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Identification of a transient subpial neurogenic zone in the developing dentate gyrus and its regulation by Cxcl12 and reelin signaling

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One striking feature of dentate gyrus development, distinct from the other cortical structures, is the relocation of neural precursors from the ventricular zone to the forming dentate pole to produce a lifelong neurogenic subgranular zone (SGZ). In this study, we demonstrate that dentate progenitors first dwell for up to 1 week in a previously unrecognized neurogenic zone intimately associated with the pial meningeal surface lining the outer edge of the forming dentate. This zone also serves as the organizational matrix for the initial formation of the dentate glial scaffolding. Timely clearance of neural precursors from their transient location depends on reelin, whereas initial formation of this transient stem cell niche requires Cxcl12-Cxcr4 signaling. The final settlement of the neural precursors at the subgranular zone relies on a pertussis toxin-sensitive pathway independent of Cxcl12-Cxcr4 signaling. Furthermore, genetic fate-mapping analysis suggests that subpial precursors contribute to the SGZ formation. These results demonstrate that the relocation of neural precursors in the dentate gyrus consists of discrete steps regulated by multiple pathways.

KEY WORDS: Dentate gyrus, Meninges, Neurogenesis

INTRODUCTION

There is tremendous interest in adult neurogenesis in the mammalian brain, both from the perspective of the normal role of new neurons in circuit plasticity and of the potential for brain repair implied by the persistence of adult neurogenesis (Gage, 2000). There are two well-established regions of adult neurogenesis in the rodent brain – the subventricular zone (SVZ) and the dentate gyrus (DG). The SVZ generates new olfactory interneurons involved in plasticity in the adult olfactory system; and in the DG, new granule neurons are believed to be involved in learning and memory.

Interestingly, there are stark contrasts in the developmental plan used to form these two adult neurogenic niches. Many studies have indicated that the SVZ and the rostral migratory stream are remnants of the embryonic SVZ (Merkle and Alvarez-Buylla, 2006; Merkle et al., 2004). By contrast, the DG uses a very distinct developmental plan to produce a durable neurogenic niche (Li and Pleasure, 2005). The multi-potential neural precursors seed the developing dentate gyrus beginning around mid-gestation from their origin in the medial cortical neuroepithelium. As the scaffolding of the DG forms around the first postnatal week, the neurogenic precursors settle at the border between the granule cell layer (GCL) and the hilus, also called the subgranular zone (SGZ). Migration of multi-potential precursors from one neurogenic zone (the dentate ventricular zone) to a nascent neurogenic zone (SGZ) has long been recognized to be a unique reorganization for the cortex, sharing some features with the migration of granule cell precursors in the cerebellum.

Previous studies have implicated the Wnt and Shh pathways as regulators of precursor behavior in the embryonic ventricular zone and SVZ, as well as the perinatal conversion of the SVZ to its adult state (Galceran et al., 2000; Machold et al., 2003; Pozniak and Pleasure, 2006; Zhou et al., 2004). In addition, both of these pathways are involved in the maintenance of precursors in the postnatal and adult DG (Lai et al., 2003; Lie et al., 2005). However, it is not clear what factors regulate the behaviors of precursors during transit to the DG. In this study, we provide evidence that en route to the DG, many precursors are localized in a specialized temporary neurogenic zone before they occupy the nascent DG. Interestingly, the organization of this zone is controlled by Cajal-Retzius cell-derived reelin and meningeally produced Cxcl12. By genetic fate-mapping analysis, we also show that subpial precursors may contribute to the SGZ formation.

MATERIALS AND METHODS

Animals

Most mouse strains (Cxcr4, reeler, Emx1$^{ires\text{-}cre}$, Rosa-*lacZ*, Rosa-YFP and Z/EG) were obtained from the Jackson Laboratory (Bar Harbor, Maine). Cxcr4-flox line was kindly provided by Dr Dan Littman (New York University), Nestin-GFP transgenic line by Dr Masahiro Yamaguchi (Japan), Gli1^{CreERT2} line by Dr Alexandra L. Joyner (Sloan-Kettering) and Rosa26-PTX was generated in Dr Shaun R. Coughlin's laboratory (UCSF). All the lines were maintained in the C57/B6 genetic background. The day of vaginal plug was considered to be embryonic day 0.5 (E0.5). Mouse colonies were housed at the University of California, San Francisco, in accordance with National Institutes of Health and UCSF guidelines.

Immunohistochemistry

Details can be provided on request.

In situ hybridization (ISH)

In situ hybridization protocol and probes (Cxcl12 and Cxcr4) were used as described (Li et al., 2008a).

In utero electroporation and DiO Injection

Details can be provided on request.

Glial process tracing

A series of overlapping thin section confocal images were taken with LSM 510 meta two-photon microscope (Carl Zeiss, Inc.). BLBP glial processes were traced with NIH ImageJ program.

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BrdU injection

Timed pregnant mice were intraperitoneally (i.p.) injected with BrdU (Roche) dissolved in 1×PBS (10 mg/ml) at the dose of 100 mg/kg animal for acute labeling or 50 mg/kg animal for birthdating analysis.

Tamoxifen induction and *lacZ* staining

Tamoxifen (TM, Sigma) was dissolved in corn oil (Sigma) at 20 mg/ml. Pregnant females were dosed i.p. with 3 mg of tamoxifen/40 g animal. Embryos were collected at the specified ages and *lacZ* staining was carried out as described (Li et al., 2008b).

Quantification

Details can be provided on request.

RESULTS

A transient subpial neurogenic zone in the dentate gyrus

Pioneering studies by Altman and Bayer (Altman and Bayer, 1990a; Altman and Bayer, 1990b) showed that proliferative dentate precursors leave the dentate neuroepithelium and form a migratory stream into the forming dentate gyrus during late embryonic development. To better characterize the spatiotemporal dynamics of the precursors in the dentate migratory stream in mice, we decided to re-examine this framework with new molecular markers. Using Nestin-GFP transgene and Tbr2 to label the dentate precursors and neurogenic transit amplifying cells, respectively (Englund et al., 2005; Yamaguchi et al., 2000), we revealed the sequential distribution of these cells during initial dentate gyrus formation.

By E14.5, the domain adjacent to the cortical hem in the ventricular zone (VZ) representing the dentate primordium was marked by strong Nestin-GFP expression (Fig. 1A,A1). Concurrently, Tbr2+ cells were abundant in the subventricular zone (SVZ) and a stream of Tbr2+ cells stretched from the SVZ above the forming fimbria to form a line of cells along the pial surface (Fig. 1A2,A4). A day later at E15.5, Nestin-GFP+ cells emanating from the dentate notch formed a narrow stream oriented toward the subpial region at the junction between the fimbria and forming dentate gyrus (Fig. 1B); we have termed this the fimbriodentate junction (FDJ). At high magnification, Tbr2+ cells were also found

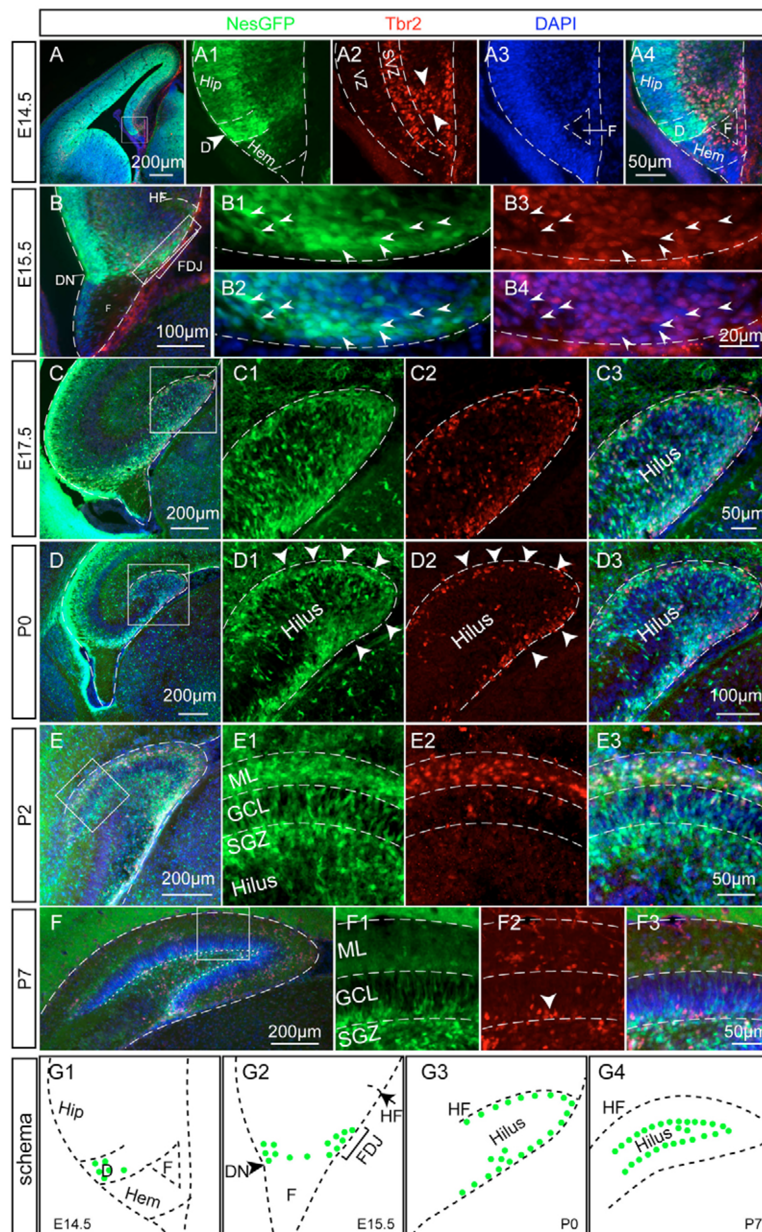

Fig. 1. Progressive development of the dentate neurogenic zones is revealed by Nestin-GFP transgene and Tbr2. (**A**) At E14.5, Nestin-GFP is upregulated in the dentate primordium abutting the cortical hem, which is shown in higher magnification in (A1), with a few Nestin-GFP+ cells leaving the dentate VZ. (A2) Tbr2+ cells, which represent neurogenic precursors, have migrated away from the SVZ (arrowheads). (A3) The fimbria has started to form in the relatively cell-free region of the cortical hem (shown by nuclei staining with DAPI). (A4) The organization of the medial wall with all the markers. Scale bar: 200 µm in A; 50 µm in A1-A4. (**B**) At E15.5, Nestin-GFP+ cells (green) formed a new neurogenic zone in the subpial region of the fimbriodentate junction (FDJ). (B1-B4) The distribution of Nestin-GFP+ and Tbr2+ cells in the subpial region of the FDJ. Some of the Nestin-GFP+ cells (arrowheads in B1 and B2) were Tbr2– (arrowheads in B3 and B4). Scale bar: 100 µm in B; 20 µm in B1-B4. (**C**) At E17.5, Nestin-GFP+ cells were distributed across the hilus. (C1-C3) The distribution of Nestin-GFP+ cells (C1,C3), Tbr2+ cells (red, C2,C3) and DAPI (blue, C3) at higher magnification. Scale bar: 200 µm in C; 50 µm in C1-C3. (**D**) At P0, the subpial neurogenic zone was established around the edge of the dentate pole. (D1-D3) Both Nestin-GFP+ and Tbr2+ cells were largely subpially localized in the forming dentate pole (arrowheads). Scale bar: 200 µm in D; 100 µm in D1-D3. (**E**) At P2, the subgranular zone (SGZ) started to take shape. (E1-E3) Nestin-GFP+ cells began to seed the nascent SGZ with the largest population being adjacent to the pia, whereas most Tbr2+ cells were distributed in the ML. Scale bar: 200 µm in E; 50 µm in E1-E3. (**F**) By P7, the permanent neurogenic niche was formed in the subgranular zone. (F1-F3) Most Nestin-GFP+ and some Tbr2+ cells were present in the SGZ. Scale bar: 200 µm in F; 50 µm in F1-F3. (G1-G4) A schematic representation of the two stages involved in neurogenic zone relocation during the development of the dentate gyrus. Green dots indicate the location of stem/progenitor cells as labeled by Nestin-GFP transgene. D, dentate; DN, dentate notch; F, fimbria; FDJ, fimbriodentate junction; GCL, granule cell layer; Hem, cortical hem; Hip, hippocampus; HF, hippocampal fissure; ML, molecular layer; SGZ, subgranular zone; SVZ, subventricular zone; VZ, ventricular zone.

in the FDJ region (Fig. 1B, B3-4) and, in fact, this structure appeared to have formed as a continuation of the subpial collection of Tbr2+ cells seen at E14.5. Interestingly, in the FDJ, the strongly stained Nestin-GFP+ cells did not overlap with the Tbr2 staining, implying that these were largely non-overlapping cell populations (arrowheads in Fig. 1B1-B4).

At E17.5, Nestin-GFP+ cells had fanned out into the hilus (Fig. 1C,C1) and lined the hippocampal fissure (HF) (Fig. 1C1). Tbr2+ cells were also distributed across the hilus and along the HF (Fig. 1C2,C3). At P0, Nestin-GFP+ cells completely covered the dentate side of the HF and the subpial region of the future lower blade, whereas few Nestin-GFP+ cells were located in the hilus (Fig. 1D,D1,D3). Tbr2+ cells continued to mirror this distribution pattern (Fig. 1D2,D3).

By P2, Nestin-GFP+ cells and processes appeared to spread toward the hilus from the marginal zone (MZ) of the granule cell layer (GCL) (Fig. 1E,E1), but by contrast, most Tbr2+ cells were still restricted to the emerging molecular layer at this stage (Fig. 1E2,E3). At the end of the first postnatal week, most Nestin-GFP+ cells populated the hilus and the subgranular zone (SGZ), and had exited the molecular layer (Fig. 1F,F1). The Tbr2+ population in the molecular layer was reduced and now found largely in the SGZ (arrowhead in Fig. 1F2,F3).

Consistent with work in the adult dentate (Yamaguchi et al., 2000), perinatal analysis of Nestin-GFP+ cells with acute BrdU labeling showed that the subpial Nestin-GFP+ cells close to the HF were actively dividing with little overlap with Prox1 (see Fig. S1A,A′,A″ in the supplementary material). Similarly, subpial Tbr2+ cells were proliferating (see Fig. S1B in the supplementary material) with clear distinction from the reelin+ Cajal-Retzius cells (see Fig. S1C in the supplementary material). In agreement with their neurogenic nature in the developing cortex (Arnold et al., 2008; Englund et al., 2005; Sessa et al., 2008), the weak Tbr2+ cells also showed low Prox1 expression in both MZ and the newly formed SGZ at P5 (see Fig. S1D-F in the supplementary material). Taken together, the spatiotemporal distribution of Nestin-GFP+ and Tbr2+ cells reveal two phases of neurogenic zone transitions (Fig. 1G1-G4): the dentate VZ-to-subpial transition and the subpial-to-subgranular transition.

Formation of the transient neurogenic zone coincides with the appearance of transhilar glial processes

Since previous studies indicate that radial glial cells regulate the morphogenesis of the dentate gyrus (Eckenhoff and Rakic, 1984; Rickmann et al., 1987), we further investigated the distribution of

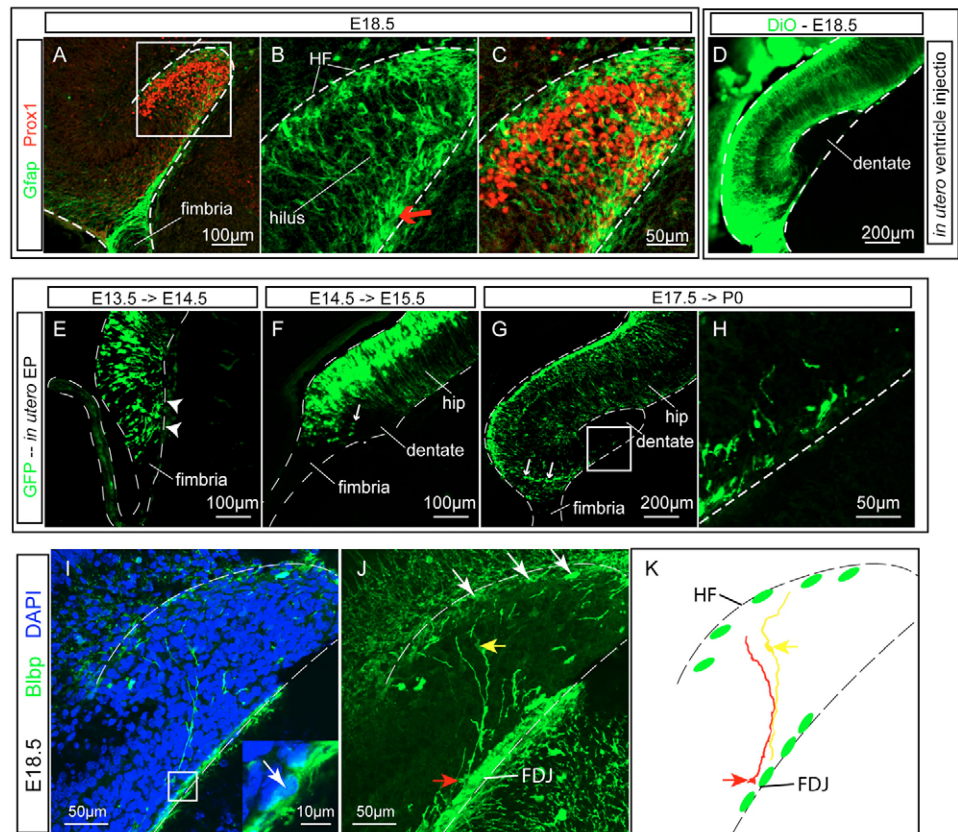

Fig. 2. Development of the transhilar glial scaffolding proceeds without evidence of VZ connection. (**A**) By E18.5, a distinct upper blade was formed as labeled by granule cell marker Prox1. The GFAP+ glial scaffolding was highly concentrated at the border of the fimbria and extended into FDJ. (**B**,**C**) GFAP+ glial orientation was correlated with the granule cell arrangement in the forming dentate plate. At the entrance of the hilus (red arrow in B), GFAP+ radial glia radiated out into HF, whereas most Prox1+ granule cells were gathered towards HF. Scale bar: 50 µm in B and C. (**D**) Labeling radial glia in the ventricular zone by DiO injected into the ventricle at E18.5 revealed that no radial glial fibers directly projected from the VZ into the forming dentate. (**E-H**) The dynamic changes of radial glial projection in the developing dentate by in utero electroporation (EP) of GFP expression vector targeting the medial wall. (E) Examined at E14.5 after EP at E13.5, GFP labeled radial glia at the dentate VZ showed the association of the endfeet with pia (arrowheads). (F) Examined at E15.5 after EP at E14.5, GFP labeled radial glia no longer extended into the field of the dentate gyrus, in contrast to the GFP labeled glial fibers radially oriented elsewhere in the hippocampal fields. A few cells (arrows) migrated toward the dentate gyrus. (G) Forty-eight hours after EP at E17.5, GFP marked the VZ of the whole medial wall. Radial glial processes in the hippocampal field were clearly labeled but not in the dentate gyrus. GFP labeled a stream of cells along the edge of the fimbria (arrows). Some of them already reached the entrance of hilus, which are shown at higher power in H. (**I-K**) The ongoing relocation of BLBP+ glia at E18.5. BLBP+ glial processes were revealed by thin section confocal imaging (I). BLBP+ cell bodies (arrow) were seen in the FDJ (inset in I). *z*-projection of image stacks revealed the long processes spanning from FDJ to HF. The location of individual cell bodies (red and yellow arrows in J) was identified through tracing serial sections. A few glial cell bodies already arrived at the HF (white arrows in J). (K) The two long processes and corresponding cell bodies (arrows), in addition to the glial cell bodies (in green) localized in the FDJ and HF. FDJ, fimbriodentate junction; HF, hippocampal fissure; Hip, hippocampus.

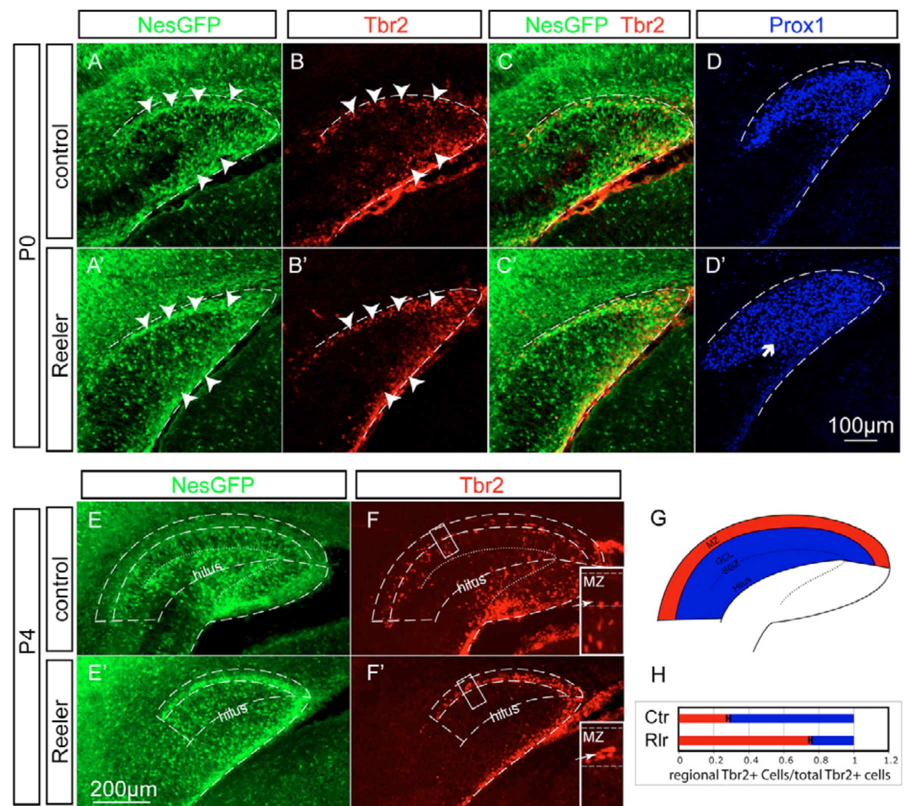

Fig. 3. Sustained subpial neurogenic zone in the reeler mice. (**A-C**,**A′-C′**) The formation of subpial neurogenic zone proceeded in reeler mutants. By P0, the Nestin-GFP+ (A,A′) and Tbr2+ (B,B′) cells were localized in the subpial region in both control and reeler mutant (arrowheads in A,B,A′,B′). Merged images for Nestin-GFP and Tbr2 in control and mutant are shown in C and C′, respectively. (**D**,**D′**) Granule cells migration relied on reelin signaling. At P0, Prox1+ granule cells were collected as a well-defined upper blade in the control (D), whereas they were evenly scattered in the dentate plate of the Reeler mutant (D′). (**E-F**,**E′-F′**) The prolonged presence of neurogenic precursors (both Nestin-GFP+ and Tbr2+ cells) in the subpial neurogenic zone in the reeler mutant at P4. By P4, as most of the Nestin-GFP+ cells left the subpial region in the control (E), many cells still persisted subpially in the reeler mutant (E′). Tbr2+ cells were loosely distributed in the molecular layer in control (F and inset). By contrast, they formed a compact subpial layer in the reeler mutant (F′ and inset). (**G**) The regional schema for quantification of Tbr2+ cells in the dorsal half of the dentate gyrus. The distribution of Tbr2+ cells at P4 was quantified in the MZ (in red) and the area (in blue) including granule cell layer and hilus. (**H**) The percentage of Tbr2+ cells in the control marginal zone (28.6±1%) was significantly lower than reeler mutant (75.4±0.9%, n=6, *P<0.001, χ^2-test). Ctr, control; Rlr, reeler; MZ, marginal zone; GCL, granule cell layer; SGZ, subgranular zone. Scale bar: 100 μm in A-D and A′-D′; 200 μm in E-F,E′-F′.

the radial glial scaffolding by GFAP staining as the Nestin-GFP+ precursors migrate from the dentate primordium to the subpial neurogenic zone. By E18.5, Prox1+ granule cells already occupied the forming upper blade, whereas GFAP+ glial fibers were enriched at the border of the fimbria (Fig. 2A). These GFAP+ fibers appeared to spread out at the entrance of the hilus and project to the pia all around the forming dentate (Fig. 2B), whereas Prox1+ granule cells were arranged in parallel to this glial scaffolding in the hilus (Fig. 2C). Previous studies assumed that these glial fibers are projecting from radial glial cells with their cell bodies located in the VZ (Eckenhoff and Rakic, 1984). To determine whether these hilar fibers directly project from the dentate VZ or the new organizing center at the FDJ (Fig. 2B, red arrow), we injected DiO solution into the ventricle of E18.5 embryos and allowed these to survive for only 3 hours to physically label all the fibers projecting from the VZ. This labeling prominently marked all the radial glial fibers spanning the whole hippocampal fields except the dentate field (Fig. 2D). It suggests that radial glial fibers in the forming dentate during this migratory phase do not directly project to the dentate from the dentate ventricular zone.

To further examine which cells contribute to this scaffolding in the developing dentate, a GFP expression construct was electroporated in utero into the medial wall, including the dentate VZ, at different ages followed by various survival times. In brains of mice electroporated at E13.5 and examined at E14.5, cells in the dentate VZ projected to the pia or cells themselves appeared to be migrating in the same trajectory (Fig. 2E, arrowheads). However, when electroporated at E14.5 and examined at E15.5, the dentate VZ showed no direct radial projection into the emerging dentate at all, despite the prominent radial glial scaffolding in the hippocampus proper (Fig. 2F). Instead, apparently migrating GFP+ cells were seen at the hilar entrance (Fig. 2F, arrow). Therefore, although it appeared that there was a direct VZ to dentate cellular connection at E14.5, this was no longer apparent by E15.5. To further address this at later stages, the medial cortex was electroporated at E17.5. Two days later, extensive labeling of radial fibers was seen in the hippocampal fields, but the dentate was completely devoid of radial fibers from the VZ (Fig. 2F). Instead, GFP+ cells formed a distinct stream from the dentate VZ along the fimbria (Fig. 2F, arrows) and some of these were again visible at the entry of the hilus (Fig. 2H).

Staining with a radial glial marker, brain lipid binding protein (BLBP), labeled a prominent subset of glial processes in the forming dentate at E18.5 (Fig. 2I). Strikingly, unlike with GFAP staining, glial cell bodies were clearly identifiable with BLBP in the FDJ (arrow in Fig. 2I inset) and some of them had already reached the HF (arrows in Fig. 2J). A collapsed z-projection of the serial sections is shown in Fig. 2J, allowing the reconstruction of two whole BLBP+ cells. The somata of the BLBP+ processes spanning the hilus were pinpointed by tracing the overlapping thin sections (Fig. 2K). Interestingly, somata were found near the FDJ pia or in the hilus (red and yellow arrows in Fig. 2J,K), suggesting that some glia were in the process of migration from the FDJ towards the HF.

Reelin is dispensable for the formation of but controls exit from the transient zone

Numerous studies have shown that reelin secreted from Cajal-Retzius cells is essential for the development of the neocortex and hippocampus by controlling the proper lamination of projection

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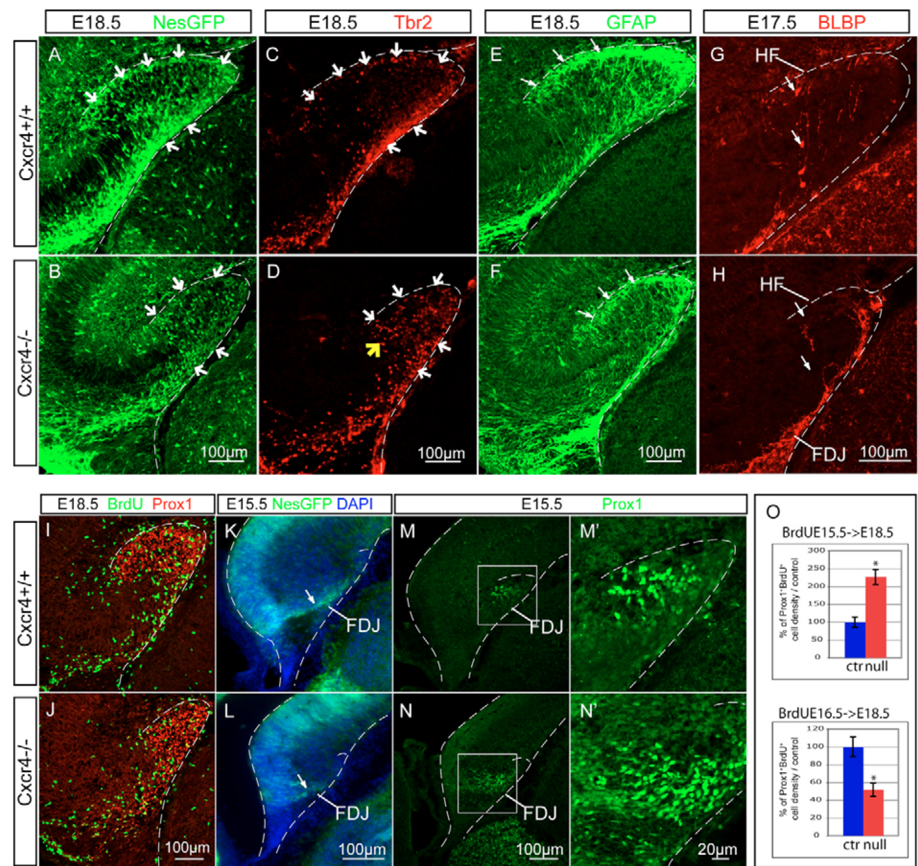

Fig. 4. Defective subpial neurogenic zone, abnormal radial glial scaffolding and premature granule cell production in Cxcr4 null. (**A-D**) Defective subpial neurogenic zone in Cxcr4 mutant. By E18.5, Nestin-GFP+ and Tbr2+ cells were scarce in the subpial region in the Cxcr4 mutant compared with the control (white arrows in A-D). Tbr2+ cells were ectopically packed in the hilus in the mutant (yellow arrow in D). (**E-H**) Aberrant development of the transhilar glial fibers in the Cxcr4 mutant. At E18.5, GFAP+ processes were more enriched in the HF in the controls compared with the mutants (arrows in E,F). A subset of transhilar glial fibers labeled by BLBP at E17.5 in the controls (G) were almost lost in the mutants (H). BLBP+ somas seen in the HF and hilus of controls were absent in the mutants (arrows in G,H). (**I-O**) Premature production of granule cells in the Cxcr4 mutant. By E18.5, acute BrdU labeling was decreased in the forming dentate plate in the mutant (J) when compared with the control (I). However, Prox1+ granule cells were not overwhelmingly affected in the mutant despite the abnormal distribution on the migratory stream (J). By E15.5, Nestin-GFP+ precursors were able to leave the dentate primordium and form a migratory stream in the mutant (arrow in L), although it was not as robust as in the control (arrow in K). At this stage, there were more Prox1+ granule cells in the mutant (N) compared with the control (M). Boxed areas in M and N are shown at higher power in M′ and N′. (O) Birthdating analysis with BrdU pulses (at E15.5 and E16.5) and quantification at E18.5 showed that the density of BrdU+/Prox1+ cells produced at E15.5 was much higher in the mutants (227±21%), but much lower at E16.5 (51.7±7.5%) compared with the controls (n=4, *P<0.01, Student's t-test).

neurons (Rice and Curran, 2001). As the dentate is also quite abnormal in reelin mutants (Forster et al., 2002), we wondered what role reelin plays in the migration of dentate precursors to the newly identified transient subpial zone. To tackle this issue, we examined the distribution of Nestin-GFP+ cells at birth in reeler mice. In both the controls and reeler mice, Nestin-GFP+ or Tbr2+ cells were properly localized to the subpial zone (arrowheads in Fig. 3A,B and Fig. 3A′,B′). Consistent with the known role of reelin in neuronal migration, Prox1+ granule cells were abnormally distributed across the dentate field in the mutants (arrow in Fig. 3D′) instead of forming a relatively compact upper blade as in the controls (Fig. 3D). Thus, reelin signaling is required for proper granule cell migration but not for the subpial localization of Nestin-GFP+ cells.

In control mice at P4, most of the Nestin-GFP+ cells were in the SGZ and hilus, whereas the subpial zone was essentially depleted of Nestin-GFP+ cells (Fig. 3E). Strikingly, in Reeler mutant mice, the subpial zone was still packed with Nestin-GFP+ cells (Fig. 3E′). As the upper blade was well-defined at this age, we quantified the distribution of Tbr2+ cells in the dorsal half of the dentate (Fig. 3H) with the regional schema shown in Fig. 3G. Only a small proportion of Tbr2+ cells (28.6±1%) were localized in the control marginal zone, with the majority (71.4±1%) scattered in the GCL, SGZ and hilus (arrow in Fig. 3F, inset), whereas in the mutants the Tbr2+ cells (75.4±0.9%) were clustered in the marginal zone (arrow in Fig. 3F′, inset) with a small number (24.6±0.9%, n=6, *P<0.001, χ^2-test, Fig. 3H) in the region outside the marginal zone. Therefore, the dentate neurogenic niche failed to undergo the subpial-to-subgranular transition in the absence of functional reelin. Although previous studies (Forster et al., 2002) suggest that reelin signaling may cell-autonomously regulate the behavior of radial glial cells, it is also possible that this role for reelin could be due to a non-autonomous role for reelin in organizing other cellular components in the dentate. However, what is clear is that reelin is dispensable for the original positioning of radial glial and transit-amplifying cells in the subpial zone but is indispensable for the later reorganization of this zone.

Formation of the subpial zone requires Cxcl12/Cxcr4 signaling

Previous studies indicate that the chemokine Cxcl12 and its cognate receptor Cxcr4 regulate the morphogenesis of the dentate gyrus (Bagri et al., 2002; Lu et al., 2002), but the mechanistic basis of this defect is not well characterized. More recent studies suggest the Cxcl12/Cxcr4 signaling plays a crucial role in regulating the positioning of neurons adjacent to the pia (Borrell and Marin, 2006; Li et al., 2008a; Lopez-Bendito et al., 2008; Paredes et al., 2006; Tiveron et al., 2006). Owing to the expression of Cxcr4 in the migratory stream and subpial zone and expression of Cxcl12 by the pial meninges (see Fig. S2 in the supplementary material) (Berger et al., 2007), we sought to determine whether Cxcl12/Cxcr4 signaling controls the concentration of Nestin-GFP+ precursors in the subpial region of the developing dentate. At E18.5 in control animals, Nestin-GFP+ cells occupied the subpial region around the

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entire profile of the forming dentate gyrus from hippocampal fissure superficial to the nascent upper blade and ventrally to the future lower blade (arrows in Fig. 4A). In stark contrast, in Cxcr4$^{-/-}$ mice the Nestin-GFP+ cells were largely scarce in the subpial region (arrows in Fig. 4B). Consistent with these findings, Tbr2+ cells no longer formed a compact subpial zone along the FDJ and HF in the Cxcr4 mutants as they did in controls (white arrows in Fig. 4C,D). Instead, Tbr2+ cells were widely dispersed in the dentate (yellow arrow in Fig. 4D). Taken together, these findings indicate that Cxcr4 is required for the proper formation of the subpial neurogenic zone.

Subpial organization of precursors correlates with the transhilar glial scaffolding and neuronal differentiation

The displacement of Nestin-GFP+ progenitors from their subpial location prompted us to ask whether this defect affects the development of the transhilar glial scaffolding. Staining for GFAP showed dense glial processes across the hilus and enriched fiber plexus at the HF in the controls (Fig. 4E). By contrast, Cxcr4 mutants showed reduced hilar GFAP+ fibers and decreased fiber plexus in the HF (Fig. 4F). In the controls, BLBP staining revealed a subset of transhilar glial fibers emanating from the FDJ and crossing the hilus (Fig. 4G). BLBP+ somata were clearly identified in three locations: FDJ, hilus and HF. However, both BLBP+ processes and somata were almost absent in the hilus and HF in mutants (arrows in Fig. 4G,H).

To determine whether the disruption of the subpial zone and the radial glial progenitors leads to any dynamic consequences for the cellular output of the stem/progenitor cells, we analyzed acute BrdU labeling at E18.5. In agreement with previous studies (Bagri et al., 2002; Lu et al., 2002), we found that the number of BrdU+ cells was significantly decreased in the dentate of Cxcr4 mutants compared with the controls (Fig. 4I,J). However, this finding cannot be simply explained by an increase in cell death or a migration defect resulting from the loss of Cxcr4, as the production of granule cells did not seem to have drastically declined despite their abnormal distribution (Fig. 4I,J). We noticed that in association with the decrease in BrdU+ cell numbers in the Cxcr4 mutants at E18.5, there was not only an overall decrease in the number of Nestin-GFP+ cells (Fig. 4A,B) but also a corresponding increase in the number of Tbr2+ cells in the dentate field of the Cxcr4 mutants compared with the controls (Fig. 4C,D). This led us to look into the possibility that dentate precursors prematurely differentiate into granule cells when they were displaced from the subpial zone. At E15.5, a robust stream of Nestin-GFP+ cells was present in the controls but it was diminished in the Cxcr4 mutants (arrows in Fig. 4K,L). Conversely, the mutants had a larger patch of Prox1+ granule cells around the FDJ than the controls. This loss of progenitors and the excess of granule cells at this early developmental stage suggested the premature differentiation of dentate progenitors upon displacement from the subpial zone.

To test this more directly, we birthdated granule cells by injecting BrdU at E15.5 and counted the number of BrdU+/Prox1+ cells at E18.5. Interestingly, the density of double-labeled cells was dramatically higher in the mutants (227±21%) compared with controls (n=4, *P<0.05, Student's t-test). However, when BrdU was administered at E16.5, the density of BrdU+/Prox1+ cells was significantly lower in mutants Cxcr4 mutants (51.7±7.5%) than controls (n=4, *P<0.01, Student's t-test) (Fig. 4O). This indicates that mutant mice have an early excessive burst of production of

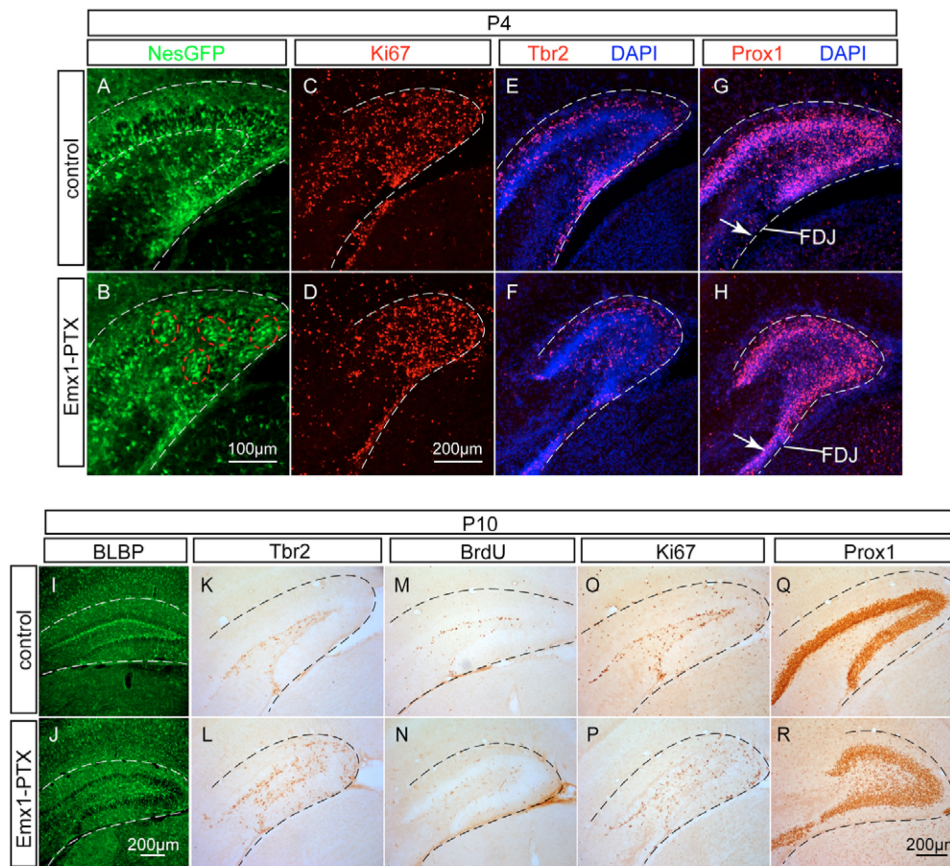

Fig. 5. Compromised organization of the subgranular niche in Emx1-PTX mice. (**A-H**) Abnormal SGZ organization at early postnatal ages in Emx1-PTX mice. By P4, Nestin-GFP+ cells started to populate the SGZ in the controls (A), whereas they showed patchy distribution in the Emx1-PTX animals (red outlines in B). Compared with the control (C and E), the Ki67+ proliferating cells tended to cluster in the hilus and Tbr2+ cells were more widely spread in the Emx1-PTX animal (D,F). In contrast to the controls (G), the granule cell layer revealed by Prox1 staining was very poorly organized in the Emx1-PTX animal, with ectopic cells in the migratory stream (arrow in H). (**I-R**) Persistent defects in the organization of subgranular zone in Emx1-PTX mice at P10. Compared with the controls (I,K,M,O), the transgranular radial glial scaffolding labeled by BLBP failed to form properly (J) and the SGZ (shown by BrdU, Ki67 and Tbr2 staining in L, N and P) was dramatically disorganized in the Emx1-PTX animals. In sharp contrast to the control (Q), the Prox1+ GCL did not form a distinct boundary with the hilar region and there were numerous ectopic granule cells in the hilus and in the migratory route in the Emx1-PTX animals (R). Scale bar: 100 μm in A,B; 200 μm in C-H; 200 μm in I,J; 200 μm in K-R.

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granule neurons but fail to produce the appropriate number of granule cells only a day later. Taken together, these data indicate that progenitors displaced from the subpial zone prematurely differentiate and the localization to the subpial transient zone may be required to maintain dentate precursors in an undifferentiated state during late embryonic stages.

SGZ formation is not affected in Emx1-Cxcr4 cKO but severely compromised in Emx1-PTX mice

To examine the SGZ formation, we used a conditional knockout model by crossing the floxed Cxcr4 allele with Emx1$^{ires\text{-}cre}$ (Gorski et al., 2002) (Emx1-Cxcr4 cKO thereafter) to bypass the prenatal lethality of null Cxcr4. The prenatal development of the dentate gyrus in Emx1-Cxcr4 cKO resembled the null mutants with Tbr2 and Prox1 staining (see Fig. S3 in the supplementary material).

By P5, there was distinct reorganization of the radial glial scaffolding in control mice shown by the Nestin-GFP+ cells at the SGZ, whereas in the cKO mice the Nestin-GFP+ cells were scattered throughout the dentate formation (see Fig. S4A,B in the supplementary material). However, the distribution patterns of Tbr2+ or Ki67+ cells showed subtle differences between controls and cKOs (see Fig. S4C-F in the supplementary material). Surprisingly, despite the prenatal abnormalities in the early GCL of the *Cxcr4*$^{-/-}$ and the Emx1-Cxcr4 cKO, Prox1+ cells formed distinct upper and lower blades of GCLs in the cKO mice (see Fig. S4G,H in the supplementary material). Our data suggests the granule cells are largely able to adopt appropriate layer positioning in Emx1-Cxcr4 cKO despite the early defects in SPZ.

At P14, Emx1-Cxcr4 cKOs showed almost normal organization of the SGZ with BLBP, Tbr2 and BrdU (see Fig. S4I-N in the supplementary material) and GCL with Prox1 (see Fig. S4O-P in the supplementary material). This recovery was sustained into adulthood (see Fig. S5 in the supplementary material). As the cre activity of Emx1$^{ires\text{-}cre}$ completely covered dentate primordium at E14.5 and showed complete recombination at P14 (see Fig. S6A-F in the supplementary material), low penetrance of Emx1$^{ires\text{-}cre}$ is unlikely to explain the developmental recovery in the cKO mice.

One possible explanation for the SGZ recovery in the Emx1-Cxcr4 cKOs is that other ligand-receptor systems may compensate for the loss of Cxcr4. To test this, pertussis toxin (PTX) expression (Regard et al., 2007) was conditionally activated by Emx1$^{ires\text{-}cre}$ (Emx1-PTX thereafter), potently blocking all the trimeric $G_{i/o}$ signaling including Cxcr4. As expected, the perinatal subpial neurogenic zone did not properly form in the Emx1-PTX animals (see Fig. S7 in the supplementary material). By P4, Nestin-GFP+ cells were distributed as patches scattered throughout the dentate (Fig. 5A,B) and both Ki67+ and Tbr2+ cells were chaotically dispersed throughout the whole dentate (Fig. 5C-F). Prox1+ granule cells also failed to assume their distinct layered organization and many were ectopically located in the subpial region of FDJ (Fig. 5G,H). Emx1-PTX animals died in the second postnatal week, so we chose P10 animals for further analysis. Compared with the controls (Fig. 5I,K,M,O), Emx1-PTX animals almost complete lost organization of the BLBP+ scaffolding in the SGZ (Fig. 5J); furthermore, Tbr2+ neurogenic precursors and the BrdU+ or Ki67+ cycling cells in the SGZ were also ectopically localized in the MZ, GCL and hilus (Fig. 5L,N,P). In addition, the border between the hilus and Prox1+ GCL was obscured owing to ectopic dispersion of granule cells into the hilus and granule cell heterotopias were visible in the remnant of the migratory stream to the dentate (Fig. 5Q,R). Therefore, the formation of SGZ appears to rely on a PTX-sensitive pathway.

Contribution of subpial progenitors to the formation of the subgranular zone

Previous genetic fate-mapping analysis with the Gli1^{CreERT2} line revealed that the self-renewing stem cells in the dentate gyrus first appear at the late embryogenesis (Ahn and Joyner, 2005). In order

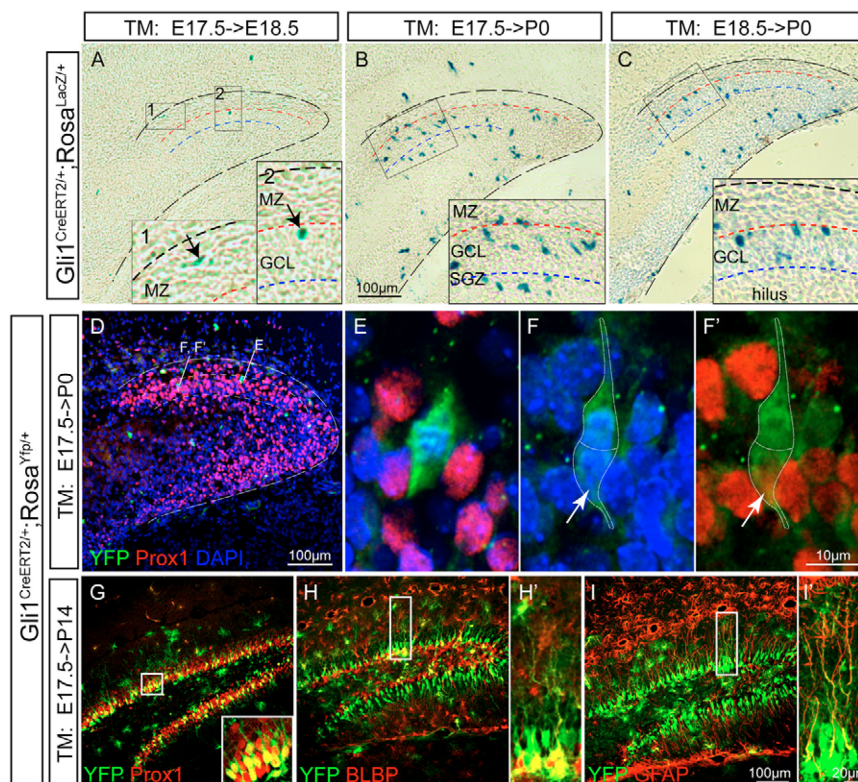

Fig. 6. Contribution of subpial precursors to the SGZ. (**A**) Tamoxifen (TM) was administered at E17.5 into Gli1^{CreERT2} line to mark the Shh-responding cells with Rosa-*lacZ* reporter. Twenty-four hours later, the labeled cells were found in the marginal zone (inset 1) and the outer edge of the upper blade (inset 2). (**B**) Forty-eight hours later, the labeled cells spread into the granule cell layer and appeared in the hilus (inset). (**C**) When tamoxifen was administered at E18.5, most labeled cells appeared 24 hours later in the upper blade (inset) and the future lower blade. (**D-F**) RosaYFP reporter was used to mark the Shh-responding cells when tamoxifen was administered at E17.5 into Gli1^{CreERT2} line. Only very few cells were GFP+. One GFP+ cell in the upper blade did not show Prox1 expression (E), whereas one of the GFP+ doublet started to have weak Prox1 expression (arrows; F,F′). (**G-I**) The Shh-responding cells marked at E17.5 gave rise to Prox1+ granule cells at P14 (G and inset). Some of them were BLBP+ (H,H′) or GFAP+ (I,I′). Boxed areas in H and I are shown at higher power in H′,I′, respectively. GCL, granule cell layer; MZ, marginal zone; SGZ, subgranular zone. Scale bar: 100 µm in A-D,G-I; 10 µm in E,F,F′; 20 µm in H′-I′.

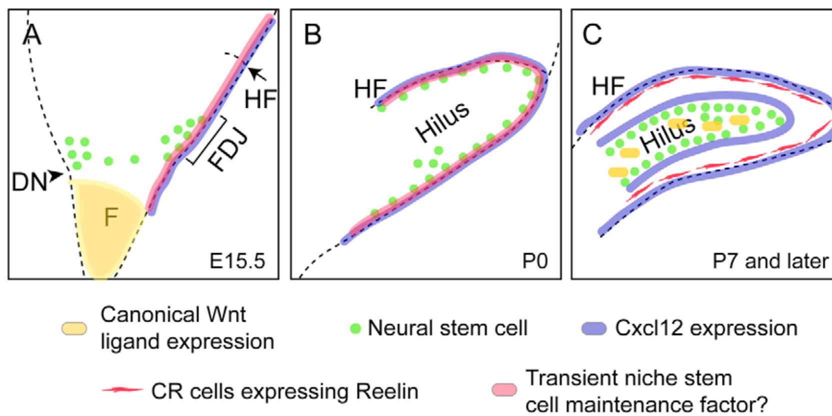

Fig. 7. Schematic representation of the progression of dentate stem cells to form the SGZ. This figure shows our conception of the events leading to the formation of the SGZ. (**A**) Canonical Wnts are embryonically expressed in the fimbria/cortical hem and support expansion of dentate progenitors. (**B**) During their migration, the dentate progenitors are located in the transient neurogenic zone, where their position is maintained by Cxcl12 signaling. (**C**) By the first postnatal week they relocate to the SGZ where they are regulated by Wnts from the hilus and Shh (not shown) from unknown sources. DN, dentate notch; F, fimbria; FDJ, fimbriodentate junction; HF, hippocampal fissure.

to test whether the SPZ progenitors may contribute to the neural stem cells settled in the SGZ, we reasoned that when tamoxifen (TM) was injected at E17.5 into the Gli1^{CreERT2} line in the presence of Rosa-*lacZ* reporter, the labeled cells would initially emerge from the subpial zone and then spread toward the GCL from there over time. If the hilar progenitors exclusively contribute to the SGZ, we would expect the opposite. Interestingly, after 24 hours, *lacZ*+ cells were first detected in the MZ (inset 1 in Fig. 6A) and the edge between MZ and GCL (inset 2 in Fig. 6A) in the upper blade. After 48 hours, *lacZ*+ spread across the GCL and SGZ in the upper blade (Fig. 6B and inset). When tamoxifen was administered at E18.5 and *lacZ* expression was analyzed 24 hours later, we found most *lacZ*+ cells were restricted in the GCL of the upper blade (Fig. 6C and inset) and others were observed in the future lower blade (Fig. 6C). To further analyze the cellular identity of cells produced after recombination induced at E17.5, we turned to the RosaYFP reporter line. The earliest GFP+ cells were detected at P0 (Fig. 6D) and did not express Prox1 (Fig. 6E). In other cases, it appeared that a cell might have just divided and Prox1 could be detected in one of the GFP+ doublet cells (arrows in Fig. 6F,F′). When cell fates were mapped in animals at P14, most recombined cells were Prox1+ granule cells (Fig. 6G, inset), and a few of them showed radial glial morphology (Fig. 6H,I) and were co-labeled with BLBP (Fig. 6H,H′) or GFAP (Fig. 6I,I′). Taken together, these findings support the idea that perinatal subpial progenitors contribute to the neural stem cells that eventually settle in the SGZ.

DISCUSSION

In this study, we described the progressive development of distinct neurogenic zones in the dentate gyrus, from the dentate VZ to the subpial zone (SPZ) and then to the subgranular zone (SGZ). We found that reelin signaling is dispensable for the formation of the SPZ but is required for the timely transition of dentate precursors from the SPZ to the SGZ. Cxcl12/Cxcr4 signaling is essential for the initial organization of the SPZ. When subpial localization of precursors is impaired, the transhilar glial scaffolding and the undifferentiated state of the dentate precursors are altered. In postnatal mice with loss of Cxcr4 signaling, the SGZ initially fails to form properly but shows gradual recovery so that it appears normal by the second week of life. A conditional PTX reporter mouse demonstrates that this recovery is likely to be due to the developmental onset of compensatory mechanisms. Fate-mapping analysis shows that the subpial precursors contribute to the neural stem cells in the SGZ. Taken together, these data provide a novel framework for understanding the development of the subgranular zone in the dentate gyrus where neurogenesis persists throughout adulthood.

A novel, temporary neurogenic zone in the developing dentate gyrus adjacent to the meninges

The most distinct feature of dentate development compared with other forebrain areas is the extended migration of neural precursors from the VZ to a newly formed region in the SGZ. The SGZ is a long-lived durable niche that allows survival and self-renewal of neural stem cells. But how do neural precursors manage to traverse the territories from VZ to ultimately form SGZ? This is not a small hurdle, because at the stage that precursors begin to exit the VZ there is no formed dentate gyrus or SGZ for them to occupy.

Our analysis of the Nestin-GFP transgene in combination with transit amplifying cell marker Tbr2 shows that neurogenic precursors initially follow a subpial migratory route to fimbriodentate junction, some then move across the hilus and take residence in the subpial region of the hippocampal fissure leaving a population of progenitors adjacent to the pia around the entire pole of the dentate (Fig. 7). From this base of operations, neurogenic precursors generate Tbr2+ transient amplifying cells and then granule neurons that form the initial structure of the dentate granule cell layer. Nestin-GFP+ and Tbr2+ cells gradually disappear from the subpial region in the first postnatal week, both cell types accordingly increase in the SGZ and hilus, suggesting there is a subpial-to-hilar transition. In agreement with our findings, Ngn2 mutant mice were recently shown to have severe neurogenic defects in the developing dentate gyrus and using a Ngn2-GFP mouse line the same authors found a similar group of neurogenic precursors was in close proximity to the subpial zone (Galichet et al., 2008).

Complex organization of the transient neurogenic zone in the developing dentate gyrus

The transient subpial zone is regulated by Cxcl12 secreted from meningeal fibroblasts and reelin from the Cajal-Retzius cells in the dentate marginal zone. It is interesting to consider what other factors may be important in the constitution of this zone and their ability to maintain dentate progenitors in an undifferentiated state (Fig. 7B). The analysis of the mutants in the canonical Wnt signaling pathway suggests that Wnts play a crucial role in the expansion and maintenance of the dentate precursor pool during this same developmental period (Galceran et al., 2000; Grove et al., 1998; Lee et al., 2000; Zhou et al., 2004). Also shown to be active and required for the formation of dentate stem cell niches is sonic hedgehog (Shh) (Machold et al., 2003). However, it is not clear which cells express Shh or Wnts in the subpial zone. There is evidence that the subgranular zone niche is intimately associated with blood vessels (Palmer et al., 2000) and that endothelial cells may provide

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important regulators to stem cell behaviors (Shen et al., 2004). These findings may have relevance to the timing and importance of the subpial zone.

The roles of Gi/o signaling pathway in stem cell migration and maintenance

The loss of Cxcr4 only transiently affects the formation of the SGZ despite the disorganization of the transient SPZ. Prominent roles for other chemokines and their receptors have been postulated in the dentate gyrus (Tran et al., 2007). The redundancy of signaling pathways is evidenced by our use of the recently developed Cre-mediated PTX expression line (Regard et al., 2007). These mice have a very dramatic developmental dentate phenotype, which suggests the search for other $G_{i/o}$-coupled receptor/ligand combinations in the dentate gyrus is likely to result in both other important developmental regulators of dentate morphogenesis but also perhaps potent molecular targets to design reagents to regulate dentate neurogenesis.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/2/327/DC1

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