A dual function of Bcl11b/Ctip2 in hippocampal neurogenesis

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The development of the dentate gyrus is characterized by distinct phases establishing a durable stem-cell pool required for postnatal and adult neurogenesis. Here, we report that Bcl11b/Ctip2, a zinc finger transcription factor expressed in postmitotic neurons, plays a critical role during postnatal development of the dentate gyrus. Forebrain-specific ablation of Bcl11b uncovers dual phase-specific functions of Bcl11b demonstrated by feedback control of the progenitor cell compartment as well as regulation of granule cell differentiation, leading to impaired spatial learning and memory in mutants. Surprisingly, we identified Desmoplakin as a direct transcriptional target of Bcl11b. Similarly to Bcl11b, postnatal neurogenesis and granule cell differentiation are impaired in Desmoplakin mutants. Re-expression of Desmoplakin in Bcl11b mutants rescues impaired neurogenesis, suggesting Desmoplakin to be an essential downstream effector of Bcl11b in hippocampal development. Together, our data define an important novel regulatory pathway in hippocampal development, by linking transcriptional functions of Bcl11b to Desmoplakin, a molecule known to act on cell adhesion.

Introduction

Hippocampal structures play an important role in memory and learning as well as in emotional behaviour. The dentate gyrus, the primary gateway for input information into the hippocampus, is one of only two brain regions with continuous neurogenesis in adult mammals. Development and postnatal function of the dentate gyrus are characterized by distinct phases involving specific molecular pathways (Kempermann et al., 2004; Frotscher et al., 2007; Li and Pleasure, 2007). While dentate gyrus morphogenesis starts early in embryonic development, the vast majority of neurons are born within the first 4 postnatal weeks (Muramatsu et al., 2007). During this time, the proliferative compartment becomes restricted to the subgranular zone (SGZ) positioned between hilus and granule cell layer (GCL) providing the stem-cell pool for continuous neurogenesis throughout adulthood (Altman and Bayer, 1990). The progression from stem/progenitor cell to mature neuron requires several steps, including migration and positioning of the newborn cells, growth and target finding of axons and dendrites of the immature neuron, and finally the synaptic integration of the mature neuron. Several transcription factors and signalling pathways, most of them commonly used in cellular differentiation, are required for the development of the hippocampus (Kempermann et al., 2004; Frotscher et al., 2007; Li and Pleasure, 2007). Previous work has demonstrated that both the transcription factors NeuroD and Prox1 are regulated by β-catenin implying the involvement of the Wnt signalling pathway in the regulation of hippocampal granule cells (Liu et al., 2000; Pleasure et al., 2000; Gao et al., 2009; Kuwabara et al., 2009; Karalay et al., 2011). Transcription factors also play an important role in proliferation and maintenance of neural stem/precursor cells as was shown for Sox2 (Ferri et al., 2004; Favaro et al., 2009). However, mechanisms underlying the phase-specific control of postnatal hippocampal development are still incompletely understood.

To further elucidate the mechanisms of postnatal neurogenesis of the dentate gyrus, we studied Bcl11b, a Kruppel-like C2H2 zinc finger transcription factor expressed in the hippocampus, neocortex (Arlotta et al., 2005; Chen et al., 2008; Seuntjens et al., 2009) and the striatum (Arlotta et al., 2008; Desplats et al., 2008). Bcl11b plays an important role in the prenatal development of corticospinal motor neurons as well as specific subsets of striatal neurons (Arlotta et al., 2005, 2008; Chen et al., 2008).

Here, we show a dual phase-specific function of Bcl11b restricted to postnatal development of the dentate gyrus. Our studies demonstrate that Bcl11b, expressed in postmitotic granule neurons, is involved in the regulation of progenitor proliferation by a feedback mechanism. As a consequence, proliferating progenitor cells are reduced, and postmitotic dentate granule cells are depleted in Bcl11b mutants. Moreover, the differentiation of postmitotic neurons depends...
on Bcl11b expression, and mutant neurons fail to integrate into hippocampal circuitry leading to impaired learning and memory. We identified Desmoplakin as a direct target gene of Bcl11b. Forebrain-specific deletion of Desmoplakin results in developmental defects similar to those observed in Bcl11b mutants as demonstrated by reduced cell proliferation and impaired neuronal differentiation. Moreover, re-introduction of Desmoplakin into the Bcl11b mutant dentate gyrus rescues the deficit in neurogenesis. Thus, our data define important functions of Bcl11b in postnatal hippocampal development. Furthermore, we identify Desmoplakin to be a critical downstream effector of Bcl11b in regulating hippocampal neurogenesis.

**Results**

**Analysis of Bcl11b expression in the hippocampus**

Bcl11b expression was first observed at embryonic stage 15 (E15), restricted to the Cornu Ammonis (CA) and expanding to the suprapyramidal blade of the developing dentate gyrus at E18 (Supplementary Figure S1A–D). During postnatal development, Bcl11b is expressed in dentate granule cells and in the CA1 and CA2 regions (Supplementary Figure S1E–H). NeuroD, a transcription factor expressed by newborn, migrating as well as early differentiating, neurons (Pearson et al. 2000), colocalizes with all Bcl11b expressing cells in the prospective suprapyramidal blade at E18 but not in cells migrating into the dentate primordium (Figure 1A and D). Postnatal dentate development results in a gradient of granule cell differentiation with newborn progenitor cells located in the SGZ and mature granule cells in the most superficial zone of the GCL (Figure 1). Expression analysis of NeuroD at P7 identified a cluster of cells co-expressing Bcl11b located in the middle of the GCL but no Bcl11b co-expressing cells were found in the SGZ (Figure 1B and E). Only a few cells residing in the outer layer of the GCL were co-expressing Bcl11b and the postmitotic marker genes, NeuN and Calbindin (Calb1) at P7 (Figure 1H and I). At P30, strong expression of NeuroD is limited to the innermost GCL and the SGZ with only very few cells co-expressing Bcl11b (Figure 1C and F). Similar results were obtained for Doublecortin (Dcx), expressed in both progenitor cells and immature neurons (Kempermann et al., 2004; Figure 1G and J). At this stage, the majority of Bcl11b-positive cells located in all parts of the GCL are co-expressing NeuN and Calb1 (Figure 1K and L). Together, this suggests that Bcl11b expression is restricted to postmitotic granule neurons of the dentate gyrus but excluded from proliferating progenitor cells. This is confirmed by the mutually exclusive expression of Bcl11b and Sox2, expressed in stage 1 stem cells and Tbr2, a marker for stage 2 and 3 progenitor cells (Ferri et al., 2004; Hodge et al., 2008; Figure 1M–O).

**Development of the dentate gyrus requires Bcl11b expression**

To determine the functions of Bcl11b in the developing dentate gyrus, we generated conditional knock-out mice by mating Bcl11b<sup>fl/−</sup>/<sup>fl/−</sup> mice (Li et al., 2010) with the forebrain-specific Emx1-Cre mouse line (Gorski et al., 2002). Expression of Emx1-Cre occurs in mitotic as well as in postmitotic cells of the telencephalon early on in development (Gorski et al., 2002; Supplementary Figure S2A and B). Homozygous Bcl11b<sup>fl/−</sup>/<sup>fl/−</sup>;Emx1-Cre offspring exhibit complete ablation of Bcl11b protein in the hippocampus (Supplementary Figure S2C and D). Histological analysis of Bcl11b mutants revealed no change in the overall architecture of the hippocampus but a reduction in size of the dentate gyrus as early as P7 as determined by analysis of dentate gyrus area and cell number (Supplementary Figure S3C–H). This size reduction is even more prominent at P30 especially in the infrapyramidal blade (Figure 2A–D). At this stage, the area of the dentate gyrus of mutant animals is 40% smaller (Figure 2E; P<0.005) and the cell number is reduced by 33% (Figure 2F; P<0.05) when compared with control animals. Examining embryonic stages did not show any obvious differences between control and mutant animals (Supplementary Figure S3A and B), which might be due to the restricted expression of Bcl11b in the hippocampus during prenatal development. In addition to the reduced cell number, the mutant dentate gyrus exhibits granule cell dispersion; granule cells of control animals are densely packed in a distinct layer while in the mutant these cells are loosely organized (Figure 2C and D; Supplementary Figure S3G and H). Furthermore, cells with abnormal morphology were increased, their nuclei appeared smaller and stained darker when compared with control cells, representing most likely immature precursor-like cells.

**Loss of Bcl11b reduces neuronal progenitor cell proliferation**

The loss of dentate granule cells in Bcl11b mutants might be either due to reduced cell survival or proliferation. TUNEL analysis at P14 revealed a total increase of apoptotic cells in the mutant dentate gyrus (Figure 3A–C; control: 2.9±0.34 s.e.m.; mutant: 12.94±1.55 s.e.m.; P<0.05). Examining the distribution of apoptotic cells demonstrated significantly increased numbers in the GCL (control: GCL, 52.4%; mutant: 64.3%; P<0.05) but no change in the SGZ of mutants (Figure 3C). To identify proliferating cells within the dentate gyrus, we performed short-pulse labelling with BrdU. At E18, Bcl11b mutant and control animals do not exhibit differences in proliferation within the dentate gyrus anlage (Supplementary Figure S4A–C). Examining proliferating cells of the postnatal dentate gyrus revealed a greatly reduced number of BrdU-positive cells at P7 (25% fewer BrdU-positive cells, P<0.05; Supplementary Figure S4D–F) and at P14 when the majority of proliferating progenitor cells are located in the newly developed SGZ (25% fewer BrdU-positive cells, P<0.0005; Figure 3D, E and K). This proliferation deficit was confined to the SGZ (44% reduction, P<0.0005; Figure 3K), while no changes in the number of BrdU-positive cells were detected in the GCL of Bcl11b mutants (Figure 3K). To further explore whether reduced proliferation was caused by a depletion of the dentate progenitor cell pool within the SGZ, we analysed numbers and distribution of Sox2-positive progenitor cells. Interestingly, while the overall numbers of Sox2 expressing progenitors were unchanged we observed a significant depletion of Sox2-positive cells in the SGZ (60.6% of control; P<0.05) with a shift of Sox2 expressing cells to the GCL of Bcl11b mutants (157% of control, P<0.05; Figure 3M). A similar depletion of progenitor cells specifically in the SGZ was observed when we used Tbr2 as a progenitor marker (24.25% of control, P<0.05; Figure 3O). Analysing BrdU incorporation by Sox2 and Tbr2 expressing cells, we found reduced numbers of BrdU-positive progenitor cells specifically in the SGZ (Figure 3I, J and O; Sox2/BrdU, ©2012 European Molecular Biology Organization
52.4% of control; Tbr2/BrdU, 44.4% of control; P<0.05 for both). Together, our data suggest that reduced progenitor cell proliferation as observed specifically in the SGZ of the Bcl11b mutant dentate gyrus might be due to a depletion of the progenitor cell compartment. In mutants, part of the Sox2-positive cells are no longer confined to their natural positions within the SGZ and aberrantly distribute to the GCL where BrdU incorporation was found unchanged (Figure 3K and M). Thus, aberrantly localized progenitor cells may have lost part of their proliferation capacity.

Bcl11b expression is restricted to postmitotic granule cells (cf. Figure 1). Accordingly, at no time colocalization of Bcl11b and BrdU was observed (Figure 3F–H; Supplementary Figure S4G–L) posing the question whether Bcl11b regulates progenitor cells through indirect mechanisms. To further examine this, we generated Bcl11b^{flox/flox};Nex^{Cre} mice where Cre is almost exclusively restricted to postmitotic granule neurons (Goebbels et al, 2006; Seuntjens et al, 2009). Analysing these mice revealed a similar phenotype as observed in Bcl11b^{flox/flox};Emx1-Cre mutants. At P14, Bcl11b^{flox/flox};Nex^{Cre} hippocampi exhibit a reduced granule cell number (Supplementary Figure S5A–C; 83.8% of control, P<0.05), increased apoptosis (Figure 3C; P<0.05) as well as decreased BrdU incorporation specifically in the SGZ (Figure 3L; 44% (total) and 58% (SGZ) fewer BrdU-positive cells; P<0.05). Similarly to Bcl11b^{flox/flox};Emx1-Cre mutants, Sox2-positive cells were reduced in the SGZ and shifted to the GCL (Figure 3N; GCL: 145% of control; 0.1 X P<0.05; SGZ: 72.8% of control; 0.1 X P<0.05) with Sox2/BrdU-positive cells specifically reduced in the SGZ of Bcl11b^{flox/flox};Nex^{Cre} mice (Figure 3O; 60.6% of control; P<0.05). Collectively, these findings strongly suggest an indirect feedback mechanism for Bcl11b in the regulation of the progenitor compartment.

**Bcl11b ablation impairs neuronal differentiation**

To determine the role of Bcl11b in the regulation of granule cell differentiation, we examined the expression of stage-specific markers in control and Bcl11b^{flox/flox};Emx1-Cre mutant animals. In controls, Dcx is localized in the cell body as well as in dendrites of cells of the innermost GCL
as well as the SGZ. Examining Bcl11b\textsuperscript{flox/flox};Emx1-Cre mutants at P30 revealed Dcx-positive cells distributed throughout the whole GCL (Figure 4A–D). In addition, while Dcx-expressing dendrites of control animals reach the outer surface of the molecular layer, Dcx-labelled mutant dendrites are much shorter ending in the inner molecular layer (Figure 4C and D). Moreover, we observed a two-fold increase of NeuroD-positive cells (Figure 4E–H; control 99.67 ± 9.34 s.e.m.; mutant 199.67 ± 7.92 s.e.m.; t-test, \( P < 0.005; n = 3 \) in Bcl11b mutants. Similarly to Dcx, NeuroD expressing cells were no longer restricted to the innermost part of the GCL, suggesting that loss of Bcl11b expression impairs early neuronal differentiation, and immature neurons are no longer confined to their natural position within the dentate gyrus (Figure 4E–H). NeuN, expressed in immature as well as in mature granule neurons, and Calb1, expressed only in mature granule neurons, identity successively but overlapping stages of dentate granule cell differentiation (Kempermann et al., 2004). We observed strongly reduced Calb1 expression starting as early as P7 (Figure 4I and J) accompanied by an increase of NeuN-positive, Calb1-negative immature neurons in the dentate gyrus of Bcl11b mutants (Figure 4K–N). Together, this indicates that the differentiation of Bcl11b mutant dentate granule neurons is interrupted at an early postmitotic stage supporting a function of Bcl11b during postnatal neuronal differentiation. To explore whether loss of Bcl11b expression causes indirect phenotypes of the dentate gyrus, we analysed the expression of markers for glial (GFAP), Cajal-Retzius (Reelin) as well as endothelial cells (Pecam). None of the markers exhibits an obvious phenotype in Bcl11b mutants (Supplementary Figure S6A–J).

To address the question whether Bcl11b regulates neuronal differentiation cell autonomously or through indirect mechanisms, we generated a mosaic deletion of Bcl11b by \textit{ex utero} electroporation of Cre recombinase into Bcl11b\textsuperscript{flox/flox} brains at E15.5 followed by organotypic slice culture up to 14 DIV (Figure 4O–S). While there was no significant change in the number of GFP (pCIG2: 6.5 ± 1.12 s.d.; pCIG2-Cre: 6.8 ± 2.3 s.d.) and GFP/NeuroD (pCIG2: 2.2 ± 0.8 s.d.; pCIG2-Cre: 2.3 ± 0.8 s.d.) positive cells, we found a significant increase of NeuroD-positive cells (pCIG2: 67.9 ± 10.5;
Figure 3 Ablation of Bcl11b results in reduced proliferation of progenitor cells, depletion of neural stem cells as well as increased apoptosis. Confocal images of coronal sections of control (A, D, F, G, I) and Bcl11b flox/flox;Emx1-Cre (B, E, J) at P14. Earlier developmental stages are presented in Supplementary Figure S4. Co-staining of TUNEL (green) and NeuroD (red) (A, B); BrdU (green) and NeuroD (red) (D, E); BrdU (green) and Bcl11b (red) (F, G); Sox2 (green), BrdU (red) and NeuN (blue) (I, J); Statistical analysis of TUNEL assays (C), BrdU-positive cells (K, L) as well as co-staining of Bcl11b/BrdU (H). (K–O) Statistical analysis of Sox2- and Tbr2-positive cells of control and Bcl11b mutant dentate gyrus. Bcl11b mutants exhibit a reduced progenitor cell pool and Sox2 cells are aberrantly located in the granule cell layer in Emx1-Cre (K, M, O) as well as NexCre (L, N, O) mutants. t-test, *P<0.05 error bars, s.e.m. (C, K–O), s.d. (H); n=3 (C, H, O (Tbr2/BrdU and Tbr2)); n=5 (K, M, O (Sox2/BrdU)); n=6 (L, N, O (NexCre)). Images taken at ×10 (A, B, D–F), ×40 (G, I, J) magnification. Scale bar: 100 μm (A); 20 μm (H).
pCIG2-Cre: 92.6 ± 10; P < 0.005) in the mosaic deletion slice cultures (Figure 4S). Thus, deletion of Bcl11b in a fraction of dentate neurons suffices to impair neuronal differentiation in surrounding wild-type cells (as determined by the piling-up of NeuroD expressing, immature cells), a phenotype that is compatible with indirect functions of Bcl11b in neuronal differentiation. This does, however, not exclude additional, direct functions of Bcl11b in this process.

We next asked whether the integration of granule cells into the hippocampal circuitry was affected in Bcl11b mutants. We used Timm staining to selectively label mossy fibre terminals emanating from the dentate gyrus. Compared with control hippocampi, we observed highly disorganized projection patterns of mossy fibres in Bcl11b mutants (Figure 5A and B), with large amounts of axons travelling aberrantly along the infrapyramidal tract and scattered over the entire pyramidal cell band of the CA3 region. In wild-type animals, mossy fibre axons terminate on characteristic spines, so-called thorny excrescences, on proximal apical dendrites of CA3 neurons (Gonzales et al., 2001). In accordance with the aberrant mossy fibre projections, we observed an almost 50% reduction (P < 0.05) of thorny excrescences on the proximal apical dendrites of Bcl11b mutant CA3 pyramidal cells (Figure 5C).
Dendritic spines of control (Amaral and Lavenex, 2007). As an indirect measure for the synaptic input to the dentate gyrus, we determined the spine numbers along granule cell dendrites. In Bcl11b mutants, dendritic spines as visualized by Golgi impregnation were shaped and distributed more irregularly as compared with controls (Figure 5D and E). In addition, numbers of spines along the first (next to the soma) and second thirds (middle) of the entire dendrite length were significantly reduced by ~20% (P<0.05) and 15% (P<0.05), respectively (Figure 5F).

**Desmoplakin functions as a direct transcriptional target of Bcl11b**

To elucidate a mechanism of Bcl11b regulation of neuronal differentiation and progenitor proliferation, we determined target genes by comparative transcriptome analysis using RNA of dentate gyrus tissue harvested from control and mutant animals at P14. We obtained 34 downregulated and 8 upregulated candidate target genes in the Bcl11b mutant (Supplementary Table 1) revealing Desmoplakin (Dsp) and Calb1 (downregulated) as well as HTR2C and TacR3 (upregulated) as the most robust target genes. We verified the microarray data for Dsp and Calb1 by RNA in-situ hybridization and immunohistochemistry, respectively (Figures 4I–N and 6A–D). Furthermore, immunohistological analysis revealed strong overlap of Dsp and Bcl11b expression in the dentate gyrus of wild-type animals at P14 but only faint expression of Dsp in part of Sox2-positive cells (Figure 6E–H). Quantitative RT-PCR determined that expression of Dsp and Calb1 decreases in the mutant by a factor of 14.7 (P<0.005) and 4.8 (P<0.05), respectively (Figure 6I). Dsp was most strongly downregulated in the mutant. Furthermore, we could demonstrate a direct involvement of Bcl11b in the regulation of Dsp expression by performing chromatin immunoprecipitation (ChiP) on hippocampus tissue of wild-type P14 animals using a Bcl11b-specific antibody (Feng and Cooper, 2009; Ganguli-Indra et al, 2009) followed by qPCR (Figure 6J). Genomic analysis of the Dsp locus by Ensembl.org revealed three potential Dsp promoter regions (region1: −315 to +238; region2: +356 to +1194 and region3: +1238 to +1819 corresponding to the transcription start site). Employing several primer pairs for these candidate promoter regions we found that Bcl11b is binding to region +479 to +724 relative to the transcription start site of Dsp (Figure 6J). The amplified promoter region contains putative Bcl11b binding sites GCCCG/AG/AGG (+539 corresponding to the transcription start site; Avram et al, 2002) and TGGGC (+539 corresponding to the transcription start site; Cismasu et al, 2006) further supporting the direct activation of Dsp expression by Bcl11b. Furthermore, employing primer sets covering either both or only the GCCCG/AG/AGG sequence of the potential binding motifs revealed a stronger binding of Bcl11b to the Dsp promoter region when both motifs were present.

**Analysis of the dentate gyrus phenotype of Desmoplakin mutant mice**

To further explore the interactions between Bcl11b and Dsp, we generated forebrain-specific Dsp mutants using a floxed Dsp allele (Vasioukhin et al, 2001). We asked whether Bcl11b and Dsp mutants exhibit overlapping phenotypes. Morphological analysis of the dentate gyrus of Dspfl/fl; Emx1-Cre mutants revealed a similar but milder phenotype when compared with Bcl11b mutants resulting in reduced size (Figure 7A–C; reduced by 19.6%, P<0.005) and cell number (Figure 7F; reduced by 24.5%, P<0.05) of the dentate gyrus.
dentate gyrus as well as granule cell dispersion but to a lesser degree as in Bcl11b mutants (Figure 7D and E). Like for Bcl11b mutants we found a decrease in the proliferation rate of Dsp mutants (Figure 7G–I; reduced by 21.5%, $P < 0.05$). Dsp mutants unlike Bcl11b mutants exhibit a decrease in apoptosis (Figure 7J–L; reduced by 69.1% at P14; $P < 0.05$) suggesting that increased apoptosis as observed in Bcl11b mutants unlike mutants (Figure 7G–I; reduced by 21.5%, $P < 0.05$) may be responsible for the milder phenotype, especially with regard
to cell dispersion. In addition, NeuroD exhibits a similar expression pattern in the Dsp mutant when compared with the Bcl11b mutant including a 1.4-fold increase of NeuroD-positive cells implying a defect in neuronal differentiation (Figure 7M–Q; $P < 0.01$). To address whether Dsp could rescue the Bcl11b phenotype we re-introduced Dsp cDNA into Bcl11b mutants by ex-utero electroporation followed by analysis of the proliferation phenotype (Figure 8A–F). The number of BrdU-positive cells in the Bcl11b mutant dentate gyrus electroporated with the empty vector pRC-CMV was reduced by $\sim 50\%$ compared with Bcl11b expressing controls electroporated either with or without empty pRC-CMV (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.; $P < 0.01$; Figure 8G). Electroporation of the Bcl11b mutant with the Dsp expression vector pRC-CMV-Dsp resulted in the complete recovery of BrdU-positive cells when compared with the controls (24 ± 5.7 s.d.; Figure 8G). Furthermore, most of the BrdU-positive cells do not colocalize with GFP, providing additional evidence for an indirect regulation of progenitor proliferation by Bcl11b/Dsp (Figure 8H).

We next analysed mossy fibre projections in Dsp mutants. Compared with controls and Bcl11b mutants, Dsp mutant animals displayed no overt changes in mossy fibre distribution (Supplementary Figure S7A and B). Taken together, major functions of Bcl11b in postnatal hippocampal development, that is, regulation of progenitor cell proliferation as well as neuronal cell differentiation are recapitulated in Dsp mutants suggesting Dsp to be a functional target of Bcl11b. Comparison of mutant phenotypes, however, further indicates that Bcl11b acts through additional downstream targets.

**Bcl11b involvement in learning and memory**

Experimental evidence from our study indicates that ablation of Bcl11b expression interferes with the formation of hippocampal circuitry and thus may have an effect on hippocampus-related cognitive and emotional learning and memory. Bcl11b control and mutant mice were exposed to the open field test to analyse locomotor (horizontal) activity as well as rearing activity, including rearing (vertical) activity. Bcl11b mutants exhibited a significantly higher locomotor and rearing activity whereas rearing activity was significantly lower, a first evidence for a deficit in processing spatial information (Figure 9A–C). Therefore, we tested the spatial learning by

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**Figure 6** Desmoplakin is a direct transcriptional target of Bcl11b. (A–D) Desmoplakin RNA *in situ* hybridization of hippocampal cryostat sections of control (A, C) and Bcl11b<sup>ex-utero</sup>Emx1-Cre (B, D) animals at P10 (A, B) and P30 (C, D). (E–H) Co-expression analysis of Dsp (green) and Bcl11b (red) (E, G) as well as Sox2 (red) (F, H) of wild-type dentate gyrus at P14. Images taken at ×20 (E, F) and ×63 (G, H) magnification. Scale bars: 100 μm (D); 20 μm (G). (I) Determination of hippocampal relative mRNA expression levels of Desmoplakin and Calbindin by quantitative RT–PCR at P14 (2–ΔΔC<sub>T</sub> method; *t*-test, $*P < 0.05$; **$P < 0.005$; ****$P < 0.0001$; error bar, s.d.; n = 4), (J) Determination of direct interaction of Bcl11b and Desmoplakin by Chromatin immunoprecipitation (ChIP). ChIP assays were performed on hippocampus tissue of P14 animals employing a Bcl11b-specific antibody (Bcl11bAB), IgG as a negative control and an RNA polymerase II (RNA Pol II AB)-specific antibody as positive control. Direct interactions were determined by qPCR using specific primer pairs for Desmoplakin regulatory regions as well as a specific primer pair for the Gapdh promoter region (2–ΔΔC<sub>T</sub> method; t-test, ***$P < 0.001$; ****$P < 0.0001$; error bar, s.d.; hippocampi of 10 animals were used per assay).
exposing control and Bcl11b mutant animals to a spatial eight-arm radial maze test. Spatial working memory capacity was determined by counting the number of errors, for example, re-entering an already entered arm. Both control and mutant animals demonstrated a significant decrease in the number of errors until day 3 of the experiment (Figure 9D; \( *P<0.05; **P<0.005; ***P<0.0005; ****P<0.0001 \)).

During the whole testing period, Bcl11b mutants exhibited significantly higher numbers of working memory errors compared with control animals. The results demonstrate a reduced learning capability of the Bcl11b mutants compared with control animals with the number of errors \(~7\) times higher for the mutant on the last 3 days of training (ANOVA: \( F\)-value \((1,16) = 28.575 \) (sample), \( P<0.001 \); \( F\)-value \((4,16) = 7.297 \) (day), \( P<0.001 \)).

This is supported by the fact that in mutants the number of new arm entries during the first eight-arm entries was not different from a random sequence, indicating low, if any, learning. In contrast, wild-type mice continuously enhanced the number of new entries during the trials indicating good spatial learning performance (Figure 9E; mutants: 5.1 ± 0.24; wild type: 6.9 ± 0.22; \( P=0.00001 \)).

Figure 7 Analysis of the hippocampus-specific Dsp\textsuperscript{lox/lox} Emx1-Cre phenotype at P14. (A–F) Morphological analysis by cresyl-violet staining of control (A, D) and Dsp\textsuperscript{lox/lox} Emx1-Cre (B, E) coronal forebrain sections as well as statistical analysis of the dentate gyrus area (C) and cell number (F). (G–I) Immunohistochemical analysis of progenitor proliferation by BrdU incorporation of control (G) and Dsp\textsuperscript{lox/lox} Emx1-Cre (H) animals. BrdU, red; NeuN, blue. (J–L) Analysis of cell death of control (J) and Dsp\textsuperscript{lox/lox} Emx1-Cre (K) by TUNEL assay. TUNEL, green; DAPI, blue; arrows indicate TUNEL-positive cells. (M–O) Immunohistochemical analysis of NeuroD (green)-positive cells in control (M, P) and Dsp\textsuperscript{lox/lox} Emx1-Cre (N, Q) animals (t-test, \( *P<0.05; **P<0.01; ***P<0.005; ****P<0.0001 \); error bar, s.d.; \( n=4 \) (C, F); \( n=3 \) (I, L, O)). Scale bar: 250 \( \mu \)m (B), 100 \( \mu \)m (H), 50 \( \mu \)m (E, Q).
Figure 8 Re-introduction of Desmoplakin expression rescues Bcl11b phenotype. (A–F) Immunohistological analysis of control (A, D) and Bcl11b\textsuperscript{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 using 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 ×20 magnification. (G) Statistical analysis of BrdU-positive cells in control and mutant hippocampal slice cultures. (H) Statistical analysis of co-expression of GFP/NeuroD- and GFP/BrdU-positive cells (t-test, *\textit{p}<0.01; error bar, s.e.m.; n = 4).
Bcl11b is also expressed in the amygdala (Leid et al., 2004) a brain region related to fear- and anxiety-memory processing. To exclude that the learning and memory deficits observed in the Bcl11b mutants are related to lack of Bcl11b expression in the amygdala, we tested the anxiety-related behaviour of Bcl11b mutants in an elevated plus maze experiment. Animals exhibiting increased anxiety avoid the entry into unprotected arms. Bcl11b mutant animals again exhibited a high locomotor activity but no significant difference in entering protected or unprotected arms between control and mutant animals, suggesting that there is no difference in anxiety-related behaviour (Figure 9F). Although Dsp is a functional target of Bcl11b with respect to cell proliferation and differentiation Dsp mutants did not exhibit behavioural impairments in open field and radial maze tests (Supplementary Figure S7C–G). Although there is no significant difference in the spatial learning behaviour of control and mutant animals Dsp mutant animals have the tendency to be more explorative but unable to consolidate the information (Supplementary Figure S7F and G). These data in accordance with the unchanged mossy fibres of Dsp mutants suggest a Dsp-independent role of Bcl11b in learning and memory.

Figure 9 Ablation of Bcl11b causes impaired learning and memory behaviour. (A–C) Open field test analysing locomotor activity (A), leaning (B) and rearing (C) behaviour (t-test, *P = 0.05; **P = 0.005; ***P = 0.0005; ****P = 0.00001; error bar, s.e.m.; n = 8). (D, E) Radial maze test analysing spatial learning behaviour by determining the number of errors at 5 successive days (t-test, *P < 0.05; **P < 0.005; ***P = 0.0005; ****P = 0.00001; error bar, s.e.m.; n = 9). (D) and number of entries into new radial arms of days 3–5 (t-test, ****P < 0.00001; error bar, s.e.m.; n = 9) (E). (F) Elevated plus maze test analysing anxiety behaviour by determining number of arm entries in relation to locomotor activity. No significant differences between control and mutant animals were observed (n = 9).
Discussion

In this study, we demonstrate that Bcl11b/Ctip2 is required for structural as well as functional development of the dentate gyrus. Functions of Bcl11b are phase specific and restricted to the postnatal period of dentate development: (i) Bcl11b controls progenitor cell proliferation and the size of the neural stem-cell compartment in the dentate gyrus through indirect mechanisms. In addition, loss of Bcl11b expression increases apoptosis of postmitotic neurons. Thus, mutation of Bcl11b leads to a hypoplastic dentate gyrus with reduced numbers of granule cells. (ii) Bcl11b is further required for cell type-specific differentiation of dentate granule neurons. In the absence of Bcl11b, newborn neurons are arrested at early differentiation levels, colonizing the residual GCL. (iii) Bcl11b is essential for functional integration of granule cells into hippocampal circuitry. In Bcl11b mutant granule cells, both synaptic input as reflected by reduced dendritic spine numbers, and synaptic output as reflected by aberrant mossy fibre projections and reduced thorny excrescences on CA3 neurons (Gonzales et al, 2001), are impaired. This results in reduced spatial learning capacities in Bcl11b mutants.

Finally, we found Desmoplakin to be a direct transcriptional target of Bcl11b in dentate granule cells, and demonstrate that Bcl11b binds directly to regulatory sequences in close proximity to the transcription start site of Dsp, which includes putative Bcl11b binding sites (Avram et al, 2002; Cismasiu et al, 2006). Furthermore, re-introducing Dsp expression into the Bcl11b mutant dentate gyrus rescues the progenitor proliferation phenotype. This is in accordance with previous reports defining a role of Dsp in stem-cell proliferation (Gallicano et al, 1998). Comparing Bcl11b and Dsp mutant hippocampi, we were able to separate functions of Bcl11b depending on Dsp expression from those independent of Dsp and likely involving additional, yet undetermined signals.

Bcl11b in hippocampal neurogenesis

We found that Bcl11b expression in the dentate gyrus is restricted to postmitotic neurons. In addition, at embryonic stages we could not detect Bcl11b expression in migrating progenitor cells contributing to the dentate primordium. Thus, impaired progenitor cell proliferation and depletion of the neural stem-cell pool, as defined by BrdU incorporation, progenitor cells contributing to the dentate primordium. stages we could not detect Bcl11b restricted to postmitotic neurons. In addition, at embryonic Bcl11b undetermined signals. Dsp independent of Bcl11b proliferation (Gallicano et al, 2006). Functions of neurogenesis in the dentate gyrus through Bcl11b-dependent regulation of p57KIP2 levels would require active repression of p57KIP2 by Bcl11b in proliferating progenitors, which is not supported by our expression analysis of Bcl11b.

Dsp mutant mice exhibit a similar although weaker phenotype when compared with Bcl11b mutants, for example, smaller dentate gyrus, and reduced proliferation rate suggesting that Dsp acts downstream of Bcl11b in these processes. Complete recovery of the proliferation phenotype by re-introducing Dsp expression into Bcl11b mutants further demonstrates an important role of Dsp in progenitor cell proliferation. This coincides with a previous report demonstrating that ablation of Dsp expression causes reduced proliferation during early embryonic development (Gallicano et al, 1998). The weaker Dsp phenotype might be due to the decrease in apoptosis in the Dsp mutant in contrast to the increase in apoptotic cells in Bcl11b mutants. However, this does not exclude additional factors to be involved.

There is emerging evidence that cell–cell adhesion is important for the control of stem-cell behaviour and the transmission of regulatory signals provided to the stem-cell niche (Marthiens et al, 2010; Redmer et al, 2011), raising the possibility that Dsp may exert similar functions in the control of hippocampal neurogenesis. As mentioned above, Dsp expression rescues the Bcl11b proliferation phenotype. In addition, we observed a misdistribution of Sox2-positive cells, for example, loss of Sox2-positive cells in the SGZ and increase of Sox2-positive cells in the GCL, suggesting that stem cells leave the stem-cell niche but are not able to further differentiate. It is possible that Dsp is required to provide cell–cell adhesion in order to establish the signalling pathway from neighbouring cells to allow neuronal differentiation (see also below).

Bcl11b in neuronal differentiation

We found that Bcl11b is required for cell type-specific differentiation of dentate granule cells. Previous reports demonstrated similar regulatory functions for Bcl11b in the development of corticospinal motor neurons and the striatum (Arllotta et al, 2005, 2008). Outside the CNS, Bcl11b is critical for the differentiation of T lymphocytes (Wakabayashi et al, 2003; Liu et al, 2010), suggesting the regulation of cellular differentiation to be a conserved function of Bcl11b. Surprisingly, our mosaic deletion data suggest a non-cell autonomous role of Bcl11b in the regulation of neuronal differentiation, and deletion of Bcl11b in a fraction of dentate neurons leads to impaired neuronal differentiation in surrounding wild-type cells. It was shown that Bcl11b plays an important non-cell autonomous role in the proliferation and differentiation of keratinocytes, suggesting that Bcl11b...
regulates transcription of secreted dermal factors including KGF, a dermal fibroblast-derived growth factor, which in turn regulates epidermal morphogenesis in a paracrine fashion (Golonzhka et al., 2009). This is also supported by a recent report demonstrating a non-cell autonomous role for \( \text{Bcl11b} \) in tissue formation during wound healing that is most likely due to impaired cell–cell adhesion and might include the regulation of E- and P-cadherin (Liang et al., 2012). From our data, we conclude that \( \text{Bcl11b} \) regulates progenitor proliferation as well as differentiation in a non-cell autonomous fashion but that different additional factors might be involved in the regulation of these processes. We cannot exclude that \( \text{Bcl11b} \), in addition, has a cell autonomous function in neuronal differentiation. Defective differentiation of dentate granule cells was also observed in \( \text{Dsp} \) mutants. \( \text{Dsp} \) interacts with desmosomal cadherins via plakoglobin connecting desmosomes with intermediate filaments of the cytoskeleton (Garrod and Chidgey, 2008). The cytoskeleton plays an important role in cell division, cell polarity and cell differentiation as well as signalling (Hoogenraad and Bradke, 2009). In the epidermis, \( \text{Dsp} \) rearranges microtubules according to the differentiation state of the cell (Lechler and Fuchs, 2007). Thus, it is possible that \( \text{Dsp} \) has a similar function in neuronal cells. Lack of \( \text{Bcl11b} \) and \( \text{Dsp} \) expression could therefore cause defects in the organization of the cytoskeleton resulting in granule cell dispersion as well as in impaired neuronal differentiation. Further, indirect evidence for an interaction between \( \text{Bcl11b} \) and \( \text{Dsp} \) was provided by \( \text{Bcl11b} \) regulation of proliferation and late differentiation in keratinocytes where \( \text{Dsp} \) expression occurs (Golonzhka et al., 2007, 2009).

Ablation of \( \text{Bcl11b} \) in the hippocampus caused aberrant mossy fibre projections and severe deficits in spatial learning and memory. None of these mutant phenotypes was recapitulated in \( \text{Dsp} \) mutants. This indicates that major functions of \( \text{Bcl11b} \) in hippocampal development occur independent of \( \text{Dsp} \). Furthermore, it suggests that the failure of \( \text{Bcl11b} \) mutant granule neurons to integrate into the hippocampal circuitry is critical for the development of learning and memory deficits (Schwegler et al., 1990; Schwegler and Crusio, 1995).

### Perspectives

Regulation of the developing dentate gyrus is of special interest because it is one of only two brain regions with ongoing neurogenesis in adulthood. Expression of \( \text{Bcl11b} \) in the dentate gyrus is sustained throughout the life (LB, RS and SB, unpublished). It will be interesting to determine whether \( \text{Bcl11b} \) executes a similar function during neurogenesis of the adult and aged hippocampus. Dysregulated neurogenesis is associated with disorders like Alzheimer’s disease and Schizophrenia (Zhao et al., 2008; Clelland et al., 2009; DeCarolis and Eisch, 2010). \( \text{Bcl11b} \) with its dual phase-specific regulatory functions during postnatal development can provide an excellent model system to gain better insight into the mechanisms of learning and memory and the pathophysiology of associated diseases.

### Materials and methods

#### Animals

For the generation of a conditional knockout allele, exons 4–6 of the \( \text{Bcl11b} \) gene and exon 2 of the \( \text{Dsp} \) gene were floxed as described previously (Vasioukhin et al., 2001; Li et al., 2010). \( \text{Bcl11b}^{\text{flox/flox}} \) as well as \( \text{Dsp}^{\text{flox/flox}} \) mice were crossed to \( \text{Emx1-Cre} \) (Gorski et al., 2002) as well as to \( \text{Nex-Cre} \) (Goebels et al., 2006) transgenic mice. To exclude \( \text{Cre} \)-related effects, homozygous mutant mice were strictly compared with heterozygous controls harbouring a \( \text{Cre} \) allele. Genotyping of the mice was performed by PCR. All animal experiments were carried out in accordance with the German law and were approved by the respective government offices in Berlin, Göttingen and Tübingen.

#### In-situ hybridization, histology, immunohistology, Timm staining and Golgi impregnation

For in-situ hybridization, forebrains were dissected from control and mutant embryos at E15 and E18 as well as animals at P7, P10, P14 and P30, fixed with 4% PFA and embedded in OCT compound (Sakura). Hybridizations were performed with DIG-labelled riboprobes on 18 \( \mu \text{m} \) cryosections. For immunofluorescence staining, brain tissues were fixed with 4% PFA in 0.1 M sodium phosphate buffer (pH 7.4). In all, 14 \( \mu \text{m} \) cryosections were obtained from matched control and mutant brains. Stained sections were examined on a confocal microscope (Zeiss LSM510, or Leica Sp5II).

For the Timm staining, mice were transcardially perfused with buffered sodium sulphate and glutaraldehyde, placed overnight in a 30% saccharose solution followed by embedding in OCT compound (Sakura). In all, 40 \( \mu \text{m} \) sections were developed in Timm’s solution and counterstained with methylene blue as was described in Schwegler and Lipp (1983). Golgi staining of hippocampal tissue was carried out according to a modified protocol published previously (Heimrich and Frotscher, 1991).

Morphological analysis was performed on 5 \( \mu \text{m} \) methacrylat sections (Technovit 7100, Heraeus-Kulzer, Wehrheim, Germany) using 0.02% cresyl violet/0.2 M Walpole buffer (Chroma-Waldeck, Münster, Germany) for 30 min at room temperature.

#### Antibodies

The following antibodies were used: Goat anti-NeuroD and anti-\( \text{Dcx} \) as well as mouse anti-NeuN (all from Santa Cruz), rabbit anti-Calbindin (Swant), rabbit anti-Sox2 as well as mouse anti-Reelin (all from Millipore), rat anti-BrdU (AbD Serotec), mouse anti-GFAP (Sigma-Aldrich), rabbit anti-Pecam and anti-Thrb (Abcam) and rabbit anti-Dsp (E Fuchs, Rockefeller University). To generate an anti-\( \text{Bcl11b} \) serum, a 519-bp fragment from the murine \( \text{Bcl11b} \) cDNA, corresponding to aa 462–634 (NM_021399) was amplified by PCR. The PCR fragment was cloned into the bacterial expression vector pET41a (Novagen), which provided coding sequences for a His6 tag. His6-Bcl11b was propagated in BL21(DE3)Plys5 cells, affinity purified on TALON metal resin (BD Biosciences) and injected into rabbits and guinea pigs (Charles River).

#### Ex-utero electroporation and slice culture

Slice cultures of \( \text{Bcl11b}^{\text{flox/flox}} \) and \( \text{Emx1-Cre} \) as well as \( \text{Bcl11b}^{\text{flox/flox}} \) embryonic brains were carried out as described (Hand et al., 2005). Briefly, 4 \( \mu \text{g} \) of DNA was electroporated into the prospective dentate gyrus area of embryonic brains at E 15.5 using five pulses at 50 V, brains were cut into 250 \( \mu \text{m} \) slices and kept in culture up to 11 DIV (rescue experiments) or 18 DIV (mosaic experiments). To determine the proliferation rate of dentate gyrus cells, BrdU (10 \( \mu \text{M} \)) was added to the culture medium for the first 20 h after electroporation. For the rescue experiments, brains were electroporated with the vectors pRC-CMV, pRC-CMV-Dsp (Kozer et al., 2003), as well as with pcAGGS for GFP expression. For mosaic experiments, pCIG2 and pCIG2-Cre vectors were used (Hand et al., 2005).

#### Determination of progenitor cell proliferation and apoptosis

Progenitor cell proliferation was determined by BrdU incorporation. Pregnant females or pups at the indicated times were injected with BrdU (100 \( \mu \text{g/g} \) body weight; Sigma-Aldrich, cat. #: B9285) 2 h before tissue dissection. Apoptotic cells were detected by TUNEL assay according to manufacturer’s manual (Millipore, cat. #: S7110).

#### Determination of dentate gyrus cell number and area

Forebrain tissues from at least three mutant and control animals were sectioned serially (14 \( \mu \text{m} \) thickness). Dentate gyrus cell number and area were determined on three or nine matched sections of the caudal, medial as well as the rostral hippocampus per animal.
and the average value of a section per animal was determined. Values were presented as means ± s.e.m (Bcl11b) or ± s.d (Dsp). Differences in values were considered to be significant at P<0.05 by Student’s t-test.

**Determination of the number of dendritic spines and thorny excrescences**

Sections of three Golgi impregnated control and Bcl11b mutant hippocampi were used to determine the dendritic spine number of the dorsal and ventral dentate gyrus granule cells as well as the thorny excrescences located in the apical dendritic tree of CA3 pyramidal neurons. At least 15 neurons and a total of 7000 spines per animal were counted. To determine the dendritic spine number, selected dendrites were divided into 50 μm sections starting from the cell soma to the end of the dendrites. The spines of 30 dendrites per animal were counted. Thorny excrescences were identified according to previously published morphological criteria (Gonzales et al, 2001) and counted within the first 50 μm from the soma of the apical dendrites of CA3 pyramidal cells corresponding to the stratum lucidum. At least 7–10 neurons per animal and 200 thorny spines per neuron were counted. Values were presented as means ± s.d. Differences in values were considered to be significant at P<0.05 by Student’s t-test.

**Microarray analysis, quantitative real-time RT–PCR and ChIP of Bcl11B target genes**

Microarray analysis, quantitative RT–PCR as well as ChIP assays are described elsewhere (Schwegler et al, 2012). For detailed information, see Supplementary Material and methods.

**Behavioural tests**

Behavioural analysis was performed employing adult male as well as female wild-type and mutant animals to open field (n=8), elevated plus maze (n=9) and radial arm maze (n=9) tests. Control and mutants were age matched and of the same genetic background (Bl6/CD1). Open field, elevated plus maze as well as radial arm maze tests are described elsewhere (Schwegler et al, 1990; Yilmazer-Hanke et al, 2004). For detailed information, see Supplementary Material and methods.

**Statistical analysis**

Results are expressed as the mean ± s.e.m. or ± s.d. Comparisons between groups were made by an unpaired two-tailed Student’s t-test or analysed using the 2-way ANOVA with dependent variables was performed for the analysis of the radial maze data.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**Author contributions**

HB performed expression analyses, morphological analyses and Bcl11B-labeling experiments on Bcl11b mutant mice. RS carried out Microarray, qPCR and ChIP analyses. SV and RS analysed Dsp mutant mice, and carried out slice culture experiments. HS, HB and RS performed behavioural studies. HS and SV performed mossy fibre tracings and Golgi stainings of the hippocampus. JA performed immunohistological analyses and cell counts. CW performed confocal studies and advised ex-tero electroporations and slice culture experiments. PL generated a floxed Bcl11b allele in the laboratory of NGC and NAJ, and floxed Bcl11b mice were provided by NGC. EF provided floxed Dsp mice. During initial phase of the project, experiments were carried out in the laboratory of CB at MDC. HS and RS discussed the data and designed experiments. SB designed experiments and supervised the work. RS and SB wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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