Development 139, 3051-3062 (2012) doi:10.1242/dev.080002

Prox1 postmitotically defines dentate gyrus cells by specifying granule cell identity over CA3 pyramidal cell fate in the hippocampus

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SUMMARY

The brain is composed of diverse types of neurons that fulfill distinct roles in neuronal circuits, as manifested by the hippocampus, where pyramidal neurons and granule cells constitute functionally distinct domains: cornu ammonis (CA) and dentate gyrus (DG), respectively. Little is known about how these two types of neuron differentiate during hippocampal development, although a set of transcription factors that is expressed in progenitor cells is known to be required for the survival of granule cells. Here, we demonstrate in mice that Prox1, a transcription factor constitutively expressed in the granule cell lineage, postmitotically functions to specify DG granule cell identity. Postmitotic elimination of Prox1 caused immature DG neurons to lose the granule cell identity and in turn terminally differentiate into the pyramidal cell type manifesting CA3 neuronal identity. By contrast, Prox1 overexpression caused opposing effects on presumptive hippocampal pyramidal cells. These results indicate that the immature DG cell has the potential to become a granule cell or a pyramidal cell, and Prox1 defines the granule cell identity. This bi-potency is lost in mature DG cells, although Prox1 is still required for correct gene expression in DG granule cells. Thus, our data indicate that Prox1 acts as a postmitotic cell fate determinant for DG granule cells over the CA3 pyramidal cell fate and is crucial for maintenance of the granule cell identity throughout the life.

KEY WORDS: Prox1, Cell fate determination, Dentate gyrus granule cell, CA3 pyramidal cell, Mouse

INTRODUCTION

The hippocampus is a medial cortical brain structure that is required for memory formation and learned behaviors. It is composed of two major regions: the cornu ammonis (CA, subdivided into CA1, CA2 and CA3) and the dentate gyrus (DG). The CA and DG contain pyramidal and granule cells, respectively. The development of the hippocampus begins during the embryonic stages and continues through the postnatal stages (Altman and Bayer, 1990b; Altman and Bayer, 1990c; Altman and Bayer, 1990a). Neurogenesis persists in the adult DG; granule cells are continuously generated and integrated into existing neural circuits throughout adulthood (Alvarez-Buylla and Lim, 2004; Zhao et al., 2008). How the distinctive regions in the hippocampus are generated and what determines the different types of neurons are fundamental questions for the understanding of the development and function of the hippocampus.

The hippocampal primordium in rodents is formed in the dorsomedial telencephalon adjacent to the cortical hem region during the mid-embryonic stage (Altman and Bayer, 1990c; Altman and Bayer, 1990b). Future CA neurons born in this neuroepithelial region migrate radially and form the CA in the mid-zone of the cortex. The formation of the dentate gyrus is peculiar; postmitotic cells and progenitors, which are born in a region more proximal to the cortical hem, migrate away from the ventricular zone and are assembled into the DG primordium (Li et al., 2009). The progenitors in the neonatal DG continue to generate neurons, resulting in a substantial increase in the size of the DG during the first 2 weeks after birth (Altman and Bayer, 1990a; Li et al., 2009; Mathews et al., 2010). The adult DG progenitors that reside in the subgranular zone (SGZ) continuously produce granule cells. The maturation of granule cells in the DG proceeds for ~4 weeks, and is accompanied by changes in morphology and gene expression profiles (Piatti et al., 2006; Zhao et al., 2008). Eventually, the granule cells that are born from the embryonic stages to adulthood form the granular layer in an outside-in fashion, according to their birthdate (Angevine, 1965; Mathews et al., 2010).

Hippocampal development is known to involve transcriptional regulation that generally functions in neurogenesis. Transcription factors such as NeuroD (*Neurod1*), neurogenin 2 and Tbr2, are expressed in the hippocampal primordia. The loss of these factors induces massive apoptosis of DG granule cells, whereas CA regions are not seriously affected (Miyata et al., 1999; Arnold et al., 2008; Galichet et al., 2008). The specification of CA1 pyramidal cells requires the zinc-finger protein Zbtb20 (Nielsen et al., 2007; Xie et al., 2010). However, the molecular mechanisms that specify other neuronal lineages during hippocampal development remain elusive.

Prox1, a prospero-related homeobox gene (Oliver et al., 1993), is expressed in neuroepithelial cells adjacent to the cortical hem and in DG granule cells throughout embryonic development and into adulthood (Galeeva et al., 2007; Lavado and Oliver, 2007). Prox1 plays a crucial role in cell fate determination and in cell cycle

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Accepted 6 June 2012

regulation during the development of the lymphatic vascular system, lens and retina (Wigle et al., 1999; Wigle and Oliver, 1999; Dyer et al., 2003; Harvey et al., 2005). Recently, knockout (and knockdown) studies of the *Prox1* gene have been reported, in which *Prox1* was conditionally depleted by using Nestin-Cre; this resulted in the loss of Prox1 from progenitor cells and also throughout their postmitotic progeny, and revealed that Prox1 is essential for the survival of intermediate progenitors and for their descendent neurons in the DG (Lavado et al., 2010; Karalay et al., 2011). However, the roles that Prox1 plays in postmitotic neuronal cells of the DG during development and adulthood remain to be investigated. In this study, we specifically focused on the postmitotic function of Prox1 in the hippocampal development. To do so, we conditionally knocked out *Prox1*, using CaMK-CreERT2, which activated Cre only in a postmitotic stage of neuronal cells, and therefore allowed us to knockout *Prox1* in postmitotic neuronal cells at any appropriate timing of the hippocampal development. We demonstrate that the DG cell has cell fate plasticity between granule cell type and CA3 pyramidal cell type in the immature stage, and that Prox1 postmitotically specifies and maintains the granule cell identity over the CA3 pyramidal cell fate.

MATERIALS AND METHODS

Generation of conditional *Prox1* knockout mice

The targeting plasmid was introduced into the TT2 (Yagi et al., 1993) mouse ES cell line. The homologous ES cell recombinants were introduced into CD1 eight-cell stage embryos, and chimeric mice were obtained from two independent ES recombinants. The targeting vector for the *Prox1* gene was based on the conditional knockout and the simultaneous *EGFP* knock-in strategy, which was designed by H. Enomoto using loxP/Cre system (Uesaka et al., 2008). The *Prox1* conditional knockout mice (Accession Number CDB0482K: http://www.cdb.riken.jp/arg/mutant%20mice%20list.html) were maintained in a C57BL/6 strain.

Animals

The *CaMK-CreER^{T2}* line [Tg(Camk2a-Cre/ERT2)2/Gsc, #EM:02125] was obtained from the European Mouse Mutant Archive (EMMA). The *Camk2a-Cre* line [Tg(Camk2a-Cre)20/Kmik, #RBRC00254] was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT (Japan). The *Prox1*-heterozygous mice induced by *CaMK-CreER^{T2}* and *Camk2a-Cre* exhibited normal fertility and CNS development, indicating that half the expression of *Prox1* is sufficient for CNS development. This finding is in contrast to the consequences of *Prox1* haploinsufficiency in the lymphatic system (Wigle and Oliver, 1999). Therefore, the heterozygous mutant mice were used as controls in this study. Two independent lines of mutant mice exhibited the same phenotypes.

All animal manipulations were performed in accordance with the guidelines for animal experiments at the RIKEN Center for Developmental Biology.

Tamoxifen and BrdU administration

Tamoxifen (Sigma) was dissolved in peanut oil (at a final concentration of 10 mg/ml). The doses (5 mg tamoxifen/35 g body weight) were injected with 28-gauge needles subcutaneously into the backs of neonatal pups (P1-P8) or intraperitoneally in young and adult mice. For BrdU labeling in the adult mice, BrdU (0.8 mg/ml) was dissolved in the drinking water. After the dosing period, the animals were given fresh water.

Immunohistochemistry

The cryostat sections were permeabilized by heating in HistoVT One solution (Nacalai Tesque). The primary antibodies used were goat polyclonal anti-Prox1 (R&D Systems), anti-NeuroD, anti-Math2 (Santa Cruz), rabbit polyclonal anti-Prox1, anti-BLBP, anti-calbindin, anti-calretinin, anti-Sox2, anti-GAD67 (Chemicon), anti-cleaved caspase 3 (Cell Signaling), anti-Tbr1 (gift from Robert Hevner, University of Washington, WA), anti-HuB (Abcam), anti-Homer3 (Cosmo-bio), anti-ZnT3 (Synaptic System), anti-Stxbp6 (Sigma), mouse monoclonal anti-NeuN (Chemicon), guinea pig polyclonal anti-doublecortin (Chemicon) and chick polyclonal anti-EGFP (Aves). The secondary antibodies were IgG conjugated to Cy2, Cy3 and Cy5 (Jackson ImmunoResearch). Nuclei were counterstained with DAPI

In situ hybridization

In situ hybridization using digoxigenin-labeled antisense RNA probe was performed using standard methods (Roche Diagnosis). Temeplate cDNA for probes were amplified by PCR primers containing the bacteriophage T7, T3 or SP6 RNA polymerase promoter sequences.

Golgi staining

The FD Rapid GolgiStain Kit (FD NeuroTechnologies) was used for the Golgi-Cox impregnation. The stained blocks were sectioned (at 100 μm) with a vibratome (VT1000S, Leica).

DiI tracing and immunostaining of DiI-labeled slices

A small, narrow triangle was cut from a NeuroVue Red Dye filter (Polysciences) and placed into the hilus region of the DG extracted from the PFA-fixed brains after the excision of the DG crest. The tissues were incubated in fixative at room temperature for 1 week. The labeled specimens were then sectioned (at 100 μm) with a vibratome in a plane transverse to the septotemporal axis of the hippocampal formation.

In utero electroporation and expression plasmids

In utero electroporation was performed as previously described (Saito and Nakatsuji, 2001). The expression plasmids used were based on pCAGGS (Niwa et al., 1991). pCAGGS-mStrawberry (Clontech, modified) (0.5 μg/μl) and pCAGGS-3Flag-Prox1 (1.0 μg/μl) were used for persistent expression. For inducible expression in the primary culture, pCAGGS-mStrawberry (0.5 μg/μl), pCAGGS-loxP-polyA-loxP-3Flag-Prox1 (1.0 μg/μl) and pCAGGS-Mer-Cre-Mer (0.5 μg/μl) (Zhang et al., 1996) plasmids were used.

Primary culture and immunofluorescence

The Prox1 expression plasmids were electroporated into E14.5 embryos. After 24 hours, the dissociated hippocampal cells were plated onto PLL-coated culture dishes (BD). For inducible Prox1 expression, we co-electroporated embryos with the *Mer-Cre-Mer* plasmid and an inducible Prox1 expression plasmid, in which a floxed poly A sequence intervened and this construct produce no observable effect without Cre recombinase. On day 3 after the start of the in vitro culture, when most of the cells had differentiated, 4-hydroxytamoxifen in DMSO was added to the medium (final concentration: 300 nM). We did not observe defects with tamoxifen treatment alone and Mer-Cre-Mer plasmid in the primary culture. The neurons were maintained for 13 days (in the constitutive Prox1 expression conditions) or 10 days (in the postmitotic Prox1 expression conditions) and subjected to antibody staining. Photographs were obtained with an FV1000 confocal microscope (Olympus).

Quantifications

P-values were determined using Student's *t*-test using Prism 4 software (MDF, Japan).

RESULTS

Postmitotic *Prox1* knockout deprives the granule cell signature of gene expression in DG cells

Prox1 is persistently and strongly expressed in the DG granule cells of the postnatal and adult hippocampus (Fig. 1A). To determine the role of Prox1 in the postmitotic neurons of the DG, we generated conditional *Prox1* knockout mice using a Cre/loxP recombination system (supplementary material Fig. S1), in which the *Egfp* gene is expressed under the control the *Prox1* promoter upon knockout (Fig. 1B). The *Prox1* gene was conditionally deleted by Cre, which was selectively activated in postmitotic neurons under the control of the calcium/calmodulin-dependent protein kinase II α (*Camk2a*) promoter. Tamoxifen-inducible *CaMK-CreER^{T2}* (Erdmann et al.,

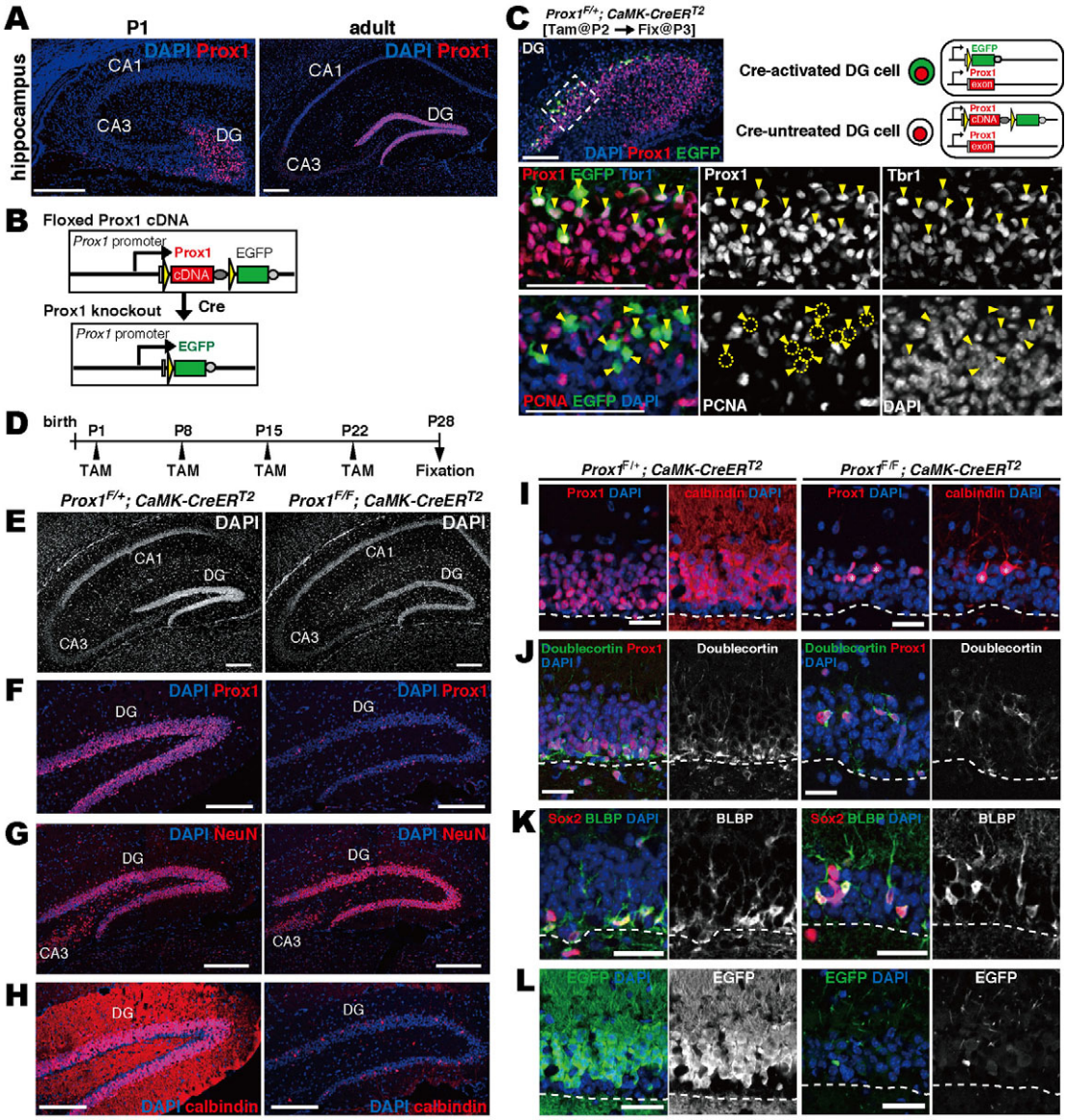

Fig. 1. Postmitotic knockout of *Prox1* results in the loss of granule cell gene expression in DG cells. (**A**) Immunostaining of the hippocampus from postnatal day 1 (P1) neonate (left) and adult mice (right) for Prox1 and DAPI. (**B**) A schematic diagram of the *Prox1* conditional knockout strategy. The full-length mouse *Prox1* cDNA (red box), flanked by loxP sequences (yellow triangles) and followed by the *EGFP* gene (green boxes), is integrated into the second exon of the *Prox1* gene (floxed *Prox1* cDNA). In this strain, Cre activation replaces *Prox1* with *EGFP* (*Prox1* knockout). (**C**) *Prox1*$^{F/+}$*; CaMK-CreER*^{T2} neonatal pups were administrated tamoxifen at P2 and analyzed at P3. The magnified DG regions were shown in the middle and lower panels, as indicated by the dotted box in the upper panel. DG cells were immunostained with EGFP, Prox1 and Tbr1 (the middle panels) and with PCNA (the lower panels). Arrowheads indicate the EGFP-positive cells in which Cre has been activated. (**D**) The experimental scheme for neonatal knockout of *Prox1* using CaMK-CreER^{T2}. (**E-H**) Staining for DAPI, Prox1, NeuN and calbindin in the hippocampus and the DG at P28. (**I-L**) Double immunostaining for calbindin and Prox1 (I), doublecortin and Prox1 (J), Sox2 and BLBP (K) and EGFP (L) in the granular layer. The asterisks in I indicate cells expressing both Prox1 and calbindin in homozygotes. The dotted lines indicate the inner periphery of the granular layer. Scale bars: 200 μm in A,E-H; 100 μm in C; 25 μm in I-L.

2007) was used to knock out the *Prox1* gene precisely during neonatal development. CaMK-CreER^{T2} can delete floxed *Prox1* gene in the Tbr1-positive postmitotic DG neurons but never in the PCNA- or Sox2-positive progenitors with the neonatal administration of tamoxifen (Fig. 1C; supplementary material Fig. S1C,D).

We weekly administrated tamoxifen to the *Prox1* conditional knockout mice (*Prox1*$^{F/+}$*; CaMK-CreER*^{T2} and *Prox1*$^{F/F}$*; CaMK-CreER*^{T2}) during neonatal development and analyzed these mice at P28 (Fig. 1D). The DG of the *Prox1*-heterozygous mice developed normally, and the DG granule cells expressed Prox1, NeuN (a pan-neuronal marker) and calbindin, a marker indicative of mature granule cells (Brandt et al., 2003) (Fig. 1E-H). The DG of the *Prox1*-homozygous mice also formed a V-shape and most of the DG cells had lost Prox1 expression (Fig. 1E,F; supplementary material Fig. S2A). Apoptosis was not significantly increased during the neonatal development (supplementary material Fig. S2B). This phenotype is in contrast to *Nestin-Cre*-driven *Prox1* knockout in progenitor cells,

which results in apoptosis and the eventual loss of DG cells (Lavado et al., 2010) (T.I., A.M., H.K., H.E. and F.M., unpublished). *Prox1*-deficient DG cells expressed NeuN but neither calbindin nor any of the markers of immature granule cells, including doublecortin, calretinin and NeuroD (Fig. 1G-J; supplementary material Fig. S2A,C). These results indicate that the postmitotic *Prox1*-deficient DG neurons had lost both mature and immature granule cell properties. There were some intact mature and immature granule cells that simultaneously expressed Prox1 with calbindin and doublecortin, respectively. They might be the cells that had escaped from Cre activation (Fig. 1I,J). However, DG progenitors, which expressed Sox2 and BLBP, did not significantly affected in terms of its population, but were intermingled in the granular layer of *Prox1*-homozygous mice, in contrast to their normal location in the SGZ of DG in *Prox1*-heterozygous mice (Fig. 1J,K; supplementary material Fig. S2A). This suggests that *Prox1*-deficent neurons affect the organization of the granular layer in a non-cell-autonomous manner.

EGFP is expressed from the conditional allele of the *Prox1*-heterozygous DG granule cells by the activation of Prox1 promoter (Fig. 1L). By contrast, the *Prox1*-deficient DG cells expressed faint or no EGFP. This indicates that the Prox1 promoter was no longer activated in *Prox1*-deficient DG cells. These observations raises the possibility that *Prox1*-deficient DG cells become a different type of neurons than granule cells in which *Prox1* promoter is activated (see also Fig. 4).

Postmitotic Prox1-deficient DG cells exhibit gene expression characteristic of the CA3 pyramidal cell type

We identified that postmitotic *Prox1* knockout deprived the granule cell property from the DG cells. Surprisingly, we found that the *Prox1*-deficient DG cells expressed the CA pyramidal cell markers HuB (expressed in CA2-3) (Okano and Darnell, 1997) and Math2 (expressed in CA1-3) (Bartholomä and Nave, 1994; Schwab et al., 2000) at high levels comparable with those of CA3 pyramidal cells (Fig. 2A-D). These cells also strongly upregulated another CA pyramidal cell marker, *Dkk3* (Diep et al., 2004), and two markers that are expressed at high levels in CA3: the kainate receptor subunit Ka1 (Grik4 – Mouse Genome Informatics) (Castillo et al., 1997) and the postsynaptic scaffolding protein Homer3 (Shiraishi et al., 2004) (Fig. 2E-G). When *Prox1* was knocked out using a non-inducible version of *Camk2a-Cre*, the size of the DG was reduced owing to apoptosis caused by leaky Cre expression in some of the proliferative cells (supplementary material Fig. S3). However, none of the cells escaped Cre activation and the subsequent loss of the *Prox1* gene. Consequently, the DG consisted completely of CA3 pyramidal cell-type neurons but no granule cells (supplementary material Fig. S3), in contrast to the case in which the *CaMK-CreER*^{T2} was used. We examined the expression of 18 genes that characterize hippocampal neurons (Table 1). The expression and levels of these markers in the *Prox1*-deficient DG cells were identical to those in the CA3 pyramidal cells but not the CA2/CA1-pyramidal cells or mossy cells. The mossy cells were present in the hilus of the early neonatal *Prox1* knockouts (*Prox1*$^{F/F}$*; Camk2a-Cre* and *Prox1*$^{F/F}$*; CaMK-CreER*^{T2}, data not shown; supplementary material Fig. S4). However, they were reduced at P28, probably owing to a reduction in the number of granule cells that form neural networks with mossy cells (Fig. 2B,E; supplementary material Fig. S3). Taken together, these data suggest that the postmitotic *Prox1* knockout results in a shift in the gene expression pattern of DG cells from that of granule cells to that of CA3 pyramidal cells.

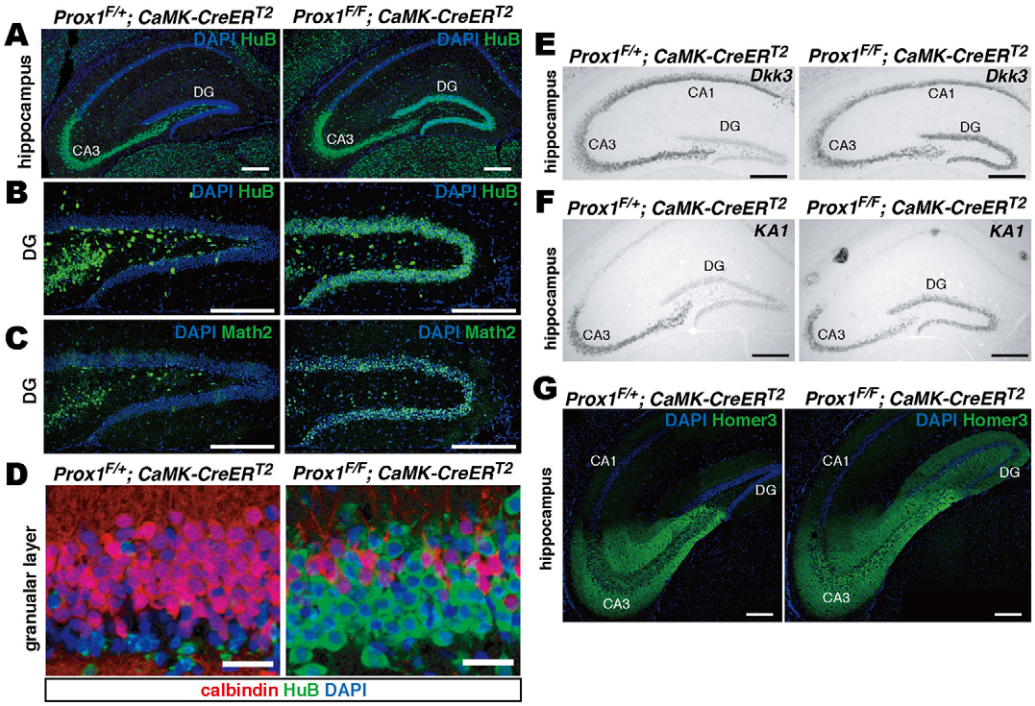

Fig. 2. Postmitotic Prox1 elimination converts granule cells into CA3 pyramidal cells in the neonatal DG. (**A-C**) Immunostaining of the hippocampus (A) and the DG (B,C) for HuB and Math2. (**D**) Double immunostaining for HuB and calbindin in the granular layer. (**E**,**F**) In situ hybridization of the *Dkk3* and *Ka1* transcripts. (**G**) Immunostaining for Homer3 is enriched in the dendrites of the CA3 pyramidal cells. Scale bars: 200 μm in A-C,E-G; 25 μm in D.

Prox1-deficient DG cells exhibit pyramidal cell-like morphology

We performed Golgi staining to determine whether *Prox1*-deficient cells also show characteristic pyramidal cell morphology. Normal granule cells exhibit monopolar dendrites, whereas pyramidal cells manifest bidirectional dendrites (Fig. 3A). Both types of neurons were observed in the DG granular layer of the *Prox1*-homozygous mice (Fig. 3B,C), indicating that a considerable proportion of the DG cells were transformed into pyramidal cells with dendrite morphology. In the DG of mice induced by Camk2a-Cre, most of the Golgi-stained cells were pyramidal in shape, consistent with the complete shift in gene expression from the granule to pyramidal cell type (supplementary material Fig. S5).

We assessed whether the pyramidal-shaped cells exhibited CA3-type gene expression in the *Prox1* knockout DG. To this end, we sparsely labeled the hippocampal cells with mStrawberry fluorescent protein using in utero electroporation. The *Prox1* mutant neonates (*Prox1*$^{F/+}$*; CaMK-CreER*^{T2} and *Prox1*$^{F/F}$*; CaMK-CreER*^{T2}) repeatedly received tamoxifen to delete the *Prox1* gene and were evaluated at P21 (Fig. 3D). In the heterozygous controls, the mStrawberry-labeled DG cells expressed no Math2 and exhibited granule cell morphology, as observed by Golgi staining (Fig. 3E,F). By contrast, the *Prox1*-deficient DG cells manifested both Math2 expression and pyramidal cell morphology, as shown by Golgi staining. These data suggest that the DG cells exhibiting the CA3 pyramidal cell gene expression pattern also manifested pyramidal-like dendrite morphology. Taken together, these results indicate that the postmitotic knockout of *Prox1* converts DG cells from a granule cell fate to a pyramidal-like cell fate in terms of both gene expression and cellular morphology.

Granule cells at late immature stages can adopt a pyramidal cell fate

Neurons in the DG granular layer pass through several developmental stages before becoming mature granule cells (Lledo et al., 2006). We examined the timing of *Prox1* knockout using CaMK-CreER^{T2} and cell fate conversion during the maturation of DG neurons. In the developing DG, the spatial pattern of gene expression is correlated with the degree of neuronal maturation; the immature neurons expressing NeuroD and calretinin are on the inside, and the mature granule cells expressing calbindin are on the outside in the granular layer (Brandt et al., 2003; Scobie et al., 2009) (Fig. 4A). We therefore activated CaMK-CreER^{T2} with tamoxifen in the heterozygous neonates (*Prox1*$^{F/+}$*; CaMK-CreER*^{T2}; Fig. 4B) and compared the spatial pattern of EGFP expression, a marker of *Prox1* gene excision (Fig. 1B), with that of NeuroD, calretinin and calbindin in the P10 granular layer (Fig. 4C-F).

We first determined the precise sequence of expression of these three markers during granule cell maturation at the neonatal stage. NeuroD expression persists throughout the immature period of granule cell differentiation, dropping to a low level at a very late immature stage. The drop in NeuroD expression overlaps with the onset of calbindin expression, but not with calretinin expression (Fig. 4A). Thus, the postmitotic maturation of DG granule cells is subdivided into three stages (Fig. 4O): the immature stage (NeuroD++, calretinin+, calbindin–), the end of the immature stage (NeuroD+, calretinin–, calbindin+) and the mature stage (NeuroD–, calretinin–, calbindin++). We then identified the onset of EGFP expression in *Prox1*-heterozygous mice during progression through these three stages. Whereas EGFP expression was undetectable in younger populations of Prox1-expressing granule cells (Fig. 4C), it was initially detected in a small population of cells that expressed low levels of both NeuroD and calbindin (arrowheads, Fig. 4D,E). EGFP expression increased as calbindin was upregulated in the mature granule cells (Fig. 4E). By contrast, the calretinin-positive immature cells never expressed EGFP (arrowheads, Fig. 4F). These observations indicate that EGFP expression is initiated in DG cells at the end of the immature stage. Interestingly, the cells at the end of the immature stage detected at the onset of EGFP expression tended to have spindle-shaped cell bodies (arrowheads, Fig. 4C-F), whereas the mature granule cells (strongly calbindin-positive) were round (Fig. 4E). The spindle-shaped immature cells expressed Math2 (Schwab et al., 2000) and calbindin at low levels (Fig. 4E,K). The

Table 1. Summary of gene expression profiles between *Prox1*-deficient DG cells induced by Camk2a-Cre and hippocampal neurons

Marker genes	Camk2a-Cre-induced Prox1-deficient cells in DG	DG granule cells	CA3 pyramidal cells	CA2 pyramidal cells	CA1 pyramidal cells	Hilar mossy cells
Prox1	–	+	–	–	–	–
Calbindin	–	+	–	–	+/–	–
Stbxp6	–	+	–	–	–	–
Dock10	–	+	–	–	–	–
Sipa1l2	–	+	–	–	–	–
C1ql2	–	+	–	–	–	–
ZnT3	–	+ (in axons)	–	–	–	–
HuB	–	–*	+	+	–	+
Math2	High	–*	High	High	High	High
Ka1	High	Low	High	Low	Low	Low
Dkk3	High	Low	High	High	High	High
PKCδ	+	–	+	–	–	+
Homer3	+ (in dendrites)	Low	+ (in dendrites)*	Low	Low	Low
Cacng5	–	–	–	+	–	–
SCIP	–	–	–	–	+	–
NeuroD	–	–*	–*	–	–	–*
Doublecortin	–	–*	–*	–	–	N.D.
Calretinin	–	–*	–	–	–	+
GluR2/3	High	Low	High	N.D.	N.D.	High

'+' or '–' indicates the presence or absence of gene expression, respectively. 'High' or 'low' indicates the level of gene expression. 'N.D.' indicates not determined. '+/–' indicates that some of the cells expressed the gene in the adult mice.
*Transient expression in immature neurons but not mature neurons.

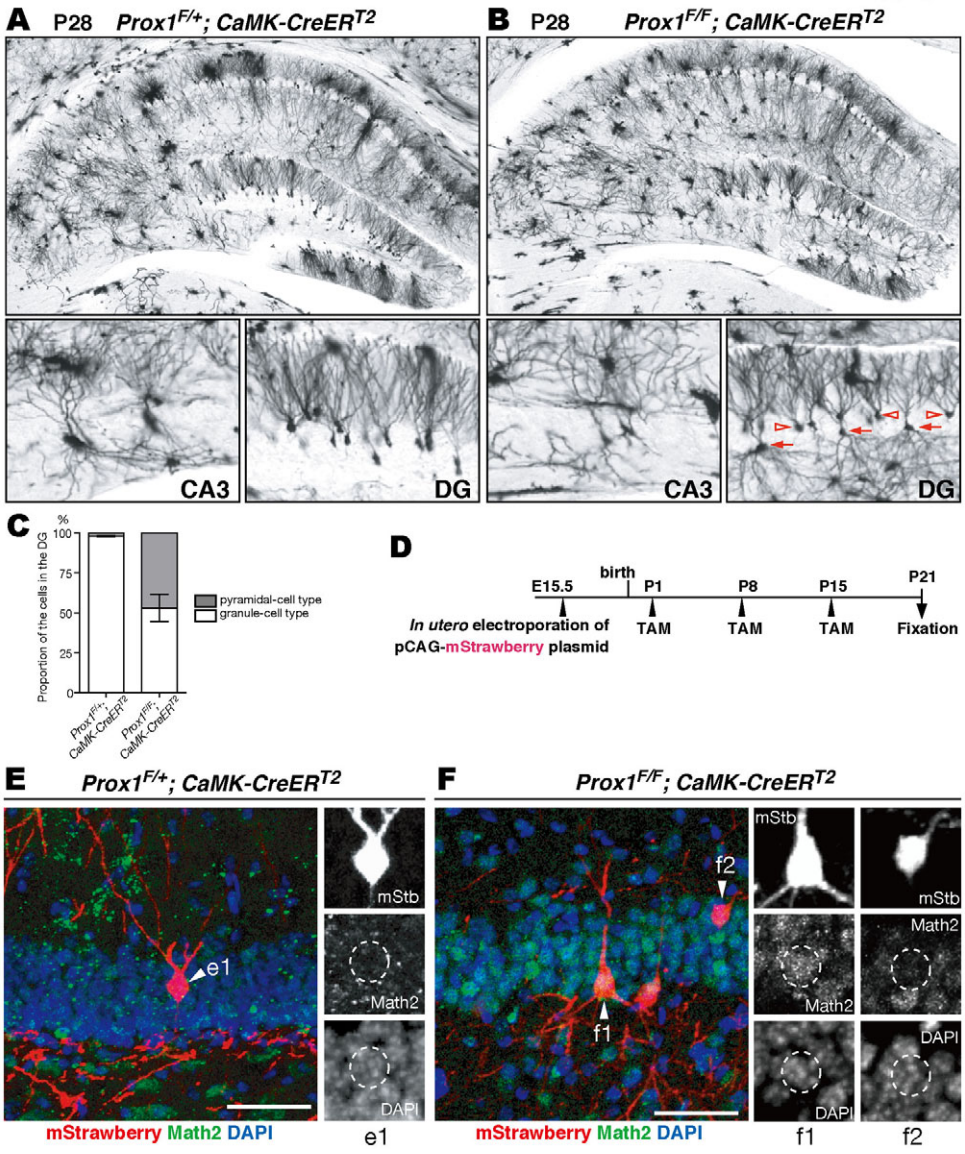

Fig. 3. Postmitotic *Prox1*-deficient DG cells exhibit pyramidal cell morphology. (**A**,**B**) Golgi staining reveals the cellular morphology of the neurons from the heterozygous controls (A) and homozygous mutants (B) at P28, after the induction of the *Prox1* knockout shown in Fig. 1D. Highly magnified images show parts of the CA3 and the DG regions. The arrows indicate cells with pyramidal morphology; the arrowheads indicate cells with granule morphology (see also Fig. 1H). (**C**) The proportion of the granule-type and pyramidal-type cells stained with Golgi's impregnation in the DG of *Prox1*$^{F/+}$*; CaMK-CreERT2* and *Prox1*$^{F/F}$*; CaMK-CreERT2* mice. Data are mean±s.d. (*n*=3 mice). (**D**) The experimental scheme for the in utero electroporation and *Prox1* knockout using CaMK-CreER^{T2}. (**E**,**F**) Math2-immunostained DG granule cell layers in the electroporated hippocampus of the heterozygous (E) and homozygous (F) mice. The individual fluorescent staining of the electroporated cells is presented separately on the right (e1, f1 and f2). The cell in f2 might be a remaining granule cell (see also Fig. 1I). The dotted circles outline the cell body. Scale bars: 50 μm.

combination of this gene expression pattern (no calretinin and low levels of NeuroD, calbindin and Math2) and cell morphology (spindle-shape) characterizes the transition of the DG neurons from the immature state to the mature state. The *Prox1* gene is depleted by CaMK-CreER^{T2} at this transition stage (Fig. 4O).

In homozygotes (*Prox1*$^{F/F}$*; CaMK-CreER*^{T2}), the cellular arrangement of the DG was disorganized. Immature neurons with high Prox1, NeuroD and calretinin expression were scattered throughout the granular layer (Fig. 4G,H,J). These cells did not express EGFP, indicating that CaMK-CreER^{T2} was not initially activated in those cells as it was in the heterozygous controls. EGFP were detected in cells that expressed little or no Prox1 (Fig. 4G), indicating a rapid decrease in Prox1 levels after knockout. Some of these EGFP-positive cells shared a characteristic gene expression pattern (low levels of NeuroD, calbindin and Math2 but no calretinin) and a spindle-shaped cell body (arrowheads, Fig. 4H-J,L). However, unlike the *Prox1* heterozygotes, 77.6±4.8% of the EGFP-positive cells in the *Prox1* homozygotes lost Prox1 and significantly upregulated Math2 and HuB expression to the levels of the CA3 pyramidal cells as the expression of EGFP decreased. Furthermore, the gene expression pattern in these cells eventually matched that of the CA3 pyramidal cell type (Fig. 4K-N). These observations suggest that switching from the granule to the CA3 pyramidal-type cell fate occurs soon after the *Prox1* knockout (Fig. 4P). Thus, the DG cells maintain the capacity to become either DG granule cells or CA3 pyramidal-like cells even at the late immature stage.

Prox1-deficient DG cells partially mimic the projections of CA3 pyramidal cells

We investigated the axonal projections of the *Prox1*-deficient DG cells that exhibited CA3 pyramidal cell gene expression patterns and morphology. In the hippocampus, the axonal collaterals of the CA3 pyramidal cells innervate the stratum radiatum (SR) and stratum oriens (SO) of CA1 and CA3, whereas the DG granule cells project mossy fibers to the apical dendrites of the CA3 cells in the stratum lucidum (SL) (Ishizuka et al., 1990; Henze et al., 2000) (Fig. 5A). When tamoxifen was administrated to the neonates at P1 and P8 (using a protocol similar to that in Fig. 4), we found that the axonal projections of the DG cells could be followed at P10 via EGFP expression that is initiated upon the excision of the *Prox1* gene (Fig. 5B). In the heterozygous controls (*Prox1*$^{F/+}$*; CaMK-CreER*^{T2}), the EGFP signals in the granule cells

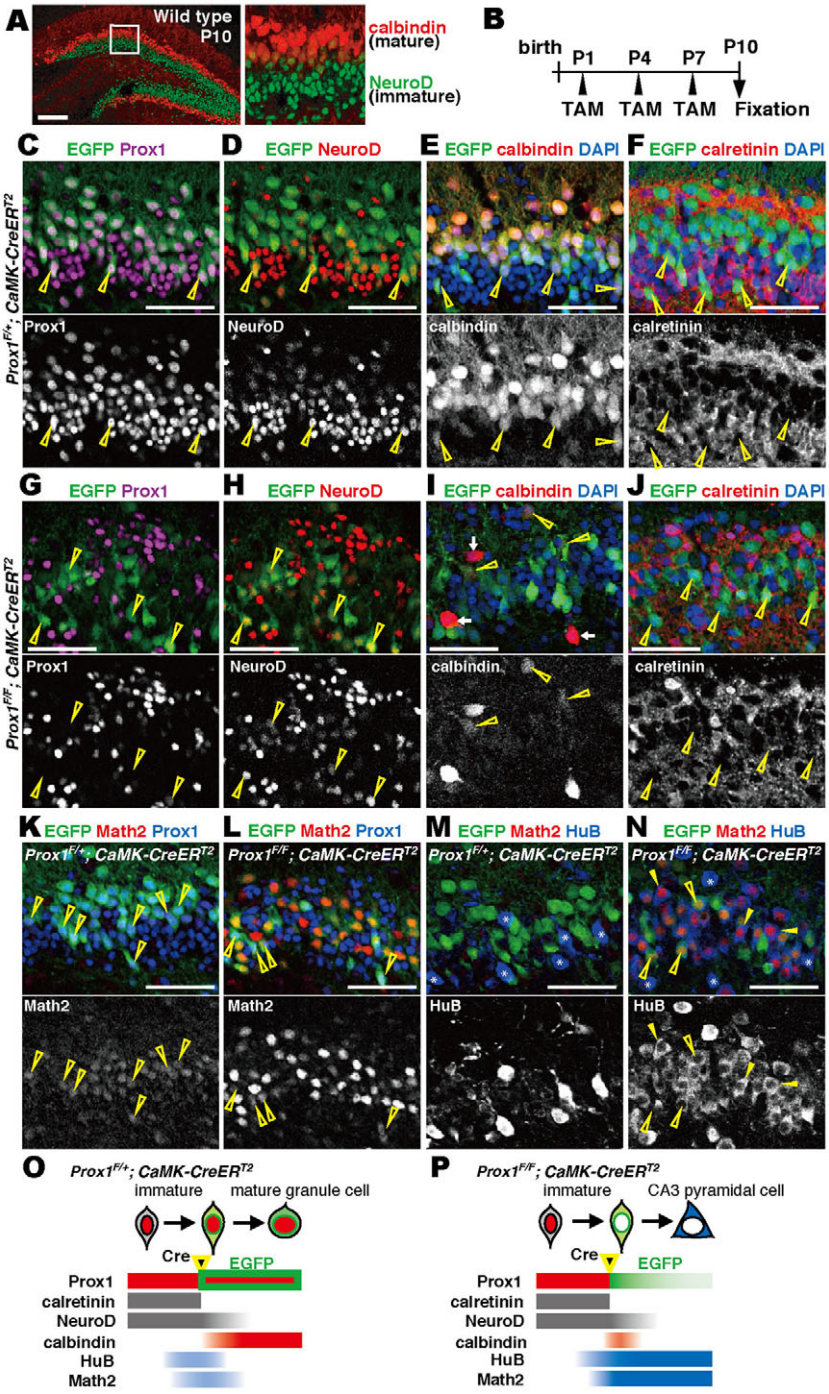

Fig. 4. Transformation of granule cells into pyramidal cells occurs just before terminal maturation and immediately after the loss of Prox1. (**A**) The maturation state of the granule cells is indicated by staining for calbindin and NeuroD in the wild-type DG at P10. The outlined granular layer is magnified on the right. (**B**) Experimental scheme for producing *Prox1*-knockout mice using CaMK-CreER^{T2} in early neonates. (**C-N**) Immunostaining for Prox1 (C,G,K,L, magenta or blue), NeuroD (D,H, red), calbindin (E,I, red), calretinin (F,J, red), Math2 (K-N, red) and HuB (M,N, blue) with EGFP (green) in the granular layer of the *Prox1* heterozygotes (C-F,K,M) and homozygotes (G-J,L,N). The open arrowheads indicate the cells at the end of the immature stage (see text). The solid arrowheads in N indicate the CA3-pyramidal-type cells that express HuB and Math2 strongly. The arrows in I indicate the mature granule cells that have escaped Cre. Asterisks in M,N indicate the interneurons. (**O**,**P**) The schemes deduced from the data shown in C-N show that the transition of gene expression and cell morphology during granule cell maturation is activated at the end of the immature stage (yellow arrowheads) upon the deletion of the *Prox1* gene in the heterozygous (O) and homozygous (P) mice containing CaMK-CreER^{T2}. In the *Prox1* homozygotes, switching from a granule cell fate to a CA3-pyramidal phenotype occurred soon after the *Prox1* depletion (P). Scale bars: 100 μm in A; 50 μm in C-N.

clearly highlighted the mossy fiber projections through the SL to CA3 (Fig. 5B). In the *Prox1*-homozygous neonates (*Prox1$^{F/F}$; CaMK-CreER^{T2}*), approximately half of the DG cells had lost Prox1 expression and retained weak EGFP signals under this condition (Fig. 4). Although reduced EGFP signals were still detected in the SL of CA3, we observed that the EGFP signals broadly extended into the SR and SO of the CA3 and CA1 regions (Fig. 5B). These fibers from the DG cells were never observed in the heterozygous controls, indicating that Prox1 is necessary for proper DG cell axonal projections.

We also investigated the axonal projections of the DG cells by directly labeling the neurons with pieces of DiI filter placed into the hilus region of the DG in the P10 neonates and the P95 adult mice. In the *Prox1*-heterozygous controls, these dye-labeled mossy fibers ran strictly within the SL of CA3 (Fig. 5C,E,F). In the *Prox1* homozygotes, the DG cells exhibited a broadly expanding pattern of axonal projections in the SR and SO of CA3, consistent with the observed EGFP signals. Furthermore, unlike in the *Prox1*-heterozygous mice, a region of the fibers from the DiI-labeled DG cells extended into the SR of the CA1 region (Fig. 5D,G,H). However, the DiI labeling did not highlight the invasion of the fibers into CA1 as clearly as the EGFP signals did. This difference might be partly because the DiI labeling was restricted to a slice of the hippocampus, whereas EGFP was expressed in the DG cells along the entire hippocampus. These observations suggest that the *Prox1*-deficient DG cells exhibited an axonal projection pattern that

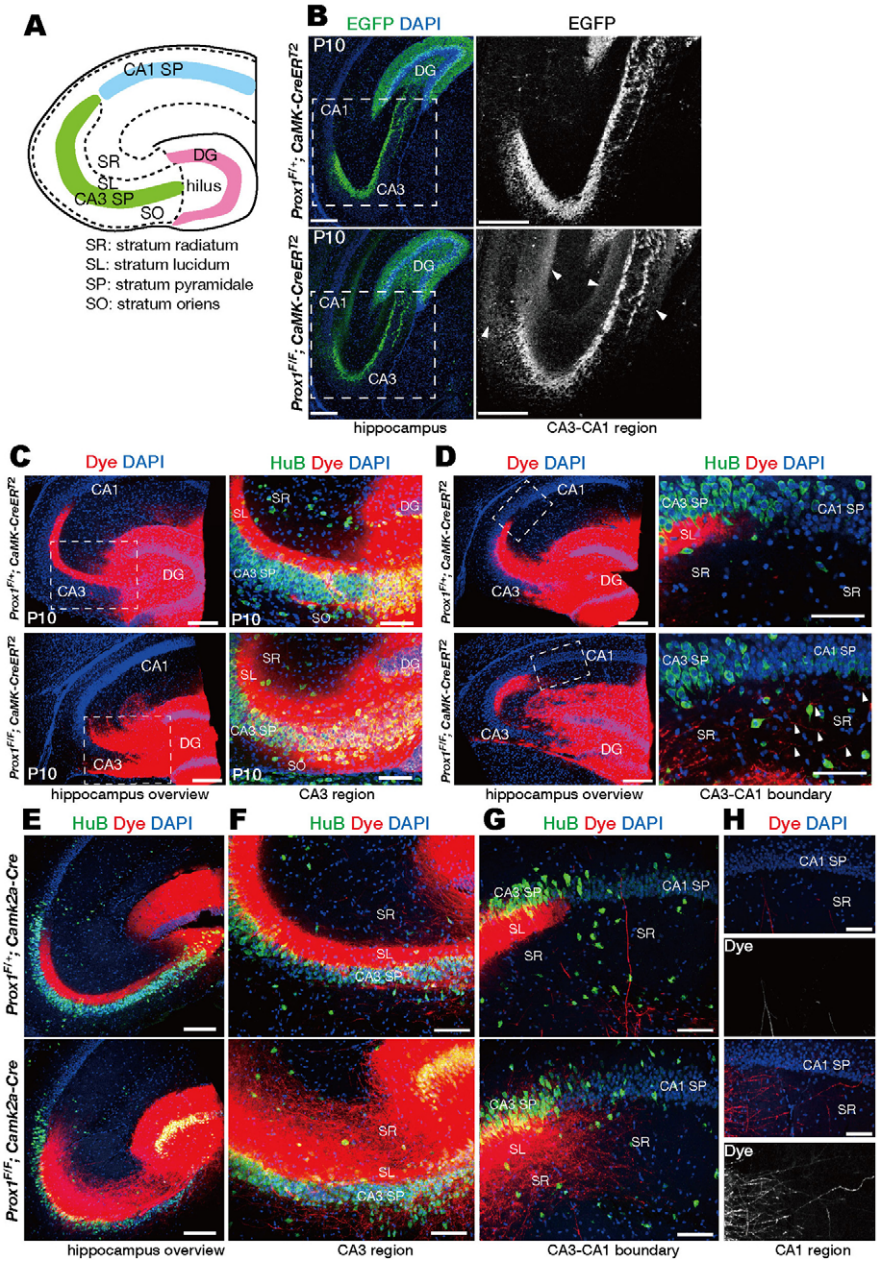

Fig. 5. *Prox1*-deficient DG cells manifesting the CA3 pyramidal cell phenotype project into CA1. (**A**) Hippocampal schematic. (**B**) The axons of the DG cells are labeled with EGFP after excision of the *Prox1* gene by CaMK-CreER^{T2}. In the homozygotes, EGFP signals were observed in the SR and SO of the CA3 and CA1 regions (arrowheads). (**C**,**D**) Hippocampus of dye-labeled slices obtained form the P10 neonates. HuB immunostaining (green) in dye-labeled sections reveals the CA3 pyramidal cell layer (CA3 SP). Dye-labeled axons in the CA3 region (C) and in the boundary between CA3 and CA1 (D) are shown in red. In the DiI-labeled slice of the *Prox1*-deficient DG cells, the projection to CA1 is minor compared with CA3 because the CA3 pyramidal fibers do not run parallel to the orthogonal plane of the hippocampus, and most of the labeled axons might have extended out of the slice. (**E**) Hippocampal overviews of the dye-labeled slices at P95 for the *Prox1* mutant mice generated by Camk2a-Cre. (**F-H**) Dye-labeled axons in the CA3 (F) and CA1 (G,H) are shown in red. Immunostaining for HuB (green) indicates the cell bodies of the CA3 pyramidal cells. Scale bars: 200 μm in B; 100 μm in C-H.

partially mimicked that of the CA3 pyramidal cells that targeted CA3 and CA1. Indeed, the giant presynaptic boutons of the granule cell mossy fibers were no longer observed on the CA3 pyramidal cells at P28, as revealed by staining for the DG granule cell synapse marker ZnT3 (Wenzel et al., 1997) (supplementary material Fig. S3L).

Prox1 overexpression favors granule cell differentiation over the pyramidal cell fate

We next examined whether Prox1 expression was sufficient to confer granule cell identity to presumptive pyramidal cells. We first tested this hypothesis in primary cultures of cells isolated from the hippocampal region of electroporated embryonic brains. When cells were cultured for 13 days after electroporation with the control plasmid, more than half of the electroporated cells expressed Math2 and HuB, and they exhibited a large cell body with thickly branched dendrites (Fig. 6A,C). The number of cells expressing calbindin was small. By contrast, in the cell cultures isolated from the embryos electroporated with the *Prox1* plasmid, more than half of the electroporated cells expressed calbindin and exhibited less branched and thinner dendrites compared with the Math2-positive cells in the controls (Fig. 6B). These cultures contained only a small population of Math2- or HuB-positive cells (Fig. 6C). These results suggest that the Prox1 overexpression promoted granule cell differentiation at the expense of the pyramidal phenotype. We also observed similar effects of the postmitotic expression of Prox1 in primary cell cultures (Fig. 6D) by using a combination of a plasmid expressing 4-hydroxytamoxifen-dependent Cre [a *Mer-Cre-Mer* (Zhang et al., 1996)] and a Cre-dependent Prox1 expression plasmid. These in vitro data indicate that postmitotic Prox1 expression efficiently diverts cells from pyramidal differentiation and favors expression of the granule phenotype.

We next tested the effects of exogenous Prox1 expression on CA3 and CA1 hippocampal pyramidal cell fate in vivo by in utero electroporation of a Prox1 expression plasmid into the

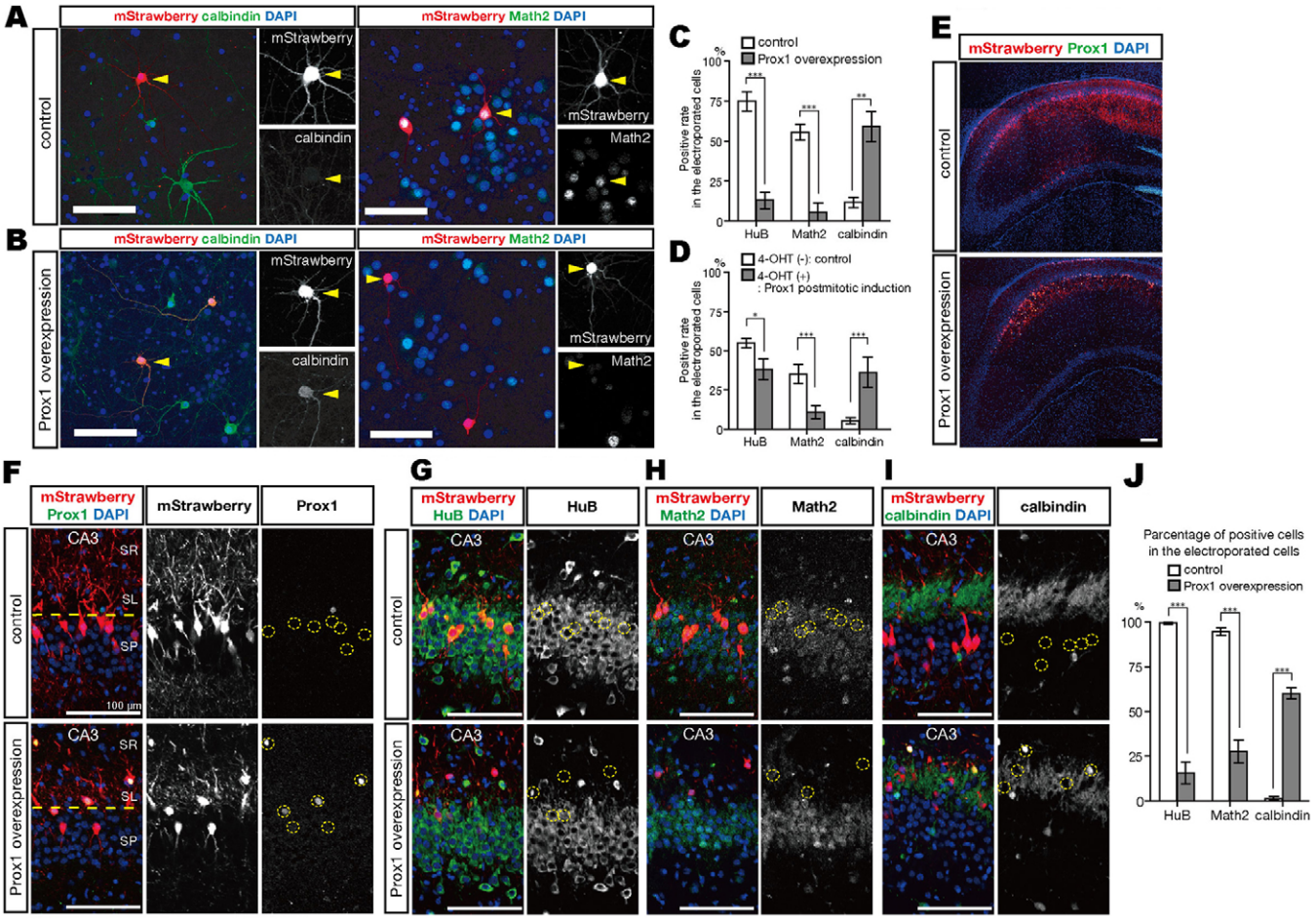

Fig. 6. Postmitotic expression of Prox1 is sufficient to specify the DG granule cell fate. (**A**,**B**) Immunostaining for calbindin (upper panels) and Math2 (lower panels) with mStrawberry fluorescence in cultured cells. The individual fluorescent channels for the cells indicated by arrowheads are separately presented on the right. (**C**,**D**) The percentage of calbindin-, Math2- or HuB-positive cells among the electroporated cells, plotted according to the scheme for constitutive expression (C) and postmitotic induction (D) of Prox1. Data are mean±s.d. (n=3 experiments). *P<0.05, **P<0.01, ***P<0.001. (**E**) The distribution of control mStrawberry- or Prox1-overexpressed cells in the hippocampus of P10 pups, which had been electroporated to overexpress the control or Prox1 on E15.5. (**F**) The distribution of the electroporated cells in the CA3 immunostained for Prox1. The dotted lines indicate the border between SP and SL. The dotted circles represent the electroporated cells. (**G-I**) Immunostaining for HuB (G), Math2 (H) and calbindin (I) in the CA3. (**J**) The percentages of HuB-, Math2- or calbindin-positive cells among the electroporated cells in the CA3 are plotted. Data are mean±s.d. (n=4 mice). ***P<0.001. Scale bars: 100 μm.

hippocampal primordium. Under our experimental condition, we observed the CA1 region was electroporated more efficiently than the CA3 region (Fig. 6E). Two weeks after electroporation, all control cells in CA3 were located in the stratum pyramidale (SP) (Fig. 6F). By contrast, 82.8±8.3% of the cells that were electroporated with the *Prox1* plasmid sustained the expression of Prox1 protein, and 63.5±17.2% of them were scattered into the SL and SR, suggesting that Prox1 expression affects neuronal migration. Most of the control electroporated cells expressed HuB and Math2 but not calbindin (Fig. 6G-J). By contrast, 60.0±3.1% of the Prox1-electroporated cells expressed calbindin, but those cells greatly reduced the expression of HuB and Math2. A similar effect of Prox1 overexpression was observed in the CA1 (supplementary material Fig. S6). The overexpression of Prox1 caused the expression of granule cell markers, calbindin, C1ql2 and Dock10 in 50.2±8.4% of the electroporated CA1 cells, and decreased the proportion of Math2-expressing cells (24.8±7.2%). Taken together, these data suggest that Prox1 is capable of inducing the granule cell phenotype in cells that would otherwise have manifested a hippocampal pyramidal cell phenotype in vivo.

Prox1 is a cell fate determinant for adult-born granule cells

We examined how Prox1 affects neurogenesis in the adult DG. Adult-generated cells in 6-week-old floxed *Prox1* mice were labeled with 5-bromo 2-deoxyuridine (BrdU), and Prox1 expression was depleted by CaMK-CreER^{T2} following the administration of tamoxifen (Fig. 7A). In the *Prox1* heterozygotes, BrdU-labeled neurons were distributed in the granular layer and expressed EGFP at 12 weeks of age. However, BrdU-labeled *Prox1*-homozygous mutant cells showed little or no expression of EGFP and calbindin, but expressed HuB and Math2 more strongly than the surrounding cells (Fig. 7B,C; supplementary material Fig. S7). These cells were different from the HuB-positive interneurons that expressed

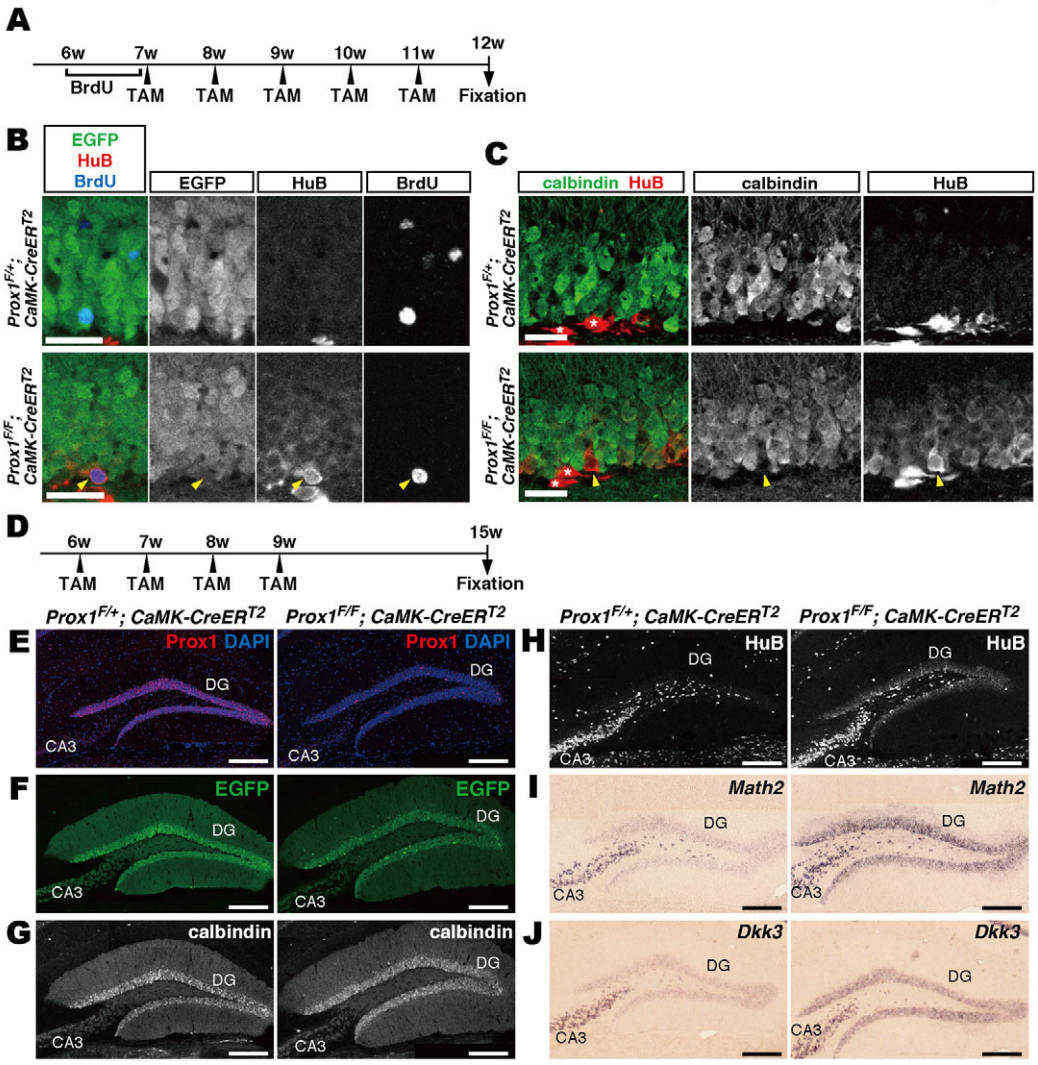

Fig. 7. Loss of Prox1 in mature granule cells partially transforms DG cells into the CA3 pyramidal cell type. (**A**) The experimental scheme for labeling adult-generated cells. (**B**,**C**) Sections from 12-week-old-mice were stained for HuB, EGFP and BrdU (B), and HuB and calbindin (C); the DG granular layers are presented. The arrowheads indicate the adult-generated cells that express HuB but not EGFP and calbindin. The cells showing strong HuB expression (asterisks) are interneurons (see also supplementary material Fig. S7). (**D**) The experimental scheme for *Prox1* knockout in the adult mice. (**E-J**) Immunostaining (E-H) and in situ hybridization (I,J) of the DG of *Prox1* mutant mice for Prox1 (E), EGFP (F), calbindin (G), HuB (H), *Math2* (I) and *Dkk3* (J). Scale bars: 25 μm in B,C; 200 μm in E-J.

GAD67 but not Math2 (supplementary material Fig. S7). These results suggest that the adult-born neurons acquired the CA3 pyramidal type gene expression pattern after the postmitotic *Prox1* knockout, as observed in the neonatal mice (Figs 1-4).

Prox1 knockout in mature granule cells causes an incomplete change in the gene expression profile

Because all of the DG granule cells continuously expressed Prox1 in the adult mice, we finally addressed the role of Prox1 in the mature granule cells of the adult DG. To deplete *Prox1* in the mature granule cells, tamoxifen was administered during postnatal weeks 6-9 to the *Prox1* floxed mice with *CaMK-CreER*^{T2} (Fig. 7D). This procedure resulted in a knockout of *Prox1* in most of the mature granule cells at 15 weeks of age (Fig. 7E). The *Prox1*-deficient DG cells in the homozygotes retained calbindin and EGFP expression at levels comparable with those of the heterozygotes (Fig. 7F,G), and Golgi staining showed a granule cell morphology for the DG cells (data not shown). However, these cells also expressed the pyramidal cell markers HuB, *Math2* and *Dkk3* (Fig. 7H-J), suggesting that *Prox1* knockout in mature DG cells results in a mixed granule cell and pyramidal cell gene expression profile. Thus, our analysis of the adult DG indicates that normal granule cell identity is guaranteed by persistent Prox1 expression but that mature granule cells in the DG lose the ability to assume the CA3 pyramidal phenotype following the loss of Prox1 expression. Interestingly, following the nearly uniform loss of Prox1 expression in the mature granule cells, the expression levels of CA3 pyramidal markers gradually decreased along the inner-outer axis in the granular layer (Fig. 7B,C,H; supplementary material Fig. S7). This progressive restriction in the flexibility of gene expression appears to correlate with the oldness of granule cells, as the more aged cells are located at more outer positions in the granular layer.

DISCUSSION

Prox1 is a postmitotic cell fate determinant of DG neurons

This study focused on the postmitotic role of Prox1 in the cell fate specification of DG granule cells in the hippocampus. *Prox1* knockout and knockdown in progenitor cells have shown that Prox1 is required for maintenance of intermediate progenitors and for postmitotic cell survival in the embryonic and adult hippocampus (Lavado et al., 2010; Karalay et al., 2011) (T.I., A.M., H.K., H.E. and F.M., unpublished). By contrast, our study revealed that the DG shows a novel phenotype when *Prox1* is postmitotically knocked out during neonatal development. When CaMK-CreER^{T2} was used to knock out *Prox1*, the DG cells survived and manifested a phenotype indistinguishable from the CA3 pyramidal cells, at least in part, in terms of gene expression patterns, dendrite morphology and axonal projections into the CA1 region. These *Prox1*-deficient DG neurons did not appear to be immature for the following reasons: they expressed no immature neuronal markers, they did express Homer3 (a dendritic component present in functional CA3 post-synapses) and they were stable throughout adulthood for at least one year (data not shown). The *Prox1*-deficient DG cells were also distinct in gene expression patterns from the DG granule cells, CA1 pyramidal cells, CA2 pyramidal cells, mossy cells (Table 1) and basket cells (interneurons in the DG) that express interneuron markers (GABA, GAD67 and parvalbumin, data not shown). Therefore, we conclude that the DG cells assume the CA3 pyramidal cell fate when deprived of Prox1 activity during the process of neuronal maturation. Conversely, the postmitotic overexpression of Prox1 in the hippocampal primordium increased the number of granule cell-type neurons at the expense of pyramidal-type neurons. This dependency of the two neuronal types on Prox1 is consistent with the notion that Prox1 is a postmitotic determinant of the two alternative cell fates. Our data thus demonstrate that Prox1 is not only a necessary component for neuronal survival and maturation, but also a postmitotic cell fate determinant that selects the DG granule cell fate over the CA3 pyramidal cell identity.

Several transcription factors act as postmitotic cell fate determinants for the neocortical pyramidal neuron subtypes (Britanova et al., 2008; Chen et al., 2008; Joshi et al., 2008). Satb2 determines the postmitotic subtype of corticospinal and cortico-cortical neurons, and Bhlhb5 postmitotically regulates the area identities of Layers II-V. Consistent with previous reports, our study indicates that postmitotic determination is a general mechanism for neuronal cell-type specification in the brain.

Morphogenesis of DG and Prox1 function in the granule cells

Our data indicate that cellular arrangement within the DG granular layer requires Prox1; postmitotic Prox1 depletion by *CaMK-CreER^{T2}* disrupts normal cell alignment and Prox1-overexpressing cells migrate abnormally in the hippocampal pyramidal layers (Figs 1, 4 and 6). This might be due to the Prox1-dependent conversion of neuronal type between the granule cell and the pyramidal cell, which forms the layer in an outside-in and inside-out manner, respectively (Angevine, 1965). Furthermore, the SGZ was disorganized in the DG of the *Prox1*-homozygous mice in spite of the postmitotic knockout of *Prox1*. This suggests that the mature granule cells direct the proper layer formation of the DG cells, including the SGZ progenitors.

Specification of hippocampal neuronal identity

It has been shown that the CA1 domain in the hippocampus requires the determinant Zbtb20 (Nielsen et al., 2007; Xie et al., 2010). This protein is expressed throughout the CA region and the DG but is necessary only for the development of CA1 pyramidal cells. Similarly, Prox1 is expressed throughout the hippocampal epithelium but does not affect from CA1 to CA3. These facts raise the possibility that there is a determinant for CA3 pyramidal cells that is expressed from CA3 to DG. Continuous Prox1 expression then confers granule cell identity on cells that would otherwise adopt a CA3 pyramidal cell fate in the DG. Alternatively, we can also suppose a determinant that expresses specifically in the CA3 pyramidal cells and acts in the mutual repressive manner against the activity of Prox1 for DG granule cells.

What causes continuous Prox1 expression in presumptive DG cells? Wnt signaling from the cortical hem, a known morphogen of hippocampal development (Lee et al., 2000; Mangale et al., 2008), is likely to play a crucial role in this process. Wnt signaling postmitotically regulates the expression of Prox1 and NeuroD during adult neurogenesis (Kuwabara et al., 2009; Karalay et al., 2011). Likewise, we observed that Prox1 expression depends on Wnt signaling in immature cells migrating towards the DG primordium (T.I., A.M., H.K., H.E. and F.M., unpublished). Given that immature DG neurons (Prox1 and NeuroD double positive) retain plasticity between the CA3 pyramidal and DG granule cell fates, Wnt signaling from the cortical hem may act as an instructive signal to sort the cell fates of postmitotic cells migrating toward the presumptive DG or CA3 (Nakahira and Yuasa, 2005).

Neuronal phenotype plasticity in postmitotic DG cells

In the hippocampus, the maturation of granule cells takes ~10 days during neonatal development or 3 weeks during adult neurogenesis (Overstreet-Wadiche et al., 2006). Our study found that the neuronal identity of DG cells was bi-potent during the late stages of these extended periods. By contrast, mature granule cells in the adult DG do not alter their identity upon Prox1 depletion, as previously suggested (Karalay et al., 2011). However, our results demonstrate that the loss of Prox1 in mature DG cells results in a mixed pattern of both DG granule cell and CA3 pyramidal cell gene expression. This ability of mature granule cells to alter gene expression becomes gradually restricted, depending on their age, and might be coupled to the integration of mature granule cells into neuronal circuits (Zhao et al., 2008). The molecular relationship between neuronal activity and the maintenance of a neuronal identity in DG granule cells is an interesting problem that should be addressed in future studies.

Acknowledgements

We thank K. Nakao (RIKEN CDB) for providing the targeting mice; K. Mikoshiba (RIKEN BSI) for providing the *Camk2a-Cre* mice; G. Schütz (DKFZ, Germany) for providing the *CaMK-CreER^{T2}* mice; Y. Shinkai (Kyoto University) for providing the pCAG-Mer-Cre-Mer plasmid; T. Seki (Tokyo Medical University, Japan), T. Namba (Nagoya University, Japan), N. Yamamoto (Osaka University, Japan), T. Hisatsune (The University of Tokyo, Japan) and G. Fishell (NYU School of Medicine) for helpful discussions and technical advice; S. Wakatsuki (National Institute of Neuroscience, Tokyo) and A. Kawaguchi (Nagoya University) for technical advice; and the members of the Matsuzaki laboratory for helpful discussions.

Funding

This work is partly supported by CREST Japanese Science and Technology Agency, Grants-in-Aid for Scientific Research of MEXT (to F.M.). Deposited in PMC for immediate release.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.080002/-/DC1

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