Expression of the transcriptional repressor Zbtb20 is confined to the hippocampal primordium of the developing dorsal midline cortex in mice. Here, we show that misexpression of Zbtb20 converts projection neurons of the subiculum and postsubiculum (dorsal presubiculum) to CA1 pyramidal neurons that are innervated by Schaffer collateral projections in ectopic strata oriens and radiatum. The Zbtb20-transformed neurons express Bcl11B, Satb2, and Calbindin-D28k, which are markers of adult CA1 pyramidal neurons. Downregulation of Zbtb20 expression by RNA interference impairs the normal maturation of CA1 pyramidal neurons resulting in deficiencies in Calbindin-D28k expression and in reduced apical dendritic arborizations in stratum lacunosum moleculare. Overall, the results show that Zbtb20 is required for various aspects of CA1 pyramidal neuron development such as the postnatal extension of apical dendritic arbors in the distal target zone and the subtype differentiation of Calbindin-D28k-positive subsets. They further suggest that Zbtb20 plays a role in arealization of the midline cortex.

Keywords: cell fate, hippocampus, neurogenesis, Sox5

Introduction

The hippocampus comprises the dentate gyrus (DG) and the hippocampus proper (or Ammon's horn). From a cytoarchitectonic perspective, the hippocampus proper, with its fields CA1, CA2, and CA3, is an archicortex with rows of pyramidal neurons compactly organized in a single cell layer, the stratum pyramidale (Amaral and Witter 1995). Hippocampal pyramidal neurons are derivatives of radial glia stem cells in the ventricular zone of the Ammonic primordium in the medial dorsal telencephalon (or prospective midline cortex). During neurogenesis, the hippocampal cortical plate (CP) splits the preplate into a marginal zone (MZ) and a vestigial subplate (the prospective stratum oriens). The MZ is organized into an inner marginal zone (IMZ), the prospective stratum radiatum, and an outer marginal zone (OMZ), the prospective stratum lacunosum moleculare (Super, Soriano, and Uylings 1998). Most afferent fiber systems enter the hippocampus via the MZ, which is in contrast to the neocortex where fibers enter the CP from the subcortical white matter (Super and Uylings 2001).

Although immature neocortical pyramidal neurons extend apical dendritic tufts that ramify at the pial surface in the MZ (Koester and O'Leary 1992; Kasper et al. 1994), there is a transient halt in the extension of apical dendrites of immature CA1 pyramidal neurons at the transition zone between the IMZ and the OMZ (Super, Martinez, et al. 1998). Outgrowth and arborization of these distal apical dendrites in the stratum lacunosum moleculare are postnatal processes linked to the maturation of CA1 pyramidal neurons and the late synaptogenesis in the CA1 field (Fiala et al. 1998). A conspicuous feature of hippocampal neurogenesis is the pronounced reduction in fate transitions of immature pyramidal neurons. Fate transition of neocortical progenitors is important for generation of distinct projection neuron subsets in cortical layers VI-II (Molyneaux et al. 2007; Fishell and Hanashima 2008; Leone et al. 2008). Conceivably, fate transitions also to some extent play roles in subtype specification of hippocampal projection neurons. Lorente de Nó used morphological criteria to reveal CA1 pyramidal neuron heterogeneity in the stratum pyramidale. He noted that pyramidal neurons in deep rows (toward the stratum oriens) are loosely packed compared with cells in upper rows (toward the stratum radiatum) (Lorente de Nó 1934). Moreover, upper row CA1 pyramidal neurons are immunoreactive for the low molecular weight calcium-binding protein Calbindin-D28k (Baimbridge et al. 1991) and electrically coupled by gap junctions (Bennett and Pereda 2006; Mercer et al. 2006). They also appear to harbor apical dendrites with a high number of terminal branches in the stratum lacunosum moleculare (Altemus et al. 2005).

The broad complex, tramtrack, bric-a-brac (BTB)-zinc finger transcription factor Zbtb20 (also known as Znf288, DPZF, and HOF) has been shown to function as a transcriptional repressor in vivo (Xie et al. 2008), and it is expressed in immature projection neurons of the hippocampal primordium in mice (Mitchelmore et al. 2002). Misexpression of Zbtb20 triggers hippocampus-like neurogenesis in mice with sojourning of multipolar progenitors in the intermediate zone (IZ) before they migrate radially to the CP (Nielsen et al. 2007). The latter is a hallmark of hippocampal pyramidal cell neurogenesis (Altman and Bayer 1990; Nakahara and Yuasa 2005). The Zbtb20-transformed neurons differentiate into pyramidal cells with large apical dendrites and displayed alterations in expression of the transcription factors Pou3f1 and Etv1, which resemble the expression patterns of these genes in wild type (WT) CA1. However, Pou3f1-expressing pyramidal neurons are also numerous in the subiculum and neocortical layer V (Frantz et al. 1994). Thus, although Zbtb20 is capable of generating a compact ectopic stratum pyramidale, it remains to be shown that Zbtb20 transforms these midline cortical neurons to pyramidal neurons with a CA1-like molecular identity. It is also not known whether expression of the endogenous Zbtb20 gene is essential for specification of pyramidal neurons in the CA1 field.

In the present study, we reveal novel roles for Zbtb20 in specification of CA1 pyramidal neurons in mice. We find that Zbtb20 expression is pronounced in upper row pyramidal neurons of the field CA1 stratum pyramidale after birth and...
demonstrate that Zbtb20 is required for subtype differentiation of Calbindin-D28k–positive pyramidal neuron subsets and for the extension of distal apical dendritic arbors in stratum lacunosum molecular. Furthermore, Zbtb20 appears to regulate stratum pyramidale fate of subsets of CA1 pyramidal neurons at occipital levels of the septotemporal hippocampal axis. Cortical misexpression of Zbtb20 converts projection neurons of the subiculum, postsubiculum (or dorsal presubiculum), and retrosplenial granular cortex to pyramidal neurons with a general CA1-like molecular identity. The converted subiculum and postsubiculum are composed of Calbindin-D28k–positive CA1 pyramidal cells and receives Schaffer collateral innervation. Overall, the data suggest that Zbtb20 regulates various aspects of CA1 pyramidal neuron development. The Zbtb20-induced enlargement of the CA1 field further implies that the gene plays a role in arealization of the murine midline cortex.

Materials and Methods

Mice

Animals were deeply anesthetized with 2,2,2-trimethoxyethanol (Avertin), and unless stated otherwise, they were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The brains were removed and postfixed in the same fixative overnight at 4 °C. Whole heads from embryos and newborn pups were fixed overnight at 4 °C in 4% PFA. All animal experiments were in accordance with Danish and European animal welfare regulations and were licensed by the Danish Animal Experimentation Inspectorate. The D6/Zbtb20 transgenic mouse strain is described elsewhere (Nielsen et al. 2007).

Downregulation of Zbtb20 Expression by RNA Interference

We used the BLOCK-iT Pol II microRNA (miR)-based RNAi system (Invitrogen, Carlsbad, CA) to downregulate Zbtb20 expression. A Zbtb20-specific oligonucleotide was cloned into the green fluorescent protein (Gfp)-encoding pCMV-2-GW/EmGFP-miR expression plasmid (Invitrogen). Subsequently, the CMV promoter was replaced with the constitutively active CMV/β-actin (CAG) promoter of the pCG2-EGFP vector (Hand et al. 2005) to produce the Zbtb20-miR<sup>Gfp</sup> vector. The ability of this vector to downregulate expression of Zbtb20 was revealed by transfecting cells from the 2 cell lines 6E12 and 2H5, which express endogenous Zbtb20 (Mitchelmore et al. 2002). The expression of Zbtb20 in Gfp-expressing cells was monitored by immunocytochemistry as previously described (Mitchelmore et al. 2002). As a control, cells were transfected with the pCMV-EmGFP/miR-neg control plasmid vector (referred to as Control-miR<sup>Gfp</sup>), which is predicted not to target any known vertebrate mRNA transcripts (Invitrogen). The miR<sup>Gfp</sup> vectors were subsequently used for in utero electroporation experiments. Briefly, the medial-dorsal telencephalon of mouse embryos was transfected by delivering 80-100 ms electric pulses of 35 V (E13) or 40 V (E14–E15) in intervals of 950 ms, as described (Nielsen et al. 2007). After in utero electroporation, the morphologies of Gfp-positive cells were revealed at P16 or P24 in 100-μm vibratome sections following Gfp immunohistochemistry (IHC) as described below.

IHC

Fixed brains were incubated overnight at 4 °C in 20% sucrose in PBS, frozen in liquid nitrogen and sectioned at 20 μm using a cryostat. Unless stated, the sections were subjected to microwave antigen retrieval in 10 mM sodium citrate, pH 6.0 and blocked for 1 h at room temperature in 5% horse serum in PBS containing 0.1% Triton X-100 (PBS-T). The sections were incubated in primary antibody for 16-40 h at 4 °C. The following primary antibodies were used: rat anti-Bcl11b/ Ctip2 (1:500; Abcam, Cambridge, United Kingdom), mouse anti-BrdU (1:100; Millipore, Billerica, MA), rabbit anti-Calbindin-D28k (1:500; Swant, Grenchen, Switzerland), rabbit anti-Calretinin (1:500; Abcam), fluorescein isothiocyanate-conjugated goat anti-Gfp (1:200; Abcam), rabbit anti-Gfp (1:500; Abcam), goat anti-Nr4A2 (1:100; R&D Systems, Minneapolis, MN), rabbit anti-Satb2 (1:1000) (Britanova et al. 2005), mouse anti-Satb2 (1:100; Abcam), goat anti-Sox5 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Tbr1 (1:500; Abcam), rabbit anti-Zbtb20 (1:200) (Mitchelmore et al. 2002), and rabbit anti-Zfp22/Fog-2 (1:200; Santa Cruz Biotechnologies). Appropriate Alexa 488- or 594-conjugated secondary antibodies raised in donkeys were used at 1:500 dilution (Molecular Probes, Eugene, OR), and sections were mounted in Vectashield mounting medium with 4',6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). For Calbindin-D28k IHC, fresh frozen brains were sectioned at 20 μm on a cryostat. The sections were incubated in methanol with 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min. After this, sections were treated for 1 h with 5% goat serum in PBS-T and were incubated overnight at 4 °C in rabbit anti-Calbindin-D28k antibody (1:500; Swant). After washing, sections were incubated for 1 h at 37 °C with goat anti-rabbit IgG biotinylated antibody (1:100; Dako, Glostrup, Denmark), followed by a streptavidin-biotin-peroxidase complex (Dako) for 30 min in PBS-T. The sections were developed with 3,3'-diaminobenzidine tetrahydrochloride), counterstained with hematoxylin, dehydrated, and mounted with Depex. Satb2 IHC was performed as previously described (Britanova et al. 2005).

Tracing of Schaffer Collateral Projections

Prior to placement of 1,1-dilinoleyl-3,3,3',3'-tetramethyliniocarbocyanine perchlorate (Dil) crystals (Molecular Probes) in the CA3 field of P<sup>7</sup> mice, coronal vibratome sections were cut in the anterior-to-posterior direction until the hippocampus was clearly visible. Brains were kept in darkness in 4% PFA for 6 weeks at 37 °C for Dil diffusion and then sectioned at 100 μm on a vibratome. The sections were mounted in Vectashield with DAPI and immediately photographed with a digital camera mounted on a fluorescence microscope.

Results

Molecular Heterogeneity of the CA1 Stratum Pyramidale: Expression of Calbindin-D28k and Zbtb20 Are Pronounced in Upper Row Pyramidal Neurons

The Zbtb20 transcription factor is expressed in the vast majority of postmitotic immature neurons of both the IZ and the CP during embryonic corticogenesis of the presumptive CA1 field (Mitchelmore et al. 2002). Postnatally, Zbtb20 expression becomes increasingly graded in the CA1 stratum pyramidale with pronounced expression of Zbtb20 in pyramidal neurons of upper rows and low-level expression in deep-row neurons (Fig. 1, A, B). Notably, in the CA1 field of adult mouse brains, the Zbtb20 expression domain appears to overlap with that of Calbindin-D28k (Fig. 1, B, C), which is a marker of upper row pyramidal neurons in the stratum pyramidale (Baimbridge et al. 1991).

The transcription factors Bcl11b (also known as Ctip2) and Satb2 are important for specification of neocortical, subcerebral, and callosal projection neurons, respectively (Arlotta et al. 2005; Alcamo et al. 2008; Britanova et al. 2008). In the adult hippocampus, Bcl11b is expressed in pyramidal neurons of field CA1 and in granule neurons of the DG (Arlotta et al. 2005) (Fig. 1D). Satb2 is expressed at low-to-moderate levels in pyramidal neurons of field CA1 in the adult hippocampus (Britanova et al. 2005) (Fig. 1E). Notably, Bcl11b and Satb2 are coexpressed in the CA1 stratum pyramidale (Fig. 1F), which is in contrast to the mutually exclusive expression patterns of these genes in the neocortex (Alcamo et al. 2008; Britanova et al. 2008; Leone et al. 2008).

Sox5 is a marker of corticofugal neurons in deep neocortical layers (Kwan et al. 2008; Lai et al. 2008). Expression of Sox5 in CA1 is confined to cells in deep rows of the stratum pyramidale.
in newborn mice (Fig. 1G). Interestingly, Zbtb20 and Sox5 display sublaminar and mutually exclusive expression patterns in the CA1 stratum pyramidale (Fig. 1G--I). Consistent with their deep-row location, cells expressing Sox5 can be labeled with BrdU at E13, demonstrating that they rank among the first-born cells of the CA1 pyramidal cell layer (Supplementary Fig. 1A). In vivo intraventricular electroporation of a Gfp-expression vector in cortical stem cells of the presumptive field CA1 at E13 shows that at least a subset of the Sox5-positive cells display an immature pyramidal neuron-like morphology at E18 (Supplementary Fig. 1B). Taken together, the data support the notion that the CA1 stratum pyramidale displays a sublaminar organization composed of molecular distinct subsets of deep and upper row pyramidal neurons.

**Zbtb20 Controls Subtype Differentiation of Calbindin-D28k-Positive CA1 Pyramidal Neurons**

Both Zbtb20 and Calbindin-D28k are expressed in upper row cells of the CA1 stratum pyramidale (Fig. 2A,C). Interestingly, overexpression of Zbtb20 triggers aberrant Calbindin-D28k expression in deep-row cells of the CA1 stratum pyramidale in D6/Zbtb20 transgenic mice (Fig. 2B,D). To test if Zbtb20 expression is essential for the normal expression of Calbindin-D28k in upper row neurons, we used RNA-interference technology to downregulate Zbtb20 expression in vivo. A Zbtb20-specific miR was inserted in the 3' nontranslated region of a Gfp reporter gene in an expression plasmid (referred to as Zbtb20-miR<sup>0</sup>), and its ability to downregulate Zbtb20 expression was confirmed by transfecting the construct into cells from 2 cell lines with endogenous expression of Zbtb20 (Supplementary Fig. 2A,E). We next expressed Zbtb20-miR<sup>0</sup> or Control-miR<sup>0</sup> plasmid vectors in pyramidal neurons destined for upper rows of the CA1 stratum pyramidale by in utero intraventricular electroporation at E15 and monitored Calbindin-D28k expression at P24. As expected, the vast majority of the in vivo transfected Gfp-positive cells have settled in upper rows of the CA1 stratum pyramidale by in utero intraventricular electroporation at E15 and monitored Calbindin-D28k expression at P24. Notably, the Zbtb20-miR<sup>0</sup>-expressing cells do not appear to coexpress Calbindin-D28k (Fig. 2F), which is in contrast to Control-miR<sup>0</sup>-expressing neurons that do coexpress Calbindin-D28k (Supplementary Fig. 3). Overall, the data suggest that Zbtb20 regulates Calbindin-D28k subtype differentiation of a subset of CA1 pyramidal neurons.

**Misexpression of Zbtb20 Converts Subiculum and Postsubiculum to CA1**

Misexpression of Zbtb20 converts the normal cytoarchitectonic organization of the subiculum, postsubiculum, and

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**Figure 1.** Calbindin-D28k and high levels of Zbtb20 expression occur in upper row cells of the CA1 stratum pyramidale. (A,B) DAPI (blue) and Zbtb20 (red). Zbtb20 expression is pronounced in upper row cells of the CA1 stratum pyramidale (arrow) in coronal hippocampal sections of WT mouse brains at P0 (A) and P24 (B). (C) Serial coronal section of (B) showing Calbindin-D28k expression in upper row pyramidal neurons of the CA1 stratum pyramidale (arrow). (D--F) DAPI (blue), Bcl11b (red), and Satb2 (green). Expression of Bcl11b (D) and Satb2 (E) coincide (F) in pyramidal cells of the CA1 stratum pyramidale in coronal hippocampal section at P24. (G--I) DAPI (blue), Sox5 (red), and Zbtb20 (green). Coronal section of CA1 of WT mice at P0 reveals Sox5 (G) expression in cells of deep rows of the CP and Zbtb20 expression (H) in cells of upper rows of the CP and in the IZ. Note the sublaminar organization of the CP with mutually exclusive expression patterns of Sox5 and Zbtb20 (I). Scale bar: 200 μm in (A), 280 μm in (B--F) and 50 μm in (G--I).
granular retrosplenial cortex to a CA1-like stratum pyramidale in D6/Zbtb20S transgenic mice (Fig. 3A--D). To reveal further details about the molecular identity of these Zbtb20 trans-formed neurons and their relationship to CA1 pyramidal neurons, we analyzed the expression of Bcl11b and Satb2 in adult D6/Zbtb20S mice. Consistent with a CA1-like identity, Bcl11b and Satb2 are coexpressed in cells of the ectopic stratum pyramidale (layer E--F*) (Fig. 3E--H) of D6/Zbtb20S brains, which is in contrast to the mutually exclusive expression patterns of these markers in corresponding areas of WT brains (Fig. 3E--H). Moreover, numerous cells in the ectopic stratum pyramidale express Calbindin-D28k (Fig. 3I), which is in contrast to the rare and sporadic appearance of Calbindin-D28k-positive cells in corresponding areas of WT brains (Fig. 3I). Contrary to the mutually exclusive expression patterns of these markers in corresponding areas of WT brains (Fig. 3E--H), Bcl11b and Satb2 are coexpressed in the ectopic stratum pyramidale of D6/Zbtb20S brains, which is in contrast to the rare and sporadic appearance of Calbindin-D28k-positive cells in corresponding areas of WT brains (Fig. 3I--L).
widespread coexpression of Bcl11b and Satb2 in the caudal dorsal midline cortex of D6/Zbtb20S mice, Calbindin-D28k expression was revealed only in the subiculum and postsubiculum but not in retrosplenial areas (Fig. 3E). Hence, the molecular marker analysis shows that Zbtb20 can transform subsets of pyramidal neurons in subicular and postsubicular areas to CA1 pyramidal neurons with a Calbindin-D28k-positive subtype.

Pyramidal neurons in the CA3 field of the hippocampus send Schaffer collateral projections that terminate specifically on basal and apical dendrites of CA1 pyramidal neurons in stratum oriens and stratum radiatum, respectively. We therefore investigated the possibility that there is Schaffer collateral innervation of the enlarged CA1 area in D6/Zbtb20S mice by placing small crystals of Dil in the CA3 field of WT (Fig. 4A) and D6/Zbtb20S mice (Fig. 4B). Because Dil is both a retrograde and anterograde tracer, there is retrograde tracing of mossy fibers projecting from the DG to CA3 in both WT and D6/Zbtb20S mice (Fig. 4A,B). There is anterograde tracing of Schaffer collaterals to stratum oriens and stratum radiatum of the CA1 field in both WT and D6/Zbtb20S mice (Fig. 4A-D).

Although Schaffer collaterals specifically target the CA1 field in WT mice (Fig. 4C), there is a pronounced innervation of both subicular and postsubicular areas in D6/Zbtb20S mice (Fig. 4D). The aberrant Schaffer collateral projections in the subiculum and postsubiculum display a hippocampal strata oriens and radiatum-like laminar organization (Fig. 4D) implying that these areas are composed of subsets of bona fide CA1 pyramidal neurons that are able to functionally integrate in the hippocampal circuit.

**Figure 4.** Dil labeling reveals aberrant Schaffer collateral projections from CA3 to the subiculum and postsubiculum of D6/Zbtb20S mice. (A–D) Coronal sections of brains from P7 WT and D6/Zbtb20S mice. (A,B) By placing small Dil crystals in the CA3 fields of WT and D6/Zbtb20S mice, there are a robust retrograde labeling of mossy fibers from the DG and anterograde labeling of Schaffer collateral projections from CA3 to the stratum oriens (SO) and stratum radiatum (SR) of the CA1 field. In the D6/Zbtb20S brains (B,D), in contrast to the WT brains (A,C), Schaffers projections aberrantly innervate the transformed subiculum (Sub) and postsubiculum (PS) both below and above the ectopic stratum radiatum (SP) in a laminar pattern that resemble the hippocampal strata oriens, pyramide and radiatum. The arrows in (A–D) indicate the border between the CA1 field and the subiculum (Sub). Scale bar: 500 μm in (A,B) and 200 μm in (C,D).
mice (Fig. 6D), in contrast to the widespread distribution of Tbr1-positive cells in deep layers of the CP in WT mice (Fig. 6E). The deficiency of Tbr1-positive subcortical neurons in D6/Zbtb20S brains was confirmed with the Zfpm2 marker (Fig. 6G,H). In the WT CA1 field, both Tbr1 and Zfpm2 are expressed in a subset of cells in deep rows of the CP (Fig. 6F,I). The orphan nuclear receptor Nr4A2 (also known as Nurr1) is expressed in subplate cells and in cortico-cortical associative projection neurons in deep layers of the neocortex (Arimatsu et al. 2003). In newborn WT mice, Nr4a2 is expressed in projection neurons of the subicular complex but not in the hippocampus (Gray et al. 2004; Britanova et al. 2006) (Fig. 6L). Nr4A2 expression is furthermore pronounced in deep layers of the postsubiculum in WT mice (Fig. 6K), in contrast to the lack of Nr4A2 expression in the postsubiculum of D6/Zbtb20S mice (Fig. 6J). Taken together, the molecular marker analyses show that misexpression of Zbtb20 dysregulates a number of transcription factors, which are important for subtype specification of neocortical projection neurons. They further support the notion that Zbtb20-transformed pyramidal neurons

![Figure 5](https://academic.oup.com/cercor/article-abstract/20/8/1904/403939/)

Cerebral Cortex August 2010, V 20 N 8 1909
display a CA1-like molecular identity in midline cortical areas of D6/Zbtb20S mice.

**Zbtb20 Is Required for Extension of Distal Apical Dendritic Arbors of CA1 Pyramidal Neurons in the Stratum Lacunosum Moleculare**

During early postnatal development of the hippocampus, apical dendrites of immature CA1 pyramidal neurons accumulate at the transition zone between the stratum radiatum and the stratum lacunosum moleculare (Super, Martinez, et al. 1998). To test the hypothesis that Zbtb20 expression is important for the formation of apical dendritic tufts of CA1 pyramidal neurons in stratum lacunosum moleculare, Zbtb20-miRgfp and Control-miRgfp plasmid vectors were expressed in developing pyramidal neurons of field CA1 from E15 to P16 in WT mice. CA1 pyramidal neurons expressing Control-miRgfp harbor basal dendrites in stratum oriens, an elaborate dendritic tree in stratum radiatum and apical dendritic tufts in stratum lacunosum moleculare (Fig. 7A,C). In contrast, CA1 pyramidal neurons expressing Zbtb20-miRgfp are deficient in extension.
of distal apical dendritic arbors in the stratum lacunosum moleculare. In these mice, there is an aberrant accumulation of apical arbors at the transition zone between the stratum radiatum and the stratum lacunosum moleculare (arrows in Fig. 7B,D). Only slender (nontufted) dendritic arbors extend into the stratum lacunosum moleculare to terminate on the pial surface (data not shown). Hence, downregulation of Zbtb20 in CA1 pyramidal neurons appears to impair dendritic extension in the distal target zone (Fig. 7E,F).

Zbtb20 Expression Is Important for Stratum Pyramidale Fate of CA1 Pyramidal Neurons at Occipital Levels of the SeptoTemporal Hippocampal Axis

Misexpression of Zbtb20 converts multilayered areas of the midline cortex to a compact CA1-like stratum pyramidale. Hence, it is possible that endogenous Zbtb20 is important for stratum pyramidale fate of CA1 pyramidal neurons. In line with this notion, Zbtb20-miR<sup>GRP</sup>-mediated downregulation of Zbtb20 expression from E14 to P16 results in dislocation of pyramidal neurons in stratum radiatum of CA1 at occipital levels of the septotemporal hippocampal axis (Fig. 8A,B). In contrast, pyramidal neurons expressing the Control-miR<sup>GRP</sup> reporter gene localize to the stratum pyramidale (Fig. 8C,D).

We did not observe dislocation of CA1 pyramidal cells when expressing Zbtb20-miR<sup>GRP</sup> at septal levels both from E14 to P16 (data not shown) and from E15 to P16 (Fig. 7B,D). Taken together, the data suggest that expression of the endogenous Zbtb20 gene is important for stratum pyramidale fate of immature CA1 pyramidal neurons at occipital levels of the septotemporal axis. It further suggests that there is functional redundancy for this phenotype along the septotemporal axis.

Discussion

A Role for Zbtb20 in Arealization of the Midline Cortex

The Zbtb20 transcriptional repressor is expressed in the presumptive hippocampus during corticogenesis in mice (Mitchelmore et al. 2002). In line with this, misexpression of Zbtb20 causes enlargement of the hippocampal territory in caudal areas of the midline cortex in mice (Nielsen et al. 2007). Here, we show that Zbtb20 aberrantly converts these areas to CA1. In the subiculum and postsubiculum, the Zbtb20 transformed neurons show signs of bona fide CA1 pyramidal neurons, that is, they are organized in a compact stratum pyramidale, express both general and subtype-specific molecular markers of CA1 pyramidal neurons and appear to integrate in the hippocampal circuit. Schaffer collaterals from CA3 aberrantly innervate these areas in a CA1-like laminar pattern with a stratum oriens below and a stratum radiatum above the...
neurogenesis (also referred to as invariant corticogenesis),

model that

Zbtb20

pal areas of the cerebral cortex. Overall, the data support the

morphological, and functional characteristics in nonhippocam-

transitions of postmitotic neural progenitors are important for

mediated repression (directly or indirectly) of

Zbtb20S transgenic mice. In the latter mice, the aberrant

the Zbtb20 transformed postsubiculum of newborn D6/

was previously found to correlate with downregulation of

CA1 pyramidal neurons (Arlotta et al. 2005). Expression of Sox5

seem to express Bcl11b, which is also normally expressed by

neurons, of Satb2-positive callosal neurons and of cells

of cortical projection neurons (Molyneaux et al. 2007; Fishell

cortical progenitors that in part may contribute to the

acquisition of mature area identity of projection neurons. The
cortical hem is a patterning center in the embryonic midline
cortex (Subramanian and Tole 2009) that appears to function as
a hippocampal organizer, in part through Wnt signaling (Lee
et al. 2000; Machon et al. 2007; Mangale et al. 2008). Our results
identify Zbtb20 as a possible downstream effector for

establishment of the hippocampal territory during arealization

of the midline cortex.

BTB-zinc finger transcription factors including Zbtb20 are

known to function as transcriptional repressors (Kelly and
Daniel 2006; Xie et al. 2008), and some of these factors have
been show to mediate repression through epigenetic modi-

fications of the chromatin structure (Barna et al. 2002;
Gearhart et al. 2006). The specification of the CA1 molecular
identity by Zbtb20 misexpression is in line with a global
chromatin modification role of the gene. The data imply that
Zbtb20 aberrantly represses area-specific features of midline
cortical projection neurons and orchestrates the expression of
transcription factors for CA1 pyramidal neuron development
resulting in coexpression of Bcl11b and Sab2 in mature
neurons. The molecular marker analyses show that Zbtb20
misexpression correlates with the repression of transcription
factors, which are normally important for subtype specification
of cortical projection neurons (Molyneaux et al. 2007; Fishell
and Hanashima 2008; Leone et al. 2008). There is a pronounced
deficiency of Nr4A2 expressing cortico-cortical associative
neurons, of Satb2-positive callosal neurons and of cells
expressing molecular markers of subplate and layer V1 neurons
(i.e., Thr1, Zifp2m, and Sox5). In their place, the majority of cells
seem to express Bcl11b, which is also normally expressed by
CA1 pyramidal neurons (Arlotta et al. 2005). Expression of Sox5
was previously found to correlate with downregulation of
Bcl11b in subsets of developing subcortical neurons (Kwan
et al. 2008) and expression of Bcl11b is further dysregulated in
the developing neocortex of Sox5−/− mice, where Bcl11b
expression aberrantly coincides with Calretinin (Lai et al.
2008). A subset of immature Bcl11b-positive pyramidal cells
coexpress Calretinin in field CA1 of newborn WT mice and in
the Zbtb20 transformed postsubiculum of newborn D6/
Zbtb20s transgenic mice. In the latter mice, the aberrant
coexpression of Bcl11b and Calretinin correlates with Zbtb20-
mediated repression (directly or indirectly) of
Sox5. Fate transitions of postmitotic neural progenitors are important for
the genesis of projection neurons with distinct molecular,
morphological, and functional characteristics in nonhippocam-
pal areas of the cerebral cortex. Overall, the data support the
model that Zbtb20 induces a hippocampal variant of cortical
neurogenesis (also referred to as invariant corticogenesis),

involving fate transition arrest in Zbtb20-expressing progeni-
tors and immature neurons (Nielsen et al. 2007).

Zbtb20 Regulates Maturation of CA1 Pyramidal Neurons

The Zbtb20 transcription factor is widely expressed in
immature CA1 pyramidal neurons of both the IZ and CP during
embryonic and early postnatal development (Mitchelmore et al.
2002). Postnatally, Zbtb20 expression becomes graded in CA1
with a pronounced expression of the gene in pyramidal
neurons in the upper 2 rows of the stratum pyramidale,
whereas pyramidal neurons in deeper rows express low to
undetectable levels of Zbtb20. The complex developmental
expression pattern of Zbtb20 suggests roles for the gene in
both embryonic neurogenesis and postnatal maturation of CA1
pyramidal neurons. The latter involves the expression of
Calbindin-D28k in a pyramidal neuron subset residing mainly
in the upper 2 rows of the stratum pyramidale toward the
stratum radiatum (Baimbridge et al. 1991). Overexpression of
Zbtb20 in CA1 pyramidal neurons induces aberrant Calbindin-
D28k expression in deep-row pyramidal cells and, conversely,
downregulation of Zbtb20 in CA1 appears to prevent expres-
sion of Calbindin-D28k in upper row CA1 pyramidal neurons.
The role of Calbindin-D28k in CA1 pyramidal neurons is not
clear. Calbindin-D28k expression is pronounced in upper row
pyramidal neurons, which may be electrically coupled by gap
junctions (Bennett and Pereda 2006; Mercer et al. 2006).
Accordingly, Calbindin-D28k might potentially buffer free
intracellular Ca2+ in these neurons (Müller et al. 2005). Mice
harboring a targeted deletion in the Calb1 gene, which
encodes Calbindin-D28k, are impaired in motor coordination,
which most likely results from the lack of Calb1 expression in
cerebellar Purkinje cells (Airaksinen et al. 1997; Schwaller
2009). Although Calb1 knockout mice have no documented
phenotype related to the cytoarchitectonic development of
pyramidal neurons in the cerebral cortex, transgenic mice with
reduced cortical expression of Calb1 displayed alterations in
long-term potentiation of CA1 pyramidal neurons, and they
were impaired in spatial learning tests (Molinari et al. 1996;
Jouveneau et al. 2002) implying that Calb1 does play a role in
memory function.

We find that downregulation of Zbtb20 expression results in
the aberrant accumulation of distal apical dendrites of affected
CA1 pyramidal neurons at the transition zone between the
stratum radiatum and the stratum lacunosum moleculare.
During early postnatal development of the CA1 field, the
growth of distal apical dendrites of immature pyramidal
neurons transiently halt at the border between the stratum
radiatum and the stratum lacunosum moleculare (Super,
Martínez, et al. 1998). The extension of these distal apical
dendrites to form tufts in the stratum lacunosum moleculare is
coincident with the late synaptogenesis in the area. Synaptic
inputs at these distal synapses are integrated differently from
inputs at more proximal synapses in the stratum radiatum
(Izumi and Zorumski 2008; Spruston 2008) and are important
for the functional integrity of the hippocampus (Brun et al.
2008). Hence, Zbtb20 seems to regulate an intrinsic genetic
program that permits distal dendritogenesis in subsets of CA1
pyramidal neurons after birth, which is in line with the general
assumption that intrinsic transcription factor-regulated signals
in combination with external diffusible cues control the
process of dendritogenesis (Parrish et al. 2007).
Reduction of Zbtb20 expression also results in ectopic pyramidal neurons in the stratum radiatum at occipital levels of the septotemporal hippocampal axis, whereas this phenotype was not observed at more septal levels of the axis. Thus, there seems to be functional redundancy for Zbtb20 functions at most levels of the septotemporal hippocampal axis, which is in line with the heterogeneity in gene expression along this axis of the hippocampus (Leonardo et al. 2006; Thompson et al. 2008). Supporting the notion of functional redundancy of pyramidal neuron specification in field CA1, downregulation of Zbtb20 from E15 does not appear to alter expression of Bcl11b and Satb2 in CA1 pyramidal neurons of adult mice (Supplementary Fig. 4). Moreover, the CA1 stratum pyramidale displays a laminar organization with a minor subset of deep-row pyramidal neurons expressing Sox5, Tbr1, and Zfpmp2 and a major subset of pyramidal cells expressing Bcl11b. The deep-row pyramidal neurons do not appear to express Zbtb20 implying that Zbtb20 could repress the deep-row molecular identity. Although the Zbtb20 misexpression results support this model, Zbtb20 does not appear to function as a simple genetic switch that regulates the deep-row molecular identity in the CA1 stratum pyramidale. Downregulation of Zbtb20 expression in progenitors born after E15, which are destined for upper rows of the stratum pyramidale, did not result in aberrant expression of Sox5 or Zfpmp2 (Blom JB, Jensen NA, unpublished data). In addition to functional redundancy, a likely explanation for this is that CA1 progenitors like neocortical progenitors get progressively restricted in their differentiation potential (Frantz and McConnell 1996) and that the progenitors for upper row CA1 neurons therefore have lost the potential to differentiate into early born deep-row neurons. Hence, additional experiments are needed to disclose regulatory genes that functionally overlap with Zbtb20 in CA1 pyramidal neuron development such as in the formation of cellular diversity in the stratum pyramidale.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Address correspondence to email: naajensen@health.sdu.dk.

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Zbtb20 in CA1 Neuron Development


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