Zbtb20-Induced CA1 Pyramidal Neuron Development and Area Enlargement in the Cerebral Midline Cortex of Mice

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; Nakahira 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 Evt1, 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 moleculare. 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-tribromoethanol (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 pcDNA6.2-GW/EmGFP-miR expression plasmid (Invitrogen). Subsequently, the CMV promoter was replaced with the constitutively active CMV/β-actin (CAG) promoter of the pCIG2-EGFP vector (Hand et al. 2005) to produce the Zbtb20-miR‡ 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 pcAG-EmGFP/miR-neg control plasmid vector (referred to as Control-miR‡), which is predicted not to target any known vertebrate mRNA transcripts (Invitrogen). The miR‡ vectors were subsequently used for in utero electroporation experiments. Briefly, the medial-dorsal telencephalon of mouse embryos was transfected by delivering 8 50-ms electric pulses of 35 V (E13) or 40 V (E14-15) 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, Bollnäs, 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-Thr1 (1:500; Abcam), rabbit anti-Zbtb20 (1:200) (Mitchelmore et al. 2002), and rabbit anti-Zfpm2/Fog-2 (1:200; Santa Cruz Biotechnologies). Appropriate Alexa 488- or 594-conjugated secondary antibodies raised in donkeys were used at 1:50 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% H2O2 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 antirabbit IgG biotinylated antibody (1:100; Dako, Glostrup, Denmark), followed by a strepavidin-biotin-peroxidase complex (Dako) for 30 min in PBS-T. The sections were developed in 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'-tetramethylinodocyclo-octane (Dil) crystals (Molecular Probes) in the CA3 field of P7 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 brains 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 Pyramidalae: 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. 1A,B). Notably, in the CA1 field of adult mouse brains, the Zbtb20 expression domain appears to overlap with that of Calbindin-D28k (Fig. 1B,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–D). 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>dp</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). An miR with no predicted target (referred to as Control-miR<sup>dp</sup>) was used as a control (Supplementary Fig. 2A,E).

We next expressed Zbtb20-miR<sup>dp</sup> or Control-miR<sup>dp</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 together with Calbindin-D28k–positive cells (Fig. 2E,F). Notably, the Zbtb20-miR<sup>dp</sup>-expressing cells do not appear to coexpress Calbindin-D28k (Fig. 2F–F’), which is in contrast to Control-miR<sup>dp</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...
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 transformed 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 (Fig. 3E, H, J), which is in contrast to the rare and sporadic appearance of Calbindin-D28k-positive cells in corresponding areas of WT brains (Fig. 3K). Contrary to the

Figure 2. Zbtb20 regulates Calbindin-D28k expression in CA1 pyramidal neurons. (A,B) DAPI (blue) and Zbtb20 (red). Coronal section of field CA1 of WT (A) and D6/Zbtb20S (B) brains at P24. (A) Zbtb20 expression is pronounced in upper rows (arrow) of the stratum pyramidale (sp), sr, stratum radiatum. (B) In D6/Zbtb20S brains, overexpression of Zbtb20 is pronounced in deep-row cells of the CA1 stratum pyramidale. (C) In a serial section of (A), Calbindin-D28k is expressed in upper row cells (arrow) of the pyramidal cell layer. (D) In a serial section of (B), Calbindin-D28k is expressed aberrantly in deep-row cells of the pyramidal cell layer. (E–F′′′) DAPI (blue), Calbindin-D28k (red) and Gfp (green). Downregulation of Zbtb20 expression by expression of the Zbtb20-miRGfp plasmid from E15 in upper row CA1 pyramidal neurons in WT mice at P24. Neurons expressing the Zbtb20-miRGfp plasmid (green) have settled together with cells expressing Calbindin-D28k in upper rows of the pyramidal cell layer. Note that cells expressing the Zbtb20-miRGfp plasmid do not appear to coexpress Calbindin-D28k (arrows). Images (E–F′′′) are higher magnifications of area indicated by white rectangle in (E). Scale bar: 100 μm in (A–D), 200 μm in (E) and 50 μm in (F′–F′′′).

Figure 3. Misexpression of Zbtb20 induces aberrant CA1-like molecular identity of projection neurons in the midline cerebral cortex. (A,B) DAPI (blue) and Zbtb20 (green). Coronal brain sections of newborn WT (A) and D6/Zbtb20S (B) mice. (A) Zbtb20 expression is confined to projection neurons of the developing hippocampus in WT brain. (B) In D6/Zbtb20S brains, Zbtb20 misexpression is pronounced in projection neurons of the neocortex. (C,D) Nissl-stained coronal brain sections showing the cytoarchitectonic organization of the subiculum (Sub), postsubiculum (PS), and granular retrosplenial cortex (Rsc) in WT (C), and D6/Zbtb20S (D) brains at P24. Note the aberrant CA1-like stratum pyramidale organization of projection neurons in Sub, PS, and Rsc in (D). (E–J) DAPI (blue), Bcl11b (red), and Satb2 (green). (F–H) The expression of Bcl11b and Satb2 in WT brains at P24 is mutually exclusive in Sub, PS, and Rsc, in contrast to CA1 where the 2 genes are coexpressed in cells of the stratum pyramidale (I). (I) Bcl11b and Satb2 coexpression in the ectopic stratum pyramidale of the Sub, PS, and Rsc is CA1-like in D6/Zbtb20S mice at P24. (K,L) Only very few Calbindin-D28k-positive cells are found in the Sub and PS of WT brains (K), in contrast to the pronounced expression of Calbindin-D28k in cells of the ectopic stratum pyramidale in these areas of D6/Zbtb20S brains (L). Scale bar: 500 μm in (A,B) and 400 μm in (C–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 DiI in the CA3 field of WT (Fig. 4A) and D6/Zbtb20S mice (Fig. 4B). Because DiI 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.

The Zbtb20-Induced CA1 Conversion of the Midline Cortex Involves Deregelation of Transcription Factors That Normally Specify Neuronal Subtypes in the Neocortex

To investigate changes in gene expression associated with the aberrant specification of CA1 pyramidal neurons by Zbtb20, we next compared the expression patterns of a number of cortical transcription factors in the postsubiculum of newborn D6/Zbtb20S mice with the expression patterns in field CA1 and postsubiculum of age-matched WT mice. Endogenous Zbtb20 is not expressed in the postsubiculum of WT mice (Fig. 5B). In contrast, the Zbtb20 transgene is expressed in immature cells of the postsubicular IZ and CP in D6/Zbtb20S mice (Fig. 5A). This expression pattern grossly resembles the expression of endogenous Zbtb20 in immature CA1 pyramidal neurons of the IZ and CP (Fig. 5C). The transcription factor Satb2 is not expressed in the postsubiculum of D6/Zbtb20S mice (Fig. 5D), whereas it is expressed in subsets of upper and deep-layer cells in the CP of WT mice (Fig. 5E).

Hence, the temporal expression pattern of Satb2 in the postsubiculum of D6/Zbtb20S brains appears to phenocopy Satb2 expression in the CA1 stratum pyramidale of WT mice, where expression of the gene is undetectable in the CP at P0 (Britanova et al. 2006) (Fig. 5F) but is detectable at low to moderate levels in the adult (Fig. 1E). Bcl11b is normally expressed in deep layers of the postsubicular CP in WT mice (Fig. 5H). In D6/Zbtb20S mice, Bcl11b is expressed in cells of both the CP and the IZ of the transformed postsubiculum (Fig. 5G). Notably, this pattern of Bcl11b expression appears to be similar to Bcl11b expression in CA1 of WT mice (Fig. 5I). The small calcium-binding protein Calretinin is normally expressed in subplate and Cajal–Retzius neurons of the neocortex (Fonseca et al. 1995; Schierle et al. 1997). Although Calretinin appears not to be expressed in immature pyramidal neurons of the postsubicular CP in newborn WT mice (Fig. 5K), Calretinin-positive cells occur aberrantly in both the IZ and the CP of the postsubiculum in D6/Zbtb20S mice (Fig. 5J). These aberrant Calretinin-positive cells have the morphological appearance of immature pyramidal neurons, and they coexpress Bcl11b in both the IZ and CP (Fig. 5M,N). Pyramidal neurons aberrantly coexpressing Bcl11b and Calretinin were also observed in Sox5+/− mice (Lai et al. 2008) implying that immature pyramidal neurons with hybrid phenotypes might also occur in the D6/Zbtb20S mice. Alternatively, because a subset of immature CA1 pyramidal neurons in the CP of newborn WT mice also coexpress Bcl11b and Calretinin (Fig. 5L,O,P), it is equally plausible that the aberrant expression of Calretinin in the postsubiculum of D6/Zbtb20S mice phenocopies CA1 pyramidal neuron development in newborn WT mice. Because the expression patterns of Sox5 and Zbtb20 in CA1 of WT mice are mutually exclusive with Sox5-expressing cells in deep rows of the CP (Fig. 1G–I), we next analyzed the pattern of Sox5 expression in the postsubiculum. Although Sox5 expression is pronounced in deep layers of the CP in the WT postsubiculum (Fig. 6B), Sox5 appears to be downregulated in the postsubiculum of D6/Zbtb20S mice (Fig. 6A). Sox5 expression is apparently mutually exclusive with that of Zbtb20, although we cannot definitively exclude the occasional appearance of Sox5-positive cells expressing low levels of Zbtb20 and vice versa (Fig. 6M).

Tbr1 and Zipm2 are both markers of subplate and layer VI neurons (Hevner et al. 2001; Kwan et al. 2008). There is a pronounced deficiency of Tbr1-positive cells in D6/Zbtb20S mice in the CA1 stratum pyramidale of WT mice, where expression of the gene is undetectable in the CP at P0 (Britanova et al. 2006) (Fig. 5F) but is detectable at low to moderate levels in the adult (Fig. 1E). Bcl11b is normally expressed in deep layers of the postsubicular CP in WT mice (Fig. 5H). In D6/Zbtb20S mice, Bcl11b is expressed in cells of both the CP and the IZ of the transformed postsubiculum (Fig. 5G). Notably, this pattern of Bcl11b expression appears to be similar to Bcl11b expression in CA1 of WT mice (Fig. 5I). The small calcium-binding protein Calretinin is normally expressed in subplate and Cajal–Retzius neurons of the neocortex (Fonseca et al. 1995; Schierle et al. 1997). Although Calretinin appears not to be expressed in immature pyramidal neurons of the postsubicular CP in newborn WT mice (Fig. 5K), Calretinin-positive cells occur aberrantly in both the IZ and the CP of the postsubiculum in D6/Zbtb20S mice (Fig. 5J). These aberrant Calretinin-positive cells have the morphological appearance of immature pyramidal neurons, and they coexpress Bcl11b in both the IZ and CP (Fig. 5M,N). Pyramidal neurons aberrantly coexpressing Bcl11b and Calretinin were also observed in Sox5+/− mice (Lai et al. 2008) implying that immature pyramidal neurons with hybrid phenotypes might also occur in the D6/Zbtb20S mice. Alternatively, because a subset of immature CA1 pyramidal neurons in the CP of newborn WT mice also coexpress Bcl11b and Calretinin (Fig. 5L,O,P), it is equally plausible that the aberrant expression of Calretinin in the postsubiculum of D6/Zbtb20S mice phenocopies CA1 pyramidal neuron development in newborn WT mice. Because the expression patterns of Sox5 and Zbtb20 in CA1 of WT mice are mutually exclusive with Sox5-expressing cells in deep rows of the CP (Fig. 1G–I), we next analyzed the pattern of Sox5 expression in the postsubiculum. Although Sox5 expression is pronounced in deep layers of the CP in the WT postsubiculum (Fig. 6B), Sox5 appears to be downregulated in the postsubiculum of D6/Zbtb20S mice (Fig. 6A). Sox5 expression is apparently mutually exclusive with that of Zbtb20, although we cannot definitively exclude the occasional appearance of Sox5-positive cells expressing low levels of Zbtb20 and vice versa (Fig. 6M). Tbr1 and Zipm2 are both markers of subplate and layer VI neurons (Hevner et al. 2001; Kwan et al. 2008). There is a pronounced deficiency of Tbr1-positive cells in D6/Zbtb20S mice.
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 Zfp2 marker (Fig. 6G,H). In the WT CA1 field, both Tbr1 and Zfp2 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. 6F). 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
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-miR(GFP) and Control-miR(GFP) plasmid vectors were expressed in developing pyramidal neurons of field CA1 from E15 to P16 in WT mice. CA1 pyramidal neurons expressing Control-miR(GFP) 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-miR(GFP) are deficient in extension.

Figure 6. Misexpression of Zbtb20 alters the expression of deep-layer cortical markers in the postsubiculum at P0. (A–C) DAPI (blue), and Sox5 (red). Sox5 is expressed in deep layers of the CP in PS of WT mice (B) but downregulated in PS of D6/Zbtb20S mice (A). Note the sporadic appearance of Sox5-positive cells (arrow) below the CP in (A) resembles the pattern of Sox5 expression in the WT CA1 field (C). (D–F) DAPI (blue) and Tbr1 (green). Tbr1 is expressed in deep layers of the CP in WT PS (E), but it is only expressed sporadically in cells (arrows) below the CP in D6/Zbtb20S PS (D). In the WT CA1 field, Tbr1 positive cells are found in the deepest rows of the CP (F). (G–I) DAPI (blue) and Zfpn2 (green). Zfpn2 is only expressed sporadically in deep-layer cells of the PS in D6/Zbtb20S mice (G) in contrast to the wide expression domain in WT mice (H). In the WT CA1 field, Zfpn2-expressing cells are found in the deepest rows of the CP (I). (J–L) Nr4A2 (red) is undetectable in the PS of D6/Zbtb20S mice and in the WT CA1 field (L), but is expressed in deep-layer cells of the CP in WT (K). (M–M′) Magnified views of area marked by rectangle in (A). Cells expressing high levels of Sox5 express low levels of Zbtb20 (M, arrowheads). Scale bar: 150 μm in (A–L) and 20 μm in (M–M′).
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<sub>Gfp</sub>-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<sub>Gfp</sub> reporter gene localize to the stratum pyramidale (Fig. 8C,D). We did not observe dislocation of CA1 pyramidal cells when expressing Zbtb20-miR<sub>Gfp</sub> 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
stratum pyramidale. Although, the aberrant CA1 molecular phenotype was also revealed in projection neurons of granular retrosplenial areas, Zbtb20 transformed retrosplenial neurons neither received pronounced Schaffer collateral innervation nor did they express the Calbindin-D28k marker. Taken together, the results show that enlargement of the CA1 field is orchestrated by the activity of the transcriptional repressor Zbtb20. Arealization or parcellation of functionally distinct areas in the cerebral cortex is controlled by a regulatory hierarchy of diffusible factors or morphogens secreted from patterning centers in the dorsal telencephalon (O’Leary and Sahara 2008; Rakic 2009). These factors are thought to initiate a complex pattern of transcription factor expression within 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 modifications 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 Satb2 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; Fishe1 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 VI neurons (i.e., Thr1, Zfp52, 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 coexpresses 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 nonhippocampal 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 progenitors 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 expression 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 Zwmp2 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 Zwmp2 (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.ercor.oxfordjournals.org/.

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