnatal lethality of unknown causes. Mice lacking one *Bcl11b* allele are susceptible to thymic lymphomas while mice with an ablation of both *Bcl11b* alleles exhibit many defects in different organs of newborn pups including the immune system, central nervous system, skin, teeth and hair cells of the cochlea (Wang et al, 2012, Golonzhka et al, 2009, Wakabayashi et al, 2003, Golonzhka et al, 2009, Arlotta et al, 2008). Previous work from our group on *Bcl11b* functions in the developing hippocampus has shown that *Bcl11b* plays a pivotal role in the development of the dentate gyrus. The initial analysis revealed that *Bcl11b*, expressed in post mitotic granule cells, is necessary for the regulation of progenitor proliferation and differentiation as well as the integration of granule cell neurons into the hippocampal circuitry (Simon et al, 2012). *Bcl11b* also regulates the expression of desmoplakin, a direct downstream target of *Bcl11b*. Deletion of *Bcl11b* results in the

loss of desmoplakin expression in postmitotic granule cells of the dentate gyrus (Simon et al, 2012).

Analyzing animals with a forebrain specific deletion of desmoplakin demonstrated a similar phenotype when compared to Bcl11b mutants (Simon et al, 2012). This phenotype includes: reduced granule cell number and area of the dentate gyrus, reduced proliferation of progenitor cells and impaired differentiation of granule cells. A direct comparison between the mutants of these mice models revealed reduction of the granule cell number (0.8 fold) and reduction of the dentate gyrus area (0.9 fold) as well as an inhibition of the progenitor proliferation rate (0.9 fold) in Desmoplakin mutants. In addition the *NeuroD* positive cells were increased by 1.4 folds in the desmoplakin mutants, indicating a defect in the differentiation of granule cells, while the Bcl11b mutants show a 2 fold increase. The weaker changes of the dentate gyrus size and number of granule cells, proliferation rate as well as defect in the differentiation of granule cells might be due to the reduced apoptosis of the desmoplakin mutants in contrast to the increased apoptosis of Bcl11b mutants. Altogether, these results indicate that desmoplakin mutants demonstrate a similar but weaker phenotype when compared to Bcl11b mutants. Moreover, Bcl11b mutant animals can be rescued by reintroducing desmoplakin cDNA into the dentate gyrus by electroporation followed by slice culture, which leads to a significant increase of proliferating cells marked by BrdU.

Integration of neurons into the hippocampal circuit is affected in Bcl11b mutants. In Bcl11b mutants, the dendritic spine number, targets for the efferent axons from the entorhinal cortex and commissural as well as associational fibers, was significantly reduced throughout the length of the dendrites (Simon et al, 2012). Furthermore, the mossy fiber tract in Bcl11b mutants displayed a highly disorganized projection pattern, scattering over the entire pyramidal cell band of the CA3 region. Additionally, the thorny excrescences, characteristic spines on the apical dendrites of CA3 pyramidal neurons, were significantly reduced in Bcl11b mutants. Contrary, desmoplakin mutants demonstrated no difference in the projection pattern of mossy fibers in CA3 region as well as no change in the number of thorny excrescences, but exhibited a reduced dendritic spine number similar to Bcl11b mutants.

The Bcl11b mutants demonstrate impaired hippocampal related cognitive and emotional learning and memory behavior. This deficit in learning and memory might

be due to the defect in the integration of neurons into the hippocampal circuit (Simon et al, 2012). Although desmoplakin mutants show a reduced dendritic spine number, these mutants do not show any memory deficit or impaired spatial learning behavior.
Taken together the phenotype analysis data of *Bcl11b* and desmoplakin mutants demonstrate that a major part of *Bcl11b* functions are elicited via desmoplakin but there are also other desmoplakin independent pathways responsible for the *Bcl11b* phenotype.

4.8 Future directions

In the present work I have shown that the structural protein desmoplakin exerts an essential function in the development of the dentate gyrus by influencing postnatal neurogenesis of granule cells of the tertiary matrix. The molecular and cellular mechanisms involved in desmoplakin dependent regulation of the proliferation of progenitor cells, differentiation of immature neurons as well as cell survival of the granule cells in the dentate gyrus remains to be determined.
One way to approach this question would be to analyze the differentially expressed genes in the dentate gyrus of desmoplakin mutants by microarray analysis. Prospective candidate genes may serve as initial hints for the future analysis of desmoplakin funtions.
I could demonstrate that overexpression of desmoplakin in the dentate gyrus increases the proliferation rate of granule cells. It would be interesting to investigate how the overexpression of desmoplakin leads to the increased proliferation and to determine the signals involved in this function of desmoplakin. It has to be seen whether overexpression of desmoplakin could rescue the reduced dendritic spine number of the granule cells. It would be interesting as well to analyze cell-cell contacts in the dentate gyrus and involvement of desmoplakin in these contacts, by electron microscopy in control and mutant dentate gyrus, as well as in hippocampal tissue overexpressing desmoplakin.
Furthermore, it would be interesting to analyze the differential expression pattern of desmoplakin in different layers (subgranular zone and granular cell layer) of the dentate gyrus. It is well established that the transcription factor Bcl11b binds to an

upstream regulatory sequence of desmoplakin allowing its expression. In the subgranular zone, where no Bcl11b is expressed, low-level expression of desmoplakin was observed co-localizing with the progenitor cell marker Sox2. One way to investigate this differential expression pattern of desmoplakin is to analyze the upstream promoter region of desmoplakin by in silico analysis followed by ChIP (Chromatin immunoprecipitation). One could also clone the *Dsp* promoter sequence, label with biotin and transfect the construct into primary neuronal cell cultures. After a period of incubation in the cells, the biotin labeled DNA can be isolated and bound protein can be subjected to mass spectrometric analysis.

In addition, analysis of the desmoplakin interacting protein partners and their subcellular localization will also give an insight into cellular functions of desmoplakin. For example, in cardiomyocytes upon deletion of desmoplakin, plakoglobin translocates into the nucleus and competes with the binding sites of β-catenin. Another desmoplakin interacting partner, Lis1 also binds to microtubules and is necessary for the polymerization of microtubules, upon deletion of desmoplakin cortical microtubule organization is perturbed.

Further analysis and elucidation of the molecular and cellular mechanisms available through desmoplakin and its control on postnatal neurogenesis in the dentate gyrus could significantly help to understand the fundamental aspects of neural developmental processes. A better understanding of the functional neurogenesis in the postnatal brain is of important clinical relevance in the treatment of neurodegenerative diseases as well as severe brain injury. This is also crucial for the development of therapeutic strategies, in which an increased loss of functional neurons can be compensated with the replacement of new nerve cells.

5. Summary

The hippocampus plays a pivotal role in learning, memory and behavior. The hippocampus, in particular the dentate gyrus, is one of two locations with neurogenesis continuing in the adult brain. The dentate gyrus, the primary gateway for inputs into the hippocampus, maintains a pool of neuronal progenitor cells in the subgranular zone. The progenitor cells go through a number of stages until they are mature, including migration and positioning of the newborn cells, growth and target finding of axons and dendrites of the immature neuron and synaptic connection with other mature neurons. Several well defined signaling pathways, like the *Wnt* signaling pathway, are involved in the maturation of the neurons in the dentate gyrus, which can be defined by the expression of specific markers. Bcl11b is one of the key transcription factors involved in the development of the dentate gyrus whose expression is restricted to postmitotic granule cells. The conditional mutation of the *Bcl11b* gene shows the following phenotypes: reduced granule cell number and area of the dentate gyrus, reduced proliferation of progenitor cells, defect in neuronal differentiation, reduced number of dendritic spines and thorny excrescences, altered distribution of mossy fibers in CA3 region as well as impaired memory and learning behavior.

Desmoplakin expression, being one of the downstream targets of Bcl11b, overlaps with the expression of Bcl11b in granule cells of the dentate gyrus. To determine the role of desmoplakin in the postnatal hippocampus, conditional mutagenesis of desmoplakin in the forebrain was employed. Analysis of these mice demonstrates that desmoplakin has essential functions during postnatal development of the dentate gyrus: desmoplakin is involved in the control of the proliferation of progenitor cells, which is evident in the conditional mutants, demonstrated by the reduced number od proliferating cells. Additionally, lack of desmoplakin in the granule cells also reduces the granule cell apoptosis. Furthermore, ablation of desmoplakin affects the differentiation of neurons resulting in an increase of immature neurons, which fails to undergo appropriate terminal differentiation. Reduced proliferation of progenitor cells and arrest of neuronal differentiation most likely are responsible for the reduced size of the dentate gyrus. In desmoplakin mutants, there is a considerable reduction in the dendritic spine number, but no significant difference in the number of thorny

excrescences on CA3 neurons. In addition, there is no obvious difference observed in the distribution of mossy fibers in CA3 region. The desmoplakin mutants do not exhibit an impairment in learning and memory behavior, which might be due to no obvious change in he number of thorny excrescences and the distribution of mossy fibers. Conditional knock out of desmoplakin in postmitotic cells of the dentate gyrus of $Dsp^{flox/flox}$;Nex-*Cre* results in a similar phenotype when compared to $Dsp^{flox/flox}$;Emx1-*Cre* in terms of reduced progenitor cell proliferation and differentiation, implying the presence of a signing pathway between postmitotic to proliferating progenitor cells.

Moreover overexpression or reintroduction of desmoplakin cDNA by electroporation into wildtype or Bcl11b mutant animals followed by slice culture, demonstrated a significant increase in the proliferation of progenitor cells of the dentate gyrus. This function of desmoplakin can be used to recue the reduced proliferation of progenitor cells in Bcl11b mutants. Taken together, these results indicate that desmoplakin is essential for the structural and functional development of the dentate gyrus.

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