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Neurogenin 2 has an essential role in development of the dentate gyrus

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The dentate gyrus (DG) of the hippocampus has a central role in learning and memory in adult rodents. The DG is generated soon after birth, although new neurons continue to be generated in the DG throughout life. The proneural factors Mash1 (Ascl1) and neurogenin 2 (Ngn2) are expressed during formation of the DG but their role in the development of this structure has not yet been addressed. Here, we show that *Ngn2* is essential for the development of the DG. *Ngn2* mutant mice have fewer DG progenitors and these cells present defects in neuronal differentiation. By contrast, the DG is normal in *Mash1* mutant mice at birth, and loss of both *Mash1* and *Ngn2* does not aggravate the defect observed in *Ngn2* single mutants. These data establish a unique role of *Ngn2* in DG neurogenesis during development and raise the possibility that *Ngn2* has a similar function in adult neurogenesis.

KEY WORDS: bHLH, Proneural, Dentate gyrus, Hippocampus, Mouse

INTRODUCTION

The dentate gyrus (DG) is, with the olfactory bulb, one of two regions of the mammalian brain where new neurons are added to existing neural circuits throughout adulthood (Altman, 1962; Altman and Das, 1965; Kempermann et al., 1997a; Kempermann et al., 1997b). Neurogenesis in the adult DG has been observed in a variety of species, including primates and humans (Eriksson et al., 1998; Gould et al., 1999a; Gould et al., 1998; Kempermann and Gage, 1998; Kuhn et al., 1996). The DG is the primary afferent pathway into the hippocampus and it has an important role in learning and memory. Maintenance of neurogenesis in the adult DG has been suggested to play a role in the acquisition of new memories (Aimone et al., 2006; Gould et al., 1999b; Lemaire et al., 2000).

Formation of the DG begins in mice at around E15 in the dorsomedial part of the telencephalic vesicles. The DG primordium is initially populated by Cajal-Retzius cells and radial glial cells that are likely to participate to its histogenesis (Alcantara et al., 1998; Borrell et al., 1999; Del Rio et al., 1997; Rickmann et al., 1987). The portion of hippocampal neuroepithelium that constitutes the DG primordium, also called primary matrix, contains stem/progenitor cells that give rise, starting at E15.5, to a stream of migratory progenitors and postmitotic neurons that has been called the secondary matrix. At the end of their migration, progenitor cells of the secondary matrix accumulate in the tertiary matrix, located in the hilar region of the hippocampus, where they give rise, from ~E17 onwards, to the granule neurons of the DG, which are organized in a V-shaped laminar structure (Altman and Bayer, 1990; Cowan et al., 1980). Although the secondary matrix starts to disappear by P5, progenitors from the tertiary matrix persist throughout life in a region located at the interface between the hilus of the hippocampus and the granular cell layer, termed the subgranular layer (SGL) (Altman and Bayer, 1990; Altman and Das, 1965). These progenitors produce throughout adulthood new granule neurons that have the same electrophysiological properties than neurons generated during embryonic and early postnatal development (Laplagne et al., 2006). Whether adult hippocampal stem cells reside in the SGL of the DG (Ming and Song, 2005) or near the lateral ventricle of the HP (Seaberg and van der Kooy, 2002) is a matter for debate.

The molecules that control the development of the DG and particularly determine cell fates in this structure remain poorly characterized. A range of defects in formation of the hippocampus has been observed in mice in which the Wnt signalling pathway is disrupted. *Wnt3a* mutants present a deletion of the whole hippocampus (Lee et al., 2000), while *Lef1* mutants lack most of the DG (Galceran et al., 2000) and *LRP6* mutants have a reduced number of DG progenitors and granule neurons (Zhou et al., 2004). Wnt signalling acts in part by promoting expression of the homeodomain protein Emx2, which is required for growth of the hippocampus and for migration of DG progenitors (Backman et al., 2005; Oldekamp et al., 2004; Pellegrini et al., 1996; Theil et al., 2002; Tole et al., 2000). Transcription factors of the basic helix-loop-helix (bHLH) class, including Neurod1 and NEX/Math2 (Neurod6 – Mouse Genome Informatics), are mainly expressed in postmitotic granule cells and have been implicated in late stages of differentiation of dentate granule neurons (Liu et al., 2000; Miyata et al., 1999; Pleasure et al., 2000; Schwab et al., 2000). By contrast, little is known of the transcription factors regulating early stages of neurogenesis in the DG and particularly the generation and initial differentiation of the different populations of progenitors involved in development of the DG.

Proneural bHLH proteins control the generation of progenitor cells and their progression through the neurogenic programme throughout the nervous system (Bertrand et al., 2002). Expression of the proneural protein Mash1 (Ascl1 – Mouse Genome Informatics) has been reported in DG progenitors at embryonic and postnatal stages (Pleasure et al., 2000), but its role in formation of the DG has not been assessed. The proneural protein neurogenin 2 (Ngn2) plays an essential role in neurogenesis in the dorsal telencephalon, where it has been shown to commit multipotent progenitors to the neuronal fate and inhibit astrocytic differentiation. Ngn2 also activates a cortical-specific differentiation programme that includes expression of transcription factors such as Neurod1 and NEX/Math2, resulting in

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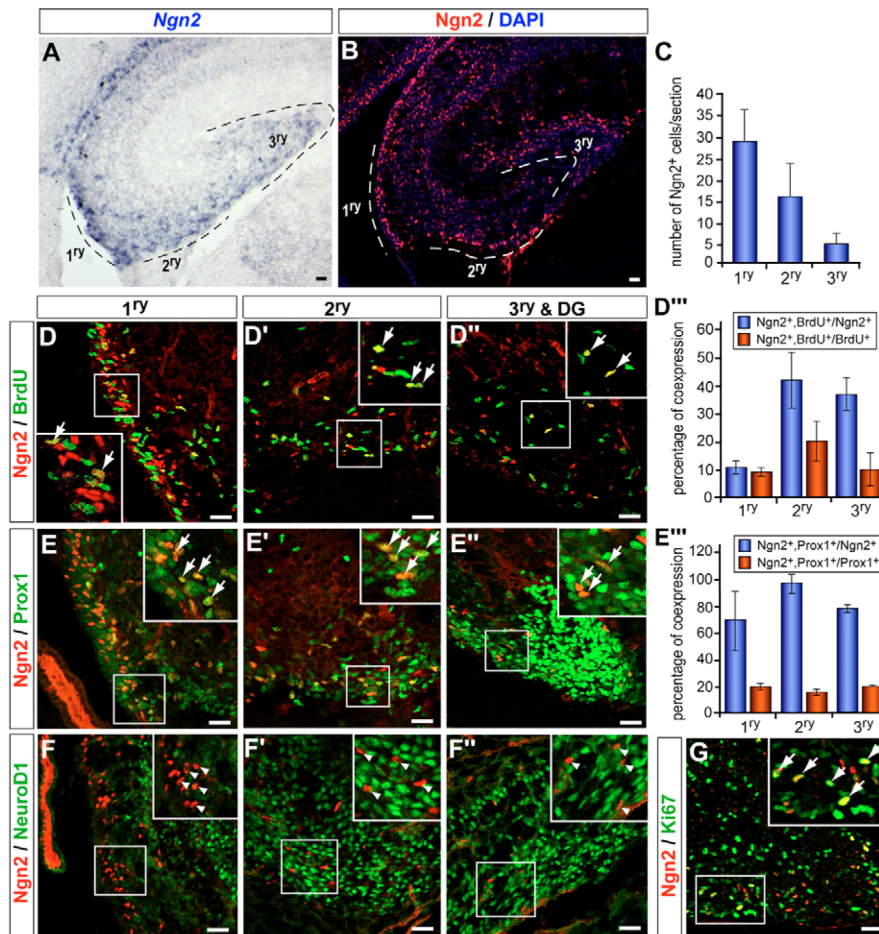

Fig. 1. Ngn2 is expressed in the different progenitor populations of the developing dentate gyrus. (**A**,**B**) Sections through the hippocampus of E18.5 wild-type embryos showing the expression of *Ngn2* transcripts by in situ hybridization (A) and of Ngn2 protein by immunohistochemistry (B). The different progenitor populations of the developing DG (1ry, primary matrix; 2ry, secondary matrix; 3ry, tertiary matrix) are outlined. (**C**) Histograms of the number of Ngn2-expressing cells in the different progenitor populations of the DG. (**D-G**) Sections through the hippocampus of E18.5 brains double-labelled for Ngn2 and BrdU following a 30 minute pulse (D-D″), Prox1 (E-E″), Neurod1 (F-F″) and Ki67 (G). Pictures were taken at the level of the primary matrix (D,E,F), secondary matrix (D′,E′,F′) or tertiary matrix (D″,E″,F″,G). Arrows indicate double-labelled cells, while arrowheads indicate Ngn2-expressing cells only. (D‴) Histogram representing the percentage of Ngn2-expressing cells having incorporated BrdU (blue bars) and the percentage of BrdU-labelled cells expressing Ngn2 (red bars). (E‴) Histogram representing the percentage of Ngn2^{+} cells expressing Prox1 at a low level (blue bars) and the percentage of Prox1^{low} cells expressing Ngn2 (red bars). Ngn2 is expressed only in progenitors expressing Prox1 at a low level and not in post-mitotic neurons expressing Neurod1 and Prox1 at a high level. Scale bars: 20 μm.

acquisition of a glutamatergic neurotransmission phenotype and pyramidal neuronal morphology (Hand et al., 2005; Nieto et al., 2001; Schuurmans et al., 2004). *Ngn2* is expressed in the developing DG (Pleasure et al., 2000) but its function in development of this structure has not yet been addressed.

In this paper, we have examined the role of Ngn2 in formation of the DG. We show that Ngn2 is expressed in the different populations of DG progenitors, and that Ngn2-expressing progenitors generate most or all dentate granule cells. Elimination of *Ngn2* function results in the loss of a large fraction of dentate granule cells and in a severe defect in DG morphogenesis. This granule cell defect reflects a unique role for *Ngn2* in the developing DG.

MATERIALS AND METHODS

Mouse breeding and genotyping

Ngn2^{KIGFP} and *Mash1*$^{\Delta}$ mice were genotyped as described by Seibt et al. (Seibt et al., 2003) and Casarosa et al. (Casarosa et al., 1999). Both lines were maintained in an outbred MF1 background. Wild-type, heterozygous and homozygous *Ngn2*^{KIGFP} mutant mice were obtained from intercrosses of *Ngn2*$^{GFP/+}$ mice. The morning of the day on which the vaginal plug was observed was termed E0.5; the day of birth was termed P0. *Mash1::GFP* mice where identified at birth by GFP expression under a UV microscope. All experiments were carried out in accordance with the UK (Scientific Procedures) Animal Act 1986.

RNA in situ hybridization

Embryonic and postnatal brains were dissected out of the skull and fixed at 4°C in paraformaldehyde (4%) overnight. Brains were then rinsed in phosphate-buffered saline (PBS), cryoprotected overnight in 20% sucrose in PBS, embedded in OCT (BDH, UK), and sectioned on a cryostat at 10 μm. RNA in situ hybridization was performed as described by Cau et al. (Cau et al., 1997). The RNA probes used in this study were the following: *Ngn1*, *Ngn2*, *Ngn3*, *Mash1* and *Neurod1* (Cau et al., 1997); *Prox1* (Torii et al., 1999).

Immunohistochemistry

Brains were dissected as mentioned above and fixed at 4°C in paraformaldehyde (4%) for 30 minutes then cut thought the midline in half and fixed in the same solution for another 30 minutes. Antigen retrieval for Ki67 antibody staining was performed by heating sections in PBS at 65°C for 5 minutes. Sections were incubated in a blocking solution (PBS plus 10% normal goat serum (Vector Laboratories) and 0.1% Tween20 or Triton X-100) and then with primary antibodies overnight at 4°C. The following primary antibodies were used: mouse monoclonal antibodies anti-GFAP (1/500, Sigma), IgG2b anti-HuC/D (1/200, Molecular Probes), IgG1 anti-Mash1 (1/10; a gift from D. J. Anderson), IgG2a anti-Ngn2 (1/20; a gift from D. J. Anderson); rat monoclonal antibodies IgG2a anti-BrdU (1/20, Oxford Biotechnology), anti-Ki67 (1/50, Novocastra) and anti-PDGFRα (1/800, DB biosciences); rabbit antisera anti-caspase 3 activated (1/1000, R&D Systems), anti-GFP (1/1000, Molecular Probes), anti-phosphohistone H3 (1/1000, Upstate), anti-Olig2 (1/1000, Chemicon) and anti-Prox1 (1/3000, Covance Research Products); goat anti-Neurod1 (1/100, Santa Cruz Biotechnology); and chicken anti-GFP (1/500, Chemicon). Corresponding secondary antibodies were incubated for 1 hour at room temperature, including Alexa Fluor 568-conjugated goat (or donkey) anti-mouse, anti-rabbit, anti-rat or anti-goat; and Alexa Fluor 488 goat (or donkey)-conjugated anti-rabbit, anti-mouse or anti-chicken (all from Molecular Probes, 1/1000). DAPI (1/5000) was used to label DNA and sections were mounted in Aquapolymount medium (Polysciences). Images were captured using SP1 and SP2 confocal microscopes (Leica, Germany), Radiance 2100 (BioRad, UK) confocal microscope and Zeiss Imager Z1 (Zeiss, Germany) with the Apotome system.

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Histology, BrdU incorporation and TUNEL experiments

For histological analysis, brains were fixed overnight in Bouin's fixative, processed for wax embedding, cut at 6 μm, and stained with Haematoxylin and Eosin. For BrdU incorporation experiments, pregnant females or P1 pups were injected intraperitoneally with 100 μg/g of body weigh of BrdU (Sigma) and sacrificed after 30 minutes. For immunohistochemistry, sections were processed as described above and BrdU incorporation was exposed by 30 minutes treatment in HCl 2N at 37°C. The TUNEL experiment was carried out following the supplier manual (ApopTag Kit, Qbiogene).

Quantification of the data and statistical analysis

Confocal images were quantified manually using Metamorph software and automated counting was performed using ImageJ software for Prox1-expressing cells, with values between 30 and 70 counted as Prox1^{low} and values between 100 and 250 counted as Prox1^{high} (see Fig. S1 in the supplementary material). All experiments were carried out in triplicate and at least three different sections were quantified for each experiment. Student's *t*-test was used for analysis of statistical significance.

RESULTS

Ngn2 is expressed by progenitors in the developing dentate gyrus

To address the role of Ngn2 in development of the DG, we first characterized the expression of *Ngn2* RNA and protein in the developing DG. At embryonic day (E) 18.5, a stage when the different progenitor populations of the DG (primary matrix, secondary matrix and tertiary matrix) can easily be distinguished (Altman and Bayer, 1990; Pleasure et al., 2000), *Ngn2* transcripts were detected in these three cell populations (Fig. 1A). Similarly, Ngn2 protein could be detected in a subset of cells in the three DG matrices (Fig. 1B). The numbers of cells expressing Ngn2 decreased from the primary to the secondary matrix and from the secondary to the tertiary matrix (Fig. 1C), in parallel with the progressive reduction in the proportion of progenitor cells present in these populations (Altman and Bayer, 1990).

In the developing neocortex, *Ngn2* is expressed in dividing progenitors and is rapidly downregulated as cells leave the cell cycle (Britz et al., 2006; Gradwohl et al., 1996; Hand et al., 2005). To determine whether this is also the case in the DG, we examined Ngn2 expression in dividing progenitors marked by a 30-minute pulse of BrdU or by Ki67 (Key et al., 1993), and in post-mitotic granule neurons marked by expression of the homeobox transcription factor Prox1 (Oliver et al., 1993; Liu et al., 2000). We found that a fraction of Ngn2^{+} cells incorporated BrdU in the three DG matrices (12%, 43% and 38% respectively; Fig. 1D-D‴) and a majority of them expressed Ki67 (e.g. 85% in the tertiary matrix; Fig. 1G), indicating that they correspond mainly to progenitor cells. By contrast, only a small fraction of Ki67^{+} or BrdU^{+} progenitor cells expressed Ngn2 in any of the three matrices (Fig. 1D-D‴,G). To determine whether Ngn2 expression is maintained in post-mitotic granule neurons, we examined the co-expression of Ngn2 and Prox1. We detected two cell populations expressing Prox1 at markedly different levels in the DG (see Fig. S1 in the supplementary material). Most cells of the primary matrix and a large fraction of cells in the secondary matrix, but fewer cells were in the tertiary matrix and the dentate gyrus itself, expressed Prox1 at a low level (Prox1^{low}) (Fig. 1E-E″). Most Prox1^{low} cells (82%) expressed the dividing cell marker Ki67, indicating that they are progenitors (see Fig. S2 in the supplementary material). By contrast, cells expressing Prox1 at a high level (Prox1^{high}) were absent from the primary matrix and sparse in the secondary matrix but constituted the main cell population of the dentate gyrus. These cells were Ki67 negative and therefore correspond to post-mitotic granule

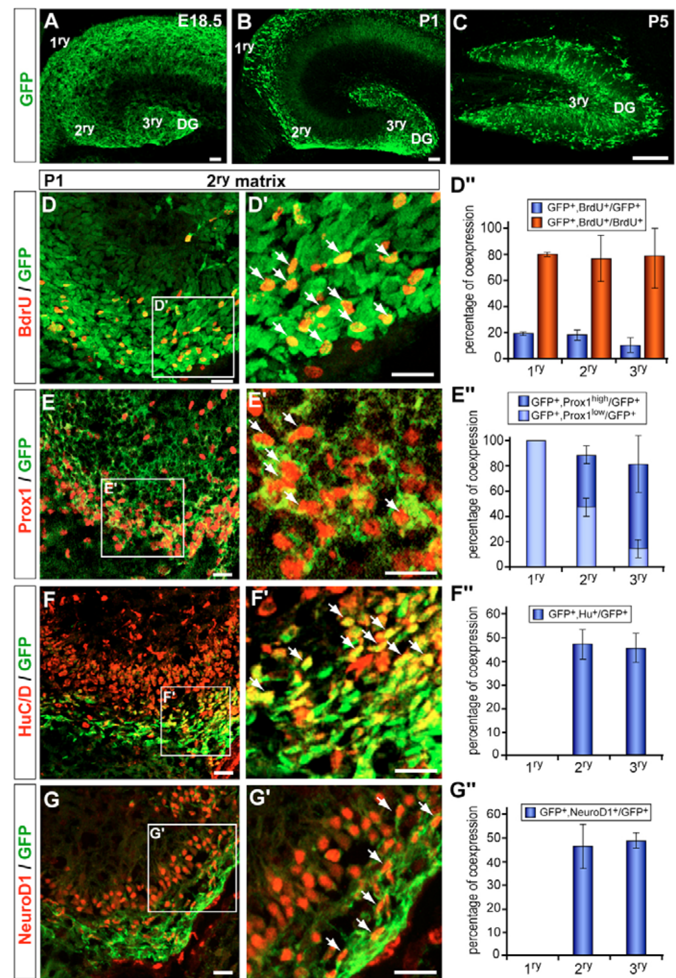

Fig. 2. Ngn2-expressing progenitors give rise to post-mitotic dentate granule neurons. (**A-C**) Sections through the hippocampus of *Ngn2*$^{KIGFP/+}$ embryos at E18.5 (A), P1 (B) and P5 (C) immunolabelled for GFP. (**D-G′**) Hippocampal sections of P1 *Ngn2*$^{KIGFP/+}$ brains showing the secondary matrix immunostained for GFP and BrdU after a 30 minutes pulse (D,D′), Prox1 (E,E′), HuC/D (F,F′) and Neurod1 (G,G′). Arrows indicate double-labelled cells. (**D″**,**E″**,**F″**,**G″**) Histograms representing (D″) the percentage of GFP-expressing cells that have incorporated BrdU (blue bars) and the percentage of BrdU-labelled cells expressing GFP (red bars), (E″) the percentage of GFP^{+} cells expressing Prox1 at a low level (light blue bars) or at a high level (blue bars) and (F″,G″) the percentage of GFP^{+} cells expressing HuC/D (F″) and Neurod1 (G″). The high level of GFP expression in *Ngn2*$^{KIGFP/+}$ mice (A) compared with Ngn2 expression (Fig. 1B) reflects the greater stability of GFP. Almost all post-mitotic dentate granule neurons are GFP^{+} and therefore originate from Ngn2-expressing progenitors. Differences in GFP labelling in E-E′ and other panels are due to the lower signal obtained with chicken anti-GFP antibody. Scale bars: 100 μm in A-C; 20 μm in D-G. 1ry, primary matrix; 2ry, secondary matrix; 3ry, tertiary matrix; DG, dentate gyrus.

neurons (see Fig. S2 in the supplementary material). Accordingly, Ngn2 was expressed only by Prox1^{low} and not by Prox1^{high} cells (Fig. 1E-E″), indicating that Ngn2 expression is downregulated when progenitors exit the cell cycle and is not maintained in post-mitotic granule neurons.

Expression of the bHLH protein Neurod1 is restricted in the DG to post-mitotic neurons (see Fig. S3B in the supplementary material), although *Neurod1* transcripts are also found in BrdU-

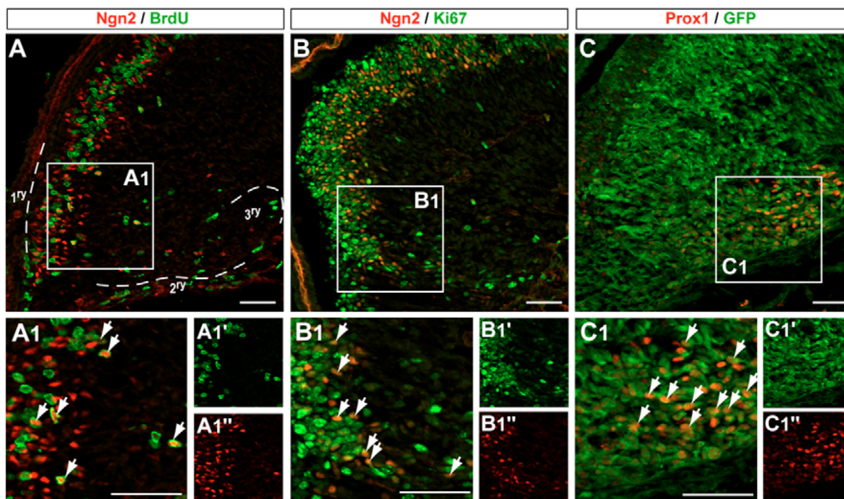

Fig. 3. Ngn2-expressing progenitors generate dentate granule neurons at the onset of dentate gyrus formation. (**A-C**) Sections through the hippocampus of wild-type (A,B) and $Ngn2^{KIGFP/+}$ (C) embryos at E16.5. (A1-C1) High magnification of the areas outlined in A-C. (**A1′-C1″**) Single-channel images of the pictures in A1-C1. Ngn2-expressing cells in primary and secondary matrices incorporate BrdU after a 30-minute pulse (A,A1), and co-express Ki67 (B,B1). (C,C1) GFP$^+$ cells in $Ngn2^{KIGFP/+}$ dentate gyrus co-express Prox1 at low or high level. Almost all dentate granule neurons (Prox1^{high}) are GFP$^+$ and therefore originate from Ngn2-expressing progenitors. Arrows indicate double-labelled cells. The different progenitor populations of the developing DG (1ry, primary matrix; 2ry, secondary matrix; 3ry, tertiary matrix) are outlined. Scale bars: 50 μm.

incorporating progenitors (see Fig. S3A in the supplementary material) (Lee et al., 2000). Double labelling for Ngn2 and Neurod1 showed that the two proteins are expressed in non-overlapping cell populations, thus confirming that Ngn2 expression in the developing DG is confined to mitotic progenitors (Fig. 1F-F″).

Ngn2-expressing progenitors give rise to granule neurons

Ngn2 is expressed by only a small fraction of progenitor cells in the three DG matrices, either because it is transiently expressed by all dentate granule progenitors or because it is expressed by a subset of progenitors with a distinct fate. To distinguish between these possibilities, we examined the fate of Ngn2-expressing progenitors using a mouse transgenic line in which GFP is expressed from the *Ngn2* locus ($Ngn2^{KIGFP}$) (Seibt et al., 2003). GFP expression in $Ngn2^{KIGFP}$ heterozygous mice recapitulates Ngn2 expression, and as the GFP protein is maintained in the recent progeny of Ngn2-expressing progenitors because of its greater stability, it can be used to trace the short-term fate of these progenitors (Fig. 2A-C) (Britz et al., 2006). Injection of BrdU in $Ngn2^{KIGFP}$ newborn mice 30 minutes before analysis revealed that most BrdU$^+$ progenitors (~80%) express GFP (Fig. 2D-D″), indicating that they had previously expressed Ngn2. Thus, Ngn2 is transiently expressed by most DG progenitors.

To confirm that Ngn2-expressing progenitors give rise to dentate granule neurons, we examined the expression of the granule neuron markers Prox1 and Neurod1, and the general neuronal marker HuC/D (Wakamatsu and Weston, 1997) in $Ngn2^{KIGFP}$ mice. Over 90% of GFP$^+$ cells expressed Prox1 in all three matrices, with an increasing fraction of cells displaying the high expression levels found in dentate neurons, as they progress from the primary to the tertiary matrix (Fig. 2E-E″). Forty to 50% of GFP$^+$ cells also expressed HuC/D in the secondary and tertiary matrix (Fig. 2F-F″) and a similar fraction expressed Neurod1 (Fig. 2G-G″). These data indicate that Ngn2-expressing progenitors give rise to postmitotic Prox1^{high}, Neurod1$^+$, HuC/D$^+$ dentate granule neurons. Over 90% of Prox1$^+$ cells expressed GFP, thus confirming that most DG neurons originate from Ngn2-expressing progenitors.

We also examined Ngn2 expression and GFP expression in $Ngn2^{KIGFP}$ mice at the onset of DG development (E16.5, Fig. 3). Ngn2-expressing cells were abundant in the primary matrix and some were also found to contribute to the emerging secondary matrix. A significant fraction of these cells incorporated BrdU after a 30-minute pulse (11% in the primary matrix and 30% in the secondary matrix; Fig. 3A) and almost all of them expressed Ki67, indicating that they are progenitors (Fig. 3B). Moreover, all Prox1^{high} granule neurons already expressed GFP in $Ngn2^{KIGFP}$ mice

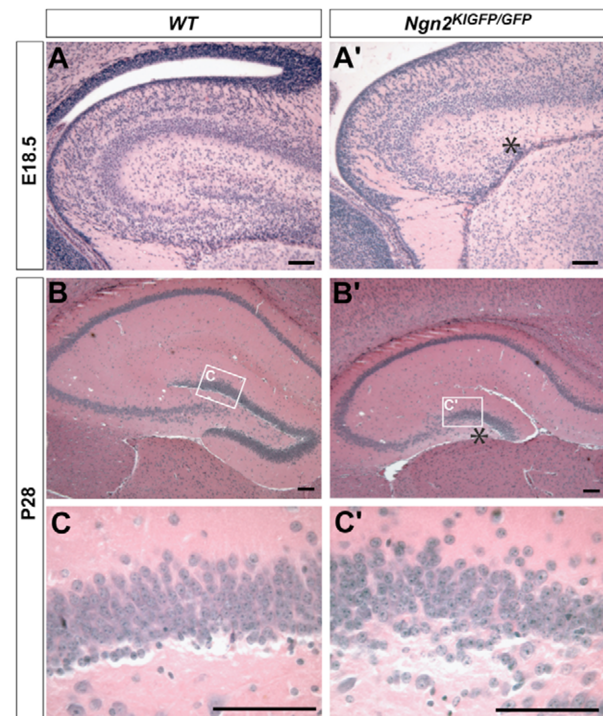

Fig. 4. Abnormal development of the dentate gyrus in *Ngn2* mutant mice. (**A**,**A′**) Haematoxylin/Eosin staining of hippocampal sections of E18.5 *Ngn2* mutant (A′) and wild-type (A) embryos show the lack of a morphologically distinct dentate gyrus (asterisk) in mutant brains at this stage. (**B**,**B′**) Sections through the hippocampus of 1-month-old (P28) *Ngn2* mutant (B′) and wild-type mice (B) show the absence of the lower blade of the DG (asterisk) in mutant brains. (**C**,**C′**) High magnification of the upper blade of the wild-type (C) and *Ngn2* mutant (C′) dentate gyrus. Note the disorganized appearance of the mutant DG. Scale bars: 100 μm.

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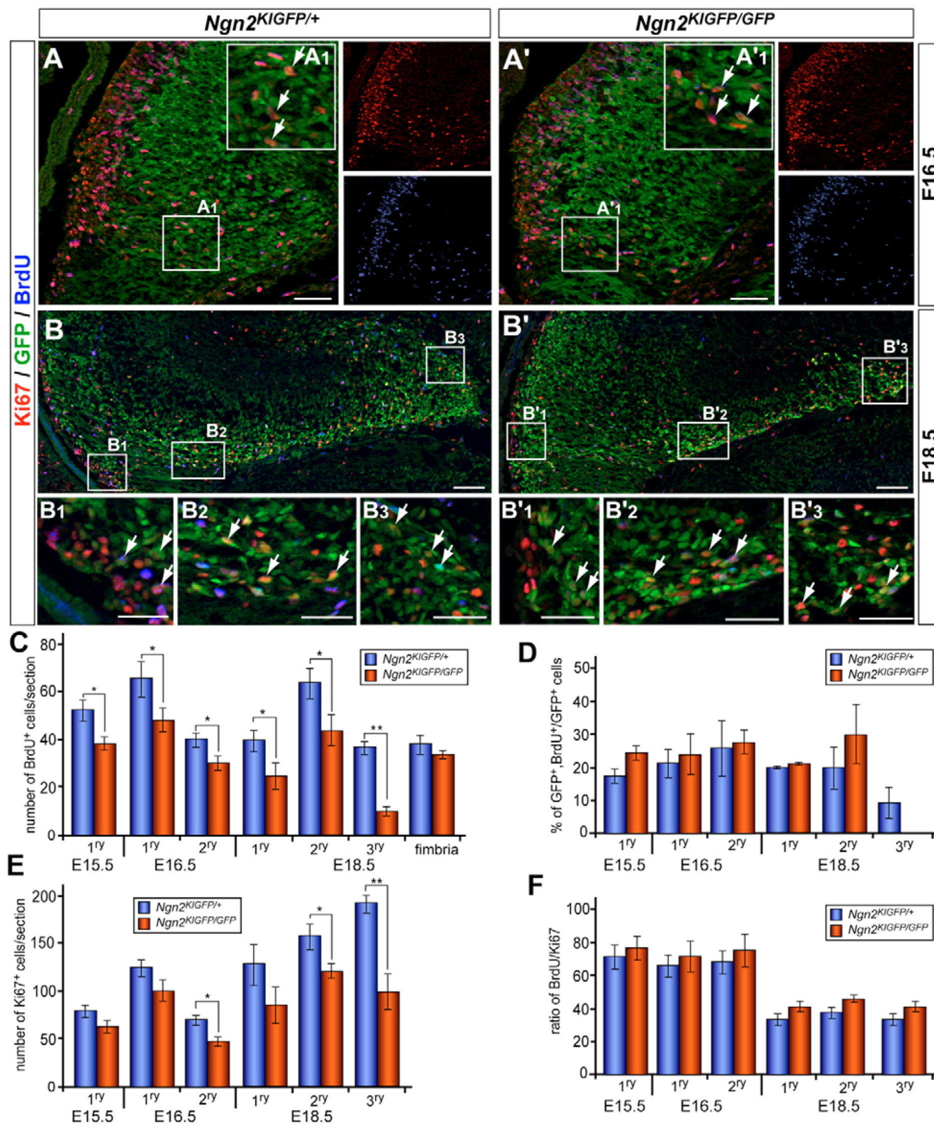

Fig. 5. Reduction in the number of progenitors in the dentate gyrus of *Ngn2* mutant mice. (**A-B′**) Hippocampal sections of E16.5 (A,A′) and E18.5 (B,B′) $Ngn2^{KIGFP/+}$ (A,B) or $Ngn2^{KIGFP/GFP}$ (A′,B′) brains immunolabelled for GFP, Ki67 and BrdU following a 30-minute pulse. Arrows indicate double labelled cells. (**A1**,**A′1**,**B1-B3**,**B′1-B′3**) High magnification of the areas outlined in A-B′. (**C**,**E**) Histograms representing the total number of BrdU-containing cells (C) or Ki67-expressing cells (E) within the different matrices of $Ngn2^{KIGFP/+}$ (blue bars) and $Ngn2^{KIGFP/GFP}$ (red bars) DG. The number of $BrdU^+$ progenitors is reduced in the three matrices of the mutant DG. The fimbria, which is not affected by the loss of *Ngn2*, is used as a control. (**D**) Histogram representing the percentage of GFP-expressing cells that have incorporated BrdU. (**F**) Histogram representing the ratio of BrdU- and Ki67-expressing cells. $*P\leqslant0.05$, $**P\leqslant0.01$. Scale bars: 50 μm.

at this early stage (Fig. 3C). Thus, Ngn2 is already expressed in dentate granule neuron progenitors at the beginning of DG development.

Defective dentate gyrus in *Ngn2* mutant mice

To assess the role of Ngn2 in development of the DG, we examined *Ngn2* null mutant mice (also named $Ngn2^{KIGFP/GFP}$). Most $Ngn2^{KIGFP/GFP}$ null mutant mice die after birth but a small fraction (4%) survive. Histological analysis of mutant brains at perinatal stages (E18.5 and P1) revealed an absence of the dentate granule layer (Fig. 4A,A′ and data not shown), while at adult stages, the upper blade of the DG was reduced in size and the lower blade was absent (Fig. 4B,B′ and not shown). Closer examination of the remaining upper blade also revealed that the granule neuron layer was disorganized when compared with control brains (Fig. 4C,C′).

Reduced generation of dentate gyrus progenitors in *Ngn2* mutant mice

We then examined whether the DG phenotype in $Ngn2^{KIGFP/GFP}$ mutant brains was due to a defect in the generation or in the survival of dentate granule neurons. At E15.5 and E16.5, the number of progenitors labelled by BrdU after a 30 minutes pulse was reduced by 20-25% in the primary and secondary matrices of *Ngn2* mutant mice when compared with wild types (Fig. 5A,A′,C). At E18.5, the number of progenitors was reduced by 30% in the primary and secondary matrices and by 65% in the tertiary matrix (Fig. 5B,B′,C). The number of GFP^+ cells (i.e. derived from $Ngn2^+$ progenitors) was also reduced in $Ngn2^{KIGFP/GFP}$ embryos, as the fraction of GFP^+ that had incorporated BrdU was similar in $Ngn2^{KIGFP/+}$ and $Ngn2^{KIGFP/GFP}$ embryos (between 20 and 30%; Fig. 5D). Ki67 labelling also revealed a reduction in number of cycling cells in $Ngn2^{KIGFP/GFP}$ at E16.5 (24% in the secondary matrix, Fig. 5A,A′,E) and at E18.5 (20%, 26% and 48% in the primary, secondary and tertiary matrix, respectively, Fig. 5B,B′,E). The ratio of cells in S-phase (BrdU+) over dividing cells (Ki67+) was similar in *Ngn2* mutant and wild-type mice, suggesting that the number of dividing progenitors rather than their cell cycle length is affected by the loss of *Ngn2*. The number of $Prox1^{low}$-expressing progenitors was also reduced in *Ngn2* mutant embryos at E16.5 (Fig. 6A,A′,C). Activated caspase 3 (Fig. 6D) and TUNEL (data not shown) labelling revealed a small but not significant increase in apoptosis in *Ngn2* mutant DG at E15.5,

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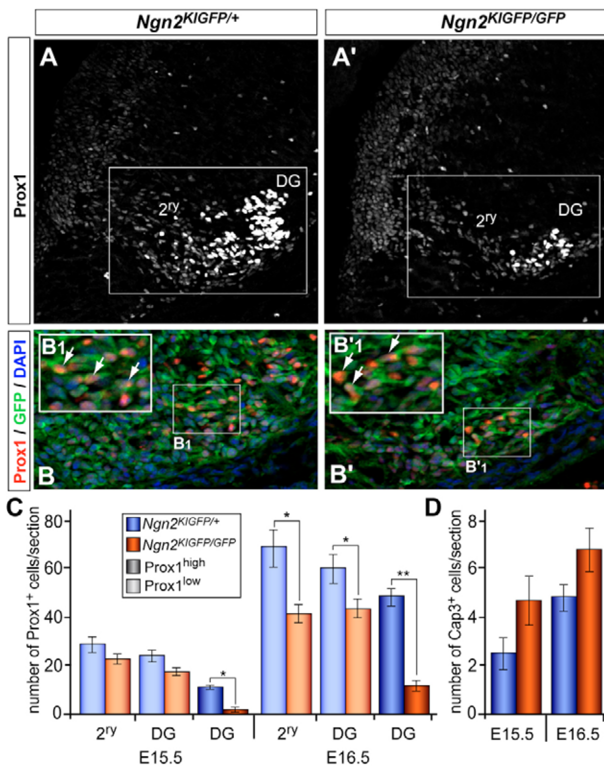

Fig. 6. Reduction in the number of granule neurons in the dentate gyrus *of Ngn2* mutant mice. (**A-B′**) Sections through the hippocampus of E16.5 *Ngn2*$^{KIGFP/+}$ (A,B) or *Ngn2*$^{KIGFP/GFP}$ (A′,B′). (A,A′) Sections showing Prox1^{high}- and Prox1^{low}-expressing cells. Both Prox1^{low} progenitors and Prox1^{high} granule neurons are reduced in *Ngn2*$^{KIGFP/GFP}$. The areas outlined in A,A′ indicate the regions shown in B,B′. (B,B′) Brains immunolabelled for GFP and Prox1. Insets B1,B′1 show Prox1^{high} granule neurons expressing GFP at a higher magnification. Arrows indicate double-labelled cells. (**C**,**D**) Histograms representing (C) the number of cells expressing Prox1^{low} (light colour) or Prox1^{high} (dark colour) and (D) the number of Casp3-positive cells at E15.5 and E16.5 in *Ngn2*$^{KIGFP/+}$ (blue bars) and *Ngn2*$^{KIGFP/GFP}$ (red bars) dentate gyrus. Fewer (Prox1^{low} progenitors and Prox1^{high} granule neurons are present in *Ngn2*$^{KIGFP/GFP}$ embryos. *$P\leqslant0.05$, **$P\leqslant0.01$.

E16.5 and E18.5 (Fig. 6D and not shown). This suggests that the reduction in progenitor cell number in *Ngn2* mutant DG reflects mainly a defect in the generation of progenitors, although a reduced ability to survive may play a minor role in this phenotype.

Abnormal differentiation of the remaining mutant dentate gyrus progenitors

We next examined the differentiation of the remaining DG progenitors in absence of *Ngn2*. In *Ngn2*$^{KIGFP/GFP}$ DG at P1, the fraction of GFP$^+$ cells in the secondary matrix that expressed the neuronal markers HuC/D and βIII-tubulin was reduced by nearly half (from 45% to 25%; Fig. 7A-A″ and data not shown). By contrast, the fraction of GFP$^+$ cells expressing Prox1^{high} in the secondary matrix was not significant reduced (Fig. 7B′,B″). Similarly, expression of Neurod1, which is regulated by *Ngn2* in other parts of the nervous system, was not significantly altered in *Ngn2* mutants, as the fraction of GFP$^+$ cells expressing Neurod1 was similar in the DG of *Ngn2* mutant and wild-type mice (Fig. 7C-C″). We examined whether other neurogenin genes (i.e. *Ngn1* and *Ngn3*) were expressed in the DG to account for the maintenance of Neurod1 expression in the absence of *Ngn2*, but found that both genes were undetectable in the DG in both

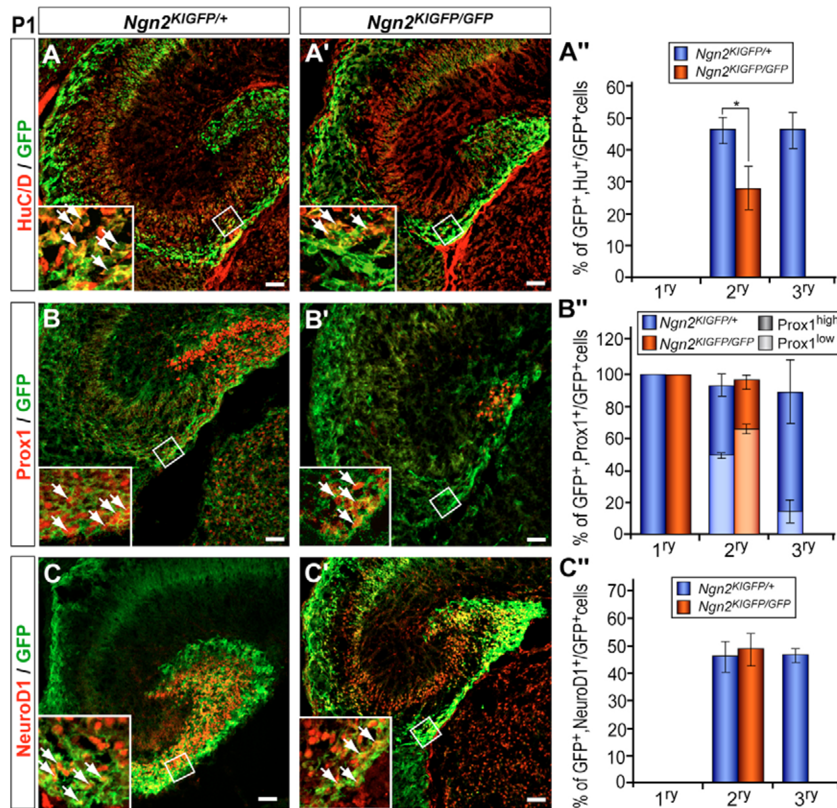

Fig. 7. Neuronal differentiation defects in *Ngn2* mutant dentate gyrus. (**A**,**A′**,**B**,**B′**,**C**,**C′**) Sections through the hippocampus of P1 *Ngn2*$^{KIGFP/+}$ (A,B,C) or *Ngn2*$^{KIGFP/GFP}$ (A′,B′,C′) brains immunolabelled for GFP and HuC/D (A,A′), Prox1 (B,B′) or Neurod1 (C,C′). The insets show a high magnification of the secondary matrix. (**A″**,**B″**,**C″**). Histograms representing (A″) the percentage of GFP$^+$ cells expressing HuC/D, (B″) the percentage of GFP$^+$ cells expressing Prox1^{low} (light colour) or Prox1^{high} (dark colour), and (C″) the percentage of GFP$^+$ cells expressing Neurod1 in *Ngn2*$^{KIGFP/+}$ (blue bars) and *Ngn2*$^{KIGFP/GFP}$ (red bars) dentate gyri. Fewer post-mitotic HuC/D, Prox1^{high}-expressing granule neurons are present in the *Ngn2*$^{KIGFP/GFP}$ DG, while *Ngn2* loss of function does not affect Neurod1 expression. *$P\leqslant0.05$, **$P\leqslant0.01$. Scale bars: 50 μm.

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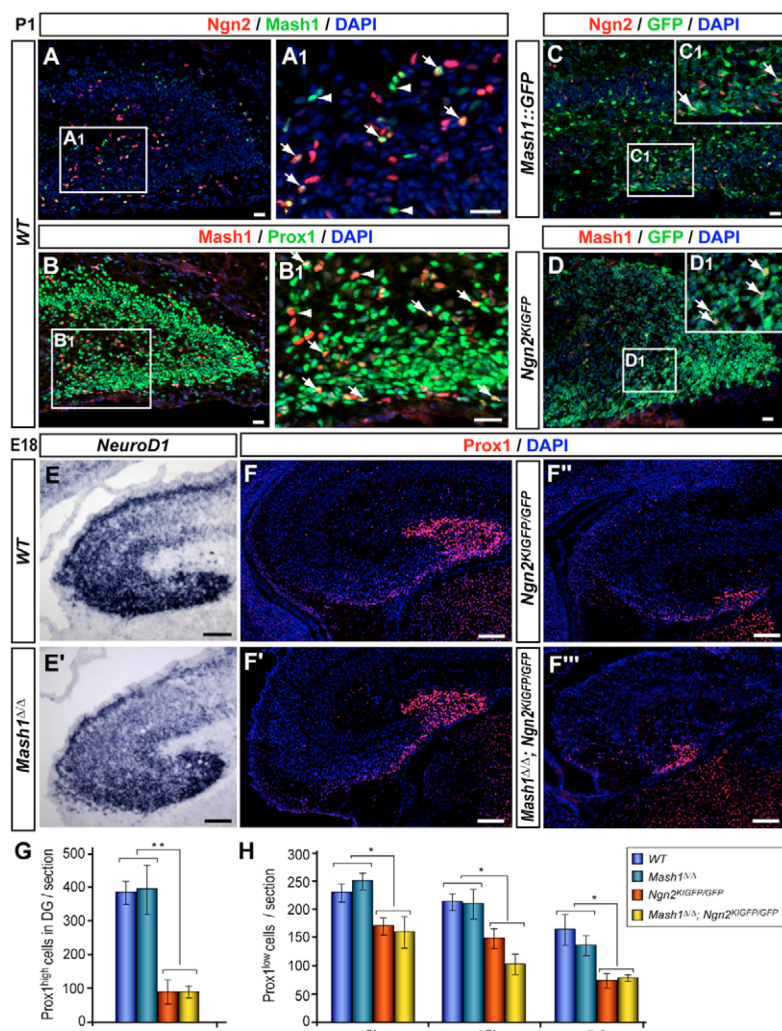

Fig. 8. Mash1 is expressed but not required in dentate granule progenitor generation. (**A-C**) Sections through the dentate gyrus of P1 wild-type mice immunolabelled for (A,A1) Ngn2 and Mash1, showing that many Mash1$^+$ progenitors co-express Ngn2 (arrows), and for (B,B1) Mash1 and Prox1, showing that most Mash1$^+$ cells co-express Prox1^{low}. (C,C1) Section through the dentate gyrus of P1 *Mash1::GFP* transgenic mice immunolabelled for Ngn2 and GFP shows that many Ngn2$^+$ progenitors co-express GFP. (**D**,D1) Section through the dentate gyrus of P1 *Ngn2$^{KIGFP/+}$* mice immunolabelled for Mash1 and GFP, show that many Mash1$^+$ progenitors co-express GFP. (A1-D1) High magnifications of the areas outlined in A-D. Arrows indicate co-expression of markers and arrowheads indicate cells expressing only one marker. (**E-F‴**) Sections through the dentate gyrus of E18.5 wild-type (E,F), *Mash1* mutant (E′,F′), *Ngn2* mutant (F″) and *Mash1*; *Ngn2* double mutant (F‴) embryos showing the expression of *Neurod1* transcripts by in situ hybridisation (E,E′) and Prox1 protein (F-F‴). *Mash1* mutant and wild-type embryos have similar patterns of *Neurod1* (E,E′) and Prox1 (F,F′) expression. (**G**,**H**) Histograms representing the number of cells expressing Prox1^{high} (G) and Prox1^{low} (H) in the different genotypes shown in F-F‴. The defect in Prox1 expression is not more severe in the dentate gyrus of *Mash1$^{Δ/Δ}$; Ngn2$^{KIGFP/GFP}$* double mutants than in *Ngn2$^{KIGFP/GFP}$* single mutants. Scale bars: 20 μm in A-D; 50 μm in E-F‴.

wild-type and *Ngn2* mutant embryos (data not shown). Altogether, our data indicate that *Ngn2* is required for both the production and correct differentiation of DG progenitors. However, some aspects of their differentiation, including the expression of Neurod1, are activated in the absence of *Ngn2*.

Mash1 does not compensate for the loss of Ngn2

Expression of another proneural gene, *Mash1*, has been reported in the developing and adult DG (Pleasure et al., 2000). We thus asked whether Mash1 is also involved in DG development and particularly if it contributes to the generation and/or differentiation of the remaining DG progenitors in *Ngn2* mutant mice. We first compared the expression of Mash1 in the developing DG with that of Ngn2. At P1, a large fraction of Ngn2$^+$ progenitors co-expressed Mash1 in the tertiary matrix (63±5% of Ngn2$^+$ cells are Mash1$^+$ and 53±8% of Mash1$^+$ cells are Ngn2$^+$; Fig. 8A,A1). A majority of Mash1-expressing cells (64±7%) co-expressed Prox^{low}, suggesting that, like Ngn2$^+$ cells, these cells are dentate granule progenitors (Fig. 8B,B1). In a *Mash1::GFP* reporter line (Gong et al., 2003), we found that a larger fraction of Ngn2$^+$ progenitors co-expressed GFP (74±6%) than Mash1 protein (61±5%), suggesting that some Ngn2$^+$ progenitors had expressed and then downregulated Mash1 (Fig. 8C). Similarly, in *Ngn2$^{KIGFP/+}$* mice, a larger fraction of Mash1$^+$ cells co-expressed GFP than Ngn2 (67±5% and 51±4%; Fig. 8A,D), suggesting that some Mash1$^+$ cells had expressed and then downregulated Ngn2.

The extensive co-expression of Mash1 and Ngn2 in DG progenitors suggested that the two factors might share some functions in DG neurogenesis, as previously reported in other parts of the telencephalon (Nieto et al., 2001). We therefore examined whether *Mash1* is required during development of the DG and/or whether it compensates for the loss of *Ngn2* in the DG progenitors that remain in *Ngn2* mutants. The expression of *Neurod1* and Prox1 (Fig. 8E-H), and the number of progenitors labelled by phophohistone H3 and Ki67 (see Fig. S5A,A′ in the supplementary material) were not affected in *Mash1* mutant embryos at E18.5, indicating that *Mash1* function is not essential for the formation of the DG at prenatal stages. *Mash1* function at postnatal stages could not be assessed owing to the death of *Mash1* mutants at birth. To determine whether *Mash1* can compensate for the loss of *Ngn2* during DG development, we examined *Ngn2*; *Mash1* double mutant mice. Prox1^{high}- and Prox1^{low}-expressing cells were similarly reduced in the DG of *Ngn2* single mutants, and *Ngn2*, *Mash1* double mutant embryos at E18.5 (Fig. 8F″-H; see Fig. S4 in the supplementary material), suggesting that *Mash1* cannot compensate for the loss of *Ngn2* during the prenatal phase of DG development.

In addition to the activation of neurogenesis, *Mash1* has been shown to inhibit astrogliogenesis (Nieto et al., 2001) and to promote oligodendroglial development (Parras et al., 2004; Parras et al., 2007; Battiste et al., 2007; Sugimori et al., 2007; Sugimori et al., 2008). However, expression of the astrocyte marker GFAP and of

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the oligodendrocyte precursor markers PDGFRα and Olig2 was not changed in the hippocampus of *Mash1* mutants at birth (see Fig. S5 and Fig. S6A,A′ in the supplementary material). Olig2 expression was also unchanged in *Ngn2* mutant and *Ngn2*, *Mash1* double mutant embryos (see Fig. S6A″,A‴ in the supplementary material).

Expression of the neural bHLH genes *Math2/Neurod6* and *Math3/Neurod4* has also been reported previously in the DG region (Pleasure et al., 2000). *Math3* appears to be expressed in DG progenitors (see Fig. S7B in the supplementary material), while *Math2* expression is confined to dentate granule neurons (Pleasure et al., 2000). *Math3* expression persists in the DG progenitors that remain in *Ngn2* mutant embryos (see Fig. S7B′ in the supplementary material), suggesting that it may promote neurogenesis to some extent in *Ngn2* mutant embryos.

Cells accumulate at the periphery of the *Ngn2* mutant DG

Analysis of GFP expression in the DG of both *Ngn2*$^{KIGFP/GFP}$ and *Ngn2*$^{KIGFP/+}$ mice revealed the presence of GFP^{+} cells at the periphery of the upper blade of the DG (Fig. 9A,A′,B,B′,E). Double labelling experiments for GFP and progenitor markers (Ngn2^{+}, BrdU^{+}, Prox1^{low}; Fig. 9C-C′1,F,G) or neuronal differentiation markers (Hu^{+}, Neurod1^{+}; Fig. 9D,D′,G) showed that these peripheral cells include both progenitors and post-mitotic neurons (Fig. 9F,G). This peripheral cell population seemed to be more packed in mutant than wild-type mice, particularly after birth (Fig. 9B,B′), and it presented a differentiation defect similar to that observed in the secondary matrix, with progenitors (i.e. Ngn2^{+}, BrdU^{+}, Prox1^{low} cells) being present in larger numbers in *Ngn2* mutant than in wild-type mice (Fig. 9G). These mutant cells may fail to migrate inwardly, from the periphery of the DG into the granule cell layer, resulting in their relative accumulation in this peripheral location (Fig. 9E; see Discussion).

Radial glial cells, which express the astrocytic marker GFAP in the DG, have been implicated in the outward migration of newborn neurons from the subgranular layer to the granule cell layer (Rickmann et al., 1987). Thick bundles of GFAP^{+} radial glial fibres were found at the periphery of the wild-type DG, and these fibres crossed the lower and upper blades of the DG with an orientation perpendicular to the long axis of the blades. The GFAP^{+} bundles were still present at the periphery of the reduced *Ngn2* mutant DG, but no fibres were found crossing the DG blades (Fig. 9C-C′1). This lack of radial glial fibres may perturb the migration of DG neurons, resulting in their accumulation at the periphery of the DG in *Ngn2* mutants at birth and in the abnormal distribution of GFP^{+} cells observed in the *Ngn2* mutant DG at later stages (see Fig. S8 in the supplementary material).

DISCUSSION

In this manuscript, we show that the proneural protein Ngn2 is transiently expressed by DG progenitors before they differentiate into granule neurons. We also demonstrate that Ngn2 is required for proper development of the DG, as loss of *Ngn2* results in a reduction in the number of progenitors, in defects in the differentiation of granule neurons and possibly in their migration to the granule cell layer of the DG.

Ngn2 expression in dentate gyrus progenitors

Nng2 protein is transiently expressed by progenitors of the DG, identified by their proliferative state and by low level of expression of the homeodomain protein Prox1. Ngn2 expression is then downregulated before progenitors become post-mitotic and

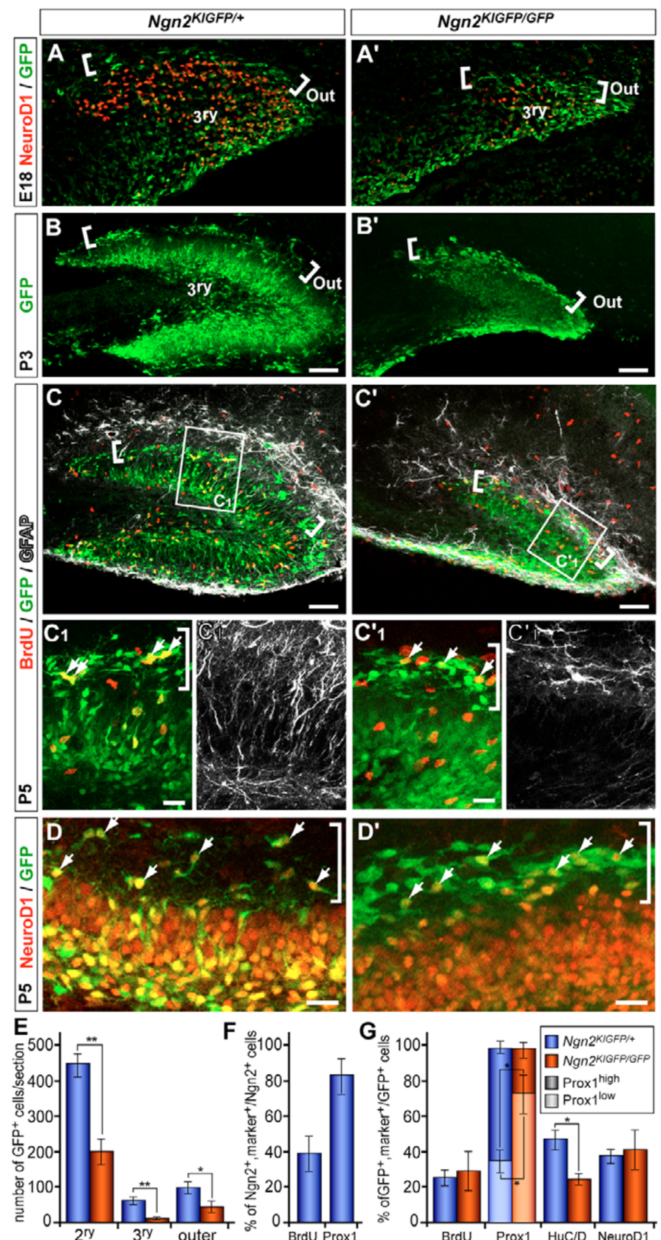

Fig. 9. Abnormal distribution of neuronal progenitors in *Ngn2* mutant dentate gyrus. (**A-D′**) Sections through the hippocampus of *Ngn2*$^{KIGFP/+}$ (A-D) or *Ngn2*$^{KIGFP/GFP}$ (A′-D′) mice at E18.5 (A,A′), P3 (B,B′) and P5 (C,C′,C1,C′1,D,D′), immunolabelled for GFP together with Neurod1 (A,A′,D,D′) or BrdU and GFAP (C,C′,C1,C′1). (C1,C1′) High magnification of the areas outlined in C,C′ showing green/red and white channels separately. (C1,C′1) The radial glial scaffold labelled by GFAP is absent from the *Ngn2*$^{KIGFP/GFP}$ DG. Note the lack of GFP-expressing cells at the position of the tertiary matrix in *Ngn2*$^{KIGFP/GFP}$ mice. Square brackets indicate cells in an outer position. (**E**) Histogram representing the number of GFP^{+} cells in secondary, tertiary and outer positions at E18.5. (**F**) Histogram representing the percentage of Ngn2-expressing cells in outer positions of the wild-type forming DG, labelled with BrdU (30-minute pulse) or Prox1^{low} at E18.5. (**G**) Histogram representing the percentage of GFP-expressing cells labelled with BrdU (30 minutes), Neurod1, Prox1^{low} (light colour) or Prox1^{high} (dark colour) in *Ngn2*$^{KIGFP/+}$ (blue bars) and *Ngn2*$^{KIGFP/GFP}$ (red bars) in outer positions of the dentate gyrus at E18.5. DG, dentate gyrus; 3ry, tertiary matrix; Out, outer position. Scale bars: 100 μm in A-C′; 20 μm in C1-D′.

differentiate, as marked by the expression of Neurod1, HuC/D and Prox1 at high level. Using a $Ngn2^{KIGFP}$ reporter mouse, we have traced the fate of $Ngn2^+$ cells and shown that they give rise to most dentate granule neurons.

Another proneural protein, Mash1, has also been found in progenitor cells of the developing and adult DG (Pleasure et al., 2000). We show here that Ngn2 and Mash1 are expressed in the same DG progenitor lineage and that the two factors are largely co-expressed in DG progenitors, suggesting that they are both involved in DG neurogenesis. Another bHLH protein Neurod1, is also present in the developing DG but its expression is restricted to postmitotic, differentiating neurons (Pleasure et al., 2000). $Neurod1^+$ cells are absent from the primary matrix, the portion of embryonic neuroepithelium from which all DG progenitors originate, but $Neurod1^+$ cells are found intermingled with $Ngn2^+$ progenitors in the secondary matrix, indicating that this migratory cell population is heterogeneous and contains cells at different stages of maturation along the dentate granule neuron lineage. The postmitotic $Neurod1^+$ neurons found in the secondary matrix might be born in the primary matrix (Pleasure et al., 2000) or in the secondary matrix from migratory progenitors that become postmitotic and begin to differentiate while migrating.

The functions of *Ngn2* in the developing dentate gyrus

In *Ngn2* mutant mice, there is a strong reduction in size of the forming DG at the end of embryonic development (E18.5). Mutant mice that escape perinatal lethality have an almost complete loss of the lower blade of the DG and a reduced upper blade (Fig. 4). This is the first report of a proneural factor being required in DG progenitors for normal DG development.

Ngn2 mutant mice present a marked reduction in number of dividing progenitors in all matrices of the DG without a major increase in cell death, suggesting that Ngn2 function is required for the generation and expansion of DG progenitors. Loss of *Ngn2* does not appear to affect the duration of the cell cycle of progenitors, as the fraction of cells in S-phase ($BrdU^+$) among all dividing progenitors ($Ki67^+$) remains the same in Ngn2 mutant and wild-type mice. Thus, loss of *Ngn2* may result in a cell cycle arrest of DG progenitors that would normally continue to proliferate. In addition, *Ngn2* mutant progenitors do not differentiate properly, as shown by the reduced fraction of $Ngn2^+$ progenitor-derived cells expressing the neuronal markers HuC/D and βIII-tubulin. It is presently unclear whether this is due to a delay or to a complete block in expression of these markers. Unexpectedly, Neurod1 appears to be normally expressed by *Ngn2* mutant DG neurons, suggesting that Ngn2 regulates only some aspects of the differentiation programme of dentate granule cells.

The proneural protein Mash1 is also expressed by DG progenitors (Pleasure et al., 2000) (Fig. 8), raising the possibility that it regulates aspects of the dentate granule neuron phenotype not controlled by Ngn2, or that it takes over some of the functions of *Ngn2* when this gene is mutated. However, analysis of *Mash1* single mutants and *Mash1*; *Ngn2* double mutants does not support a significant role for *Mash1* in DG neurogenesis in a wild-type or *Ngn2* mutant context up to birth, when these mice die. Other neurogenin genes (*Ngn1* and *Ngn3*) are not detectably expressed in the developing DG, but the bHLH gene *Math3/Neurod4* is expressed in both wild-type and, at a reduced level, *Ngn2* mutant DG, suggesting that it may partially compensate for the absence of *Ngn2* and drive DG neurogenesis in *Ngn2* mutants. Alternatively, another yet unidentified factor that may not belong to the bHLH transcription factor family (e.g. Jafar-Nejad et al., 2006) may share some of Ngn2 activities, including the regulation of Neurod1 and be involved in DG development along with *Ngn2*.

Ngn2 has been shown to specify several aspects of the subtype identity of projection neurons in the cerebral cortex, including their glutamatergic neurotransmission phenotype and their pyramidal morphology (Hand et al., 2005; Schuurmans et al., 2004). Interestingly, although DG granule cells also originate from Ngn2-expressing progenitors, they have very different characteristics from cortical projection neurons, and in particular present a mixed glutamatergic and GABAergic phenotype (Gutierrez, 2003; Gutierrez, 2005), and a granule cell morphology very distinct from that of pyramidal cortical neurons. Although there is no overt defect in expression of glutamatergic and GABAergic markers in the DG of *Ngn2* mutant embryos (see Fig. S9 in the supplementary material), it is tempting to speculate that both Ngn2 [a glutamatergic neuron determinant (Schuurmans et al., 2004)] and Mash1 [a GABAergic neuron determinant (Fode et al., 2000)] may contribute to the specification of the unique identity of DG granule cells. Testing this hypothesis will require to examine the phenotype of DG granule cells at postnatal stages in conditional *Mash1* and *Ngn2* mutant mice.

A new route for the migration of DG progenitors?

We have found progenitor cells located in the external part of the dentate granular layer in the developing DG. Altman and Bayer (Altman and Bayer, 1990) previously described the presence of proliferating cells in this outer region and assumed that these were glial progenitors, but our molecular analysis identifies them instead as dentate granule cell progenitors ($BrdU^+$, $Ngn2^{KIGFP+}$ and $Prox1^{low}$). This suggests that, at least at embryonic stages, a fraction of granule neuron progenitors could reach the DG by migrating along the outer border of the lower and upper blades of the DG, rather than along the inner side of the lower blade towards the tertiary matrix, as usually assumed. Once located at the periphery of the DG, the outer progenitors presumably produce granule neurons (some of them found in a peripheral position, Fig. 9) that may reach their final location in the granule cell layer via an inward migration route. Testing this model will require tracing the migration of DG progenitors and granule neurons by timelapse imaging. Moreover, GFP^+ cells expressing either progenitor ($Prox1^{low}$, BrdU or Ki67) or postmitotic neuronal markers ($Prox1^{high}$, Neurod1 or HuC/D) appear to accumulate in *Ngn2* mutant mice at the periphery of the remaining DG blade, suggesting that mutant neurons may fail to migrate inwards and into the granule cell layer. If a migration defect indeed takes place in the *Ngn2* mutant DG, the disruption of the radial glia scaffold revealed by GFAP staining may be involved in this phenotype.

Although the mechanism by which loss of *Ngn2* results in disruption of radial glial cells is unclear, it is noteworthy that a similar phenotype has been observed in mice mutant for the Wnt co-receptor Lrp6 (Zhou et al., 2004). The similarity in phenotype between *Lrp6* and *Ngn2* mutant mice extends to a reduction in number of dentate granule neurons. *Ngn2* has been shown to be directly regulated by Wnt signalling (Hirabayashi et al., 2004; Israsena et al., 2004), suggesting that it may mediate some of the functions of the Wnt signalling pathway in DG development. Wnt signalling has also been implicated in the regulation of neurogenesis in the adult DG (Lie et al., 2005), and *Ngn2* expression is maintained in progenitor cells in the subgranular layer of the postnatal DG (Ozen et al., 2007), thus raising the exciting possibility that a Wnt signalling-Ngn2 pathway controls both the development of the DG and the maintenance of neurogenesis in the adult structure.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/11/2031/DC1

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