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The chemokine SDF1 regulates migration of dentate granule cells

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SUMMARY

The dentate gyrus is the primary afferent pathway into the hippocampus, but there is little information concerning the molecular influences that govern its formation. In particular, the control of migration and cell positioning of dentate granule cells is not clear. We have characterized more fully the timing and route of granule cell migration during embryogenesis using in utero retroviral injections. Using this information, we developed an in vitro assay that faithfully recapitulates important events in dentate gyrus morphogenesis. In searching for candidate ligands that may regulate dentate granule cell migration, we found that SDF1, a chemokine that regulates cerebellar and leukocyte migration, and its receptor CXCR4 are expressed in patterns that suggest a role in dentate granule cell migration. Furthermore, CXCR4 mutant mice have a defect in granule cell position. Ectopic expression of SDF1 in our explant assay showed that it directly regulates dentate granule cell migration. Our study shows that a chemokine is necessary for the normal development of the dentate gyrus, a forebrain structure crucial for learning and memory.

Key words: Hippocampus, Neuronal migration, Chemokine, Dentate granule cell, Mouse

INTRODUCTION

The dentate gyrus is the chief route of excitatory input into the hippocampal formation and is thus crucial for appropriate function of the hippocampus in learning and memory. Many pathological states associated with histological abnormalities in the dentate are likely to have their origins during development. These disorders include generalized abnormalities of neuronal migration, e.g. reeler and associated mutants (Frotscher, 1998; Stanfield and Cowan, 1979a; Stanfield and Cowan, 1979b), and defects that are more restricted to the dentate gyrus, e.g. humans with temporal lobe epilepsy with apparent migration abnormalities of dentate granule neurons (Houser, 1990; Houser et al., 1992).

It is now clear that the dentate gyrus is one of the few sites in the rodent brain that undergoes continued neurogenesis throughout life with continued production of granule cell neurons from precursor cells that reside at the border between the dentate granule cell layer and the hilus (the subgranular zone – SGZ) (Altman and Bayer, 1990a; Altman and Das, 1965a; Gage, 2000; Pleasure et al., 2000b). Recent experiments have shown that dentate granule cell neurogenesis is increased by physical activity and the performance of learning paradigms and that continued dentate neurogenesis is likely to be necessary for the ability to perform specific types of learning tasks (Gould et al., 1999; Kempermann et al., 1997; Shors et al., 2001; van Praag et al., 1999).

During development, an important event in the formation of the dentate granule cell layer is the migration of cells from the dentate neuroepithelium to take up residence in the dentate gyrus anlage. Traditional neuroanatomical methods have shown that the early stages of migration consist of a mixture of postmitotic neurons and precursor cells that form the primary dentate granule cell layer and populate the hilus (Altman and Bayer, 1990a; Altman and Bayer, 1990b; Pleasure et al., 2000b). As development proceeds, the precursor cells in the hilus proliferate and produce large numbers of granule cells through the first month of postnatal life before settling in the SGZ and gradually reducing their output of new granule cells to a lower basal rate through adulthood (Altman and Bayer, 1990a; Altman and Bayer, 1990b; Altman and Das, 1965a; Altman and Das, 1965b; Pleasure et al., 2000b).

We have been examining factors that regulate the development of the dentate granule cell lineage in order to better understand the developmental organizing principles that govern the initial migration of granule cells to the dentate gyrus. As part of these studies, we have become particularly interested in the regulation of the initial migration of cells that

populate the dentate anlage from the neuroepithelium because perturbations in these initial events are likely to have dramatic consequences for the later organization of the dentate gyrus. Indeed, analysis of *Neurod1* mutants (previously known as β2 and NeuroD) bears out this assumption. These mice have a defect in the initial production of granule cells that leads to the failure to properly form a morphologically distinct dentate gyrus (Liu et al., 2000; Miyata et al., 1999). This early failure to produce granule cells leads to a lifelong absence of these cells even though certain features of the mutant phenotype are rescued by later developmental expression of closely related homologs of *Neurod1* (Liu et al., 2000). In addition, the formation of heterotopic clusters of granule-like cells in the *Neurod1* mutant mice, which have some histological similarities to the dentate gyri of individuals with epilepsy (Houser, 1990; Houser et al., 1992), are associated with limbic epilepsy in these mice (Liu et al., 2000).

Recent studies have shown that a chemokine, SDF1, regulates migration of cerebellar granule neurons (Klein et al., 2001; Ma et al., 1998; Zou et al., 1998). In the cerebellum, SDF1 is believed to act as an attractant, not allowing the granule cells to leave the zone of high SDF1 expression until the appropriate developmental timepoint. These studies have shown direct effects of SDF1 on regulating granule cell chemotaxis in dissociated cell cultures, but there have been no studies showing direct regulation of migratory behavior in assays closely related to the in vivo situation (Klein et al., 2001).

In this study we examine the migratory behavior of cells from the neuroepithelium to the dentate anlage using several techniques both in vivo and in vitro. From these studies we are able to show the normal migratory route of these cells, the time that cells take to reach the dentate gyrus and to validate a robust assay for migration of these cells in vitro. Using this assay and mutant mice, we show that SDF1, a chemokine, directly regulates migration of dentate granule cells and that mice without SDF1 receptors have granule cell migration abnormalities.

MATERIALS AND METHODS

Mouse breeding, genotyping and tissue preparation

All animals were treated according to protocols approved by the Committee on Animal Research at the University of California, San Francisco. Tissue was obtained from E14.5, E15.5 and E17.5 embryos and P0 wild-type mice and E18.5 *Cxcr4*$^{+/-}$ and *Cxcr4*$^{-/-}$ embryos (*Cmkar4* – Mouse Genome Informatics) from matings between two *Cxcr4*$^{+/-}$ mutant animals. Embryonic tissue was obtained by Caesarian section, anesthetized by cooling, while P0 mice were anesthetized by Nembutal. The animals were then perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and postfixed in PFA for 2-8 hours. Genotyping for *Cxcr4* animals was performed as described previously (Zou et al., 1998). After fixation, brains were either cryoprotected in 30% sucrose and frozen in embedding medium and cut in a cryostat (wild-type tissue) or embedded in 5% agarose and cut on a vibrating microtome (*Cxcr4*$^{+/-}$ and *Cxcr4*$^{-/-}$ tissue).

In situ hybridization

A 584 bp coding fragment of the murine CXCR4 cDNA and a 180 bp coding fragment of murine SDF1 gene obtained by PCR were cloned into Bluescript (ks) (Promega Life Sciences, Madison, WI) and used for probe synthesis after confirmatory sequence was obtained. In situ hybridization of E14.5 and E18.5 wild-type tissue sections with digoxigenin probes was performed as described (Pleasure et al., 2000b). Other chemokine and chemokine receptors examined were: ligands, MDC, eotaxin, TECK, ELC, Rantes, SLC, TARC, neurotactin, MCP3, MCP5, MIP1α, MIP1β and MIP3α; receptors, CXCR1, CXCR2, CXCR3, CXCR5, CX3CR1, CCXCKR, CCR1, CCR2A, CCR2B, CCR4, CCR5, CCR6, CCR7 and CCR9.

Prox1 antisera

The Prox1 antiserum was generated against the C-terminal 15 amino acids of mouse Prox1 (completely conserved between mouse and human) in New Zealand White rabbits exactly as described previously (Torii et al., 1999). The rabbits were immunized every 3 weeks and the titer checked by ELISA. After the fifth boost, the antiserum was specific for dentate granule cells in adult mouse brain sections.

Immunohistochemistry

Immunohistochemistry was performed on 40 μm free-floating sections (E18.5 and P0) or on 12 μm cryostat sections mounted onto glass slides (all other ages). Free-floating sections were preincubated in 5% normal serum of the species in which the secondary antibody was raised, 1% bovine serum albumin (BSA) and 0.3% TX in PBS for 1 hour at room temperature, and subsequently incubated with the primary antisera for 24-36 hours at 4°C in 2% normal serum and 0.3% TX in PBS. The following antibodies were used: rabbit anti-Prox1 (diluted 1:5000), rabbit anti-Phosphohistone-H3 (diluted 1:1000; Upstate Biotechnology) and rabbit anti-nestin (gift of R. McKay; diluted 1:1000). Sections were then incubated in biotinylated secondary antibodies (Vector; diluted 1:200) and processed by the ABC histochemical method (Vector). The sections were then mounted onto Superfrost Plus slides (Fisher), dried, dehydrated and coverslipped with Permount (Fisher). In each experiment, sections from homozygous mutants and their heterozygous littermates were processed together. Primary antiserum omission controls and normal mouse, rabbit and goat serum controls were used to confirm further the specificity of the immunohistochemical labeling. Immunohistochemistry was performed on cryostat sections on glass slides using essentially the same protocol.

Generation of pseudotyped retroviruses

Retroviral plasmid vectors (nls*lacZ* or PLAP) with internal CMV promoters were transfected into the 293GPG stable packaging line to produce pseudotyped retrovirus (Ory et al., 1996). Virus was collected over 5-6 days and concentrated by ultracentrifugation to produce high-titer retroviral stocks. Virus was titered by infection of NIH3T3 cells as described by others (Ory et al., 1996).

In utero intraventricular injections

All experimental animals were treated according to protocols approved by the University of California, San Francisco. Briefly, time-pregnant rats were fully anesthetized with ketamine (90 mg/kg), xylazine (5mg/kg) and acetylpromazine (2.5 mg/kg) intramuscularly. A 2 cm vertical midline incision was made through the abdomen to expose the uterus. A fiber-optic transilluminator was used to identify the cerebral ventricles of fetuses and each embryo in the litter received approximately 1 μl of the retroviral solution (containing ~1×10^8 infective particles/μl and 0.1 mg/ml polybreme) injected intraventricularly via Hamilton syringe with a 30-gauge needle. The uterus was replaced into the abdominal space and the abdominal muscle and skin was then closed using 3-0 silk suture. The animal was then given an analgesic (buprenorphine 0.01 mg/kg for rats) to minimize peri-operative wound pain and monitored for recovery.

Animals were allowed to survive for various length of time and then the embryos were harvested as described above. A select group of animals were allowed to deliver pups. Tissue was harvested from these animals at various postnatal time-points as described above. To

visualize cells, tissue was embedded in 5% agarose and cut on a vibrating microtome into 50 μm sections. The tissue was then processed for X-gal histochemistry to detect the β-gal marker, counterstained with nuclear Fast Red, mounted onto Superfrost Plus slides (Fisher), dried, dehydrated and coverslipped with Permount (Fisher). For tissue infected with retrovirus expressing PLAP, alkaline phosphatase (AP) histochemistry was performed (Leighton et al., 2001) and the tissue was mounted onto superfrost slides (Fisher) and mounted with Aquamount.

We performed several types of controls to be sure that the patterns of distribution of labeled cells represent true migration rather than artefact. Retroviral particles lose infectivity within hours at 37°C and therefore continued infection of cells at the ventricular zone at long periods after introduction into the ventricles seems unlikely. However, to rule out this senario further, we incubated retrovirus at 37°C for 12 hours and then injected the virus into ventricles of E18 embryos. After 3 days, no infected cells in the ventricular zone or migratory route were found in any of the embryos (n=5), confirming the idea that labeling of cells in the ventricular zone occurs soon after introduction of the virus into the ventricles. Additionally, to ensure that cells infected with nls*lacZ*- and PLAP-expressing viruses behaved similarly, a small number of embryos were injected with a mixture of the two retroviruses and allowed to develop for 3 days in utero. Serial sections from these embryos were processed for X-gal and AP histochemistry. Migratory patterns for both viruses appeared similar, indicating that cells infected with either virus behaved similarly.

Slice culture

The slice migration assay was performed as described previously (Anderson et al., 1997). Briefly, brains from E15.5 CD-1 mouse embryos were embedded in 4% low-melting agarose and 250 μm sections cut in the coronal plane on a Leica VT-1000 vibrating microtome. The slices were transferred to polycarbonate culture membranes and cultured at 37°C.

In a small number of cultures, 50 nl of nls*lacZ* virus (1×10^8 virions/ml titer with 0.1 mg/ml polybreme) was injected into the ventricular zone of the hippocampus using a pulled glass microcapillary tube and picospritzer (General Valve). Cultures were allowed to develop for 24-96 hours in vitro and then processed for X-gal histochemistry.

Overexpression experiments were also performed in slice cultures using focal electroporation to ectopically express SDF1 using an approach similar to that described previously (Marin et al., 2001; Stühmer et al., 2002). SDF1α expression construct was generated by cloning the 180 bp of coding sequence into the pSecTag-A (Invitrogen) vector backbone, sequenced and confirmed by western blot analysis. Slice cultures were placed on a piece of agarose gel laying on an platinum electrode. Another piece of agarose soaked in 1 mg/ml DNA [GFP (Marin et al., 2001) or SDF1/GFP] was placed on a second electrode and allowed to contact the surface of the hippocampus. Three square wave electrical pulses of 20-80 V lasting 50 mseconds each were delivered across the electrodes. The cultures were then allowed to develop in vitro for 96 hours and were fixed with 4% PFA, cryoprotected and resectioned to 20 μm sections on a cryostat. Sections were then double immunostained with mouse anti-GFP (Molecular Probes; diluted 1:2000), and either rabbit anti-Prox1 or rabbit anti-nestin antibodies. Using this approach, others have found correspondence of expression for GFP and an additional expression construct to be greater than 95% when individual cells are counted (Stühmer et al., 2002).

RESULTS

Intraventricular injections of virus during late gestation label granule cells

In order to gain a better understanding of the molecular cues that regulate dentate granule cell migration, it is important to better understand the timing, route and origin of cells migrating to the dentate gyrus. Previous studies on this issue have used classical neuroanatomical methods (Altman and Bayer, 1990a; Altman and Bayer, 1990b; Eckenhoff and Rakic, 1984; Eckenhoff and Rakic, 1988; Nowakowski and Rakic, 1979; Nowakowski and Rakic, 1981; Rakic and Nowakowski, 1981; Rickmann et al., 1987). BrdU and ^{3}[H]-thymidine labeling have proven very valuable for examining the migratory behavior of cells that become postmitotic in the vicinity of the ventricular zones, but the usefulness of this method is reduced when studying the dentate gyrus because the dentate migration consists of a mixture of postmitotic immature granule neurons and a cohort of mitotic dentate granule precursor cells (Altman and Bayer, 1990a; Altman and Bayer, 1990b). The migration of these mitotic precursor cells means that cells may continue to be labeled by nucleotide analogs during migration, making it difficult to understand the timing of migration of labeled cells as intermediate cellular pools are also labeled. Therefore, despite the previous neuroanatomic descriptions of dentate granule cell migration, significant questions remain as to the site of origin, migratory route and rate of migration of cells to the dentate gyrus. We wished to design another means of labeling cells migrating to the dentate gyrus that would allow us to characterize the timing of various phases of migration and the phenotype of the migrating cells. This type of information is crucial prior to being able to develop in vitro assays that faithfully represent the in vivo state.

Studies using retroviruses injected into the in utero ventricular system have been used for lineage analysis and to transduce functional molecules into dividing cells in the ventricular zone (Grove et al., 1992; Parnavelas et al., 1991; Rakic, 1988; Walsh and Cepko, 1993). The retroviruses used in this approach are only capable of infecting dividing cells and the viral genome is inherited as a lineage marker. We decided to exploit features of this system to label cells in the migratory stream to the dentate gyrus (Fig. 1A). After injection of retrovirus encoding a nuclear-localization signal containing *lacZ* (nls*lacZ*) into rat embryos at various times (the rats were then allowed to survive to P15), we found extensive labeling of dentate granule neurons after injection on E16, E18 and E20 (Fig. 1C-E). When we examined the relative distributions of cells in the hippocampal formation that are either in the dentate granule cell layer, the pyramidal cell layer or elsewhere, we observed that injections on E18 were the most efficient at labeling dentate granule neurons (Fig. 1B). In order to determine if this approach would be useful to examine cells during migration, we examined rats at shorter intervals after initial retroviral injection. These experiments showed the presence of many labeled cells in the migratory stream from the ventricular zone to the dentate gyrus (Fig. 1F-H). We conclude that intraventricular retroviral injections at E18 are most efficient for introduction of a heritable label into a cohort of cells migrating to the dentate gyrus. As we found that the numbers of cell infected tended to vary widely from animal to animal, probably because of variability of the number of progenitor cells labeled in the ventricular zone, we analyzed the percentages of different cell types labeled in each animal in order to compare different animals rather than the actual number of cells (Fig. 1B). Using this approach, the percentages of glial cells, granule neurons and pyramidal neurons labeled

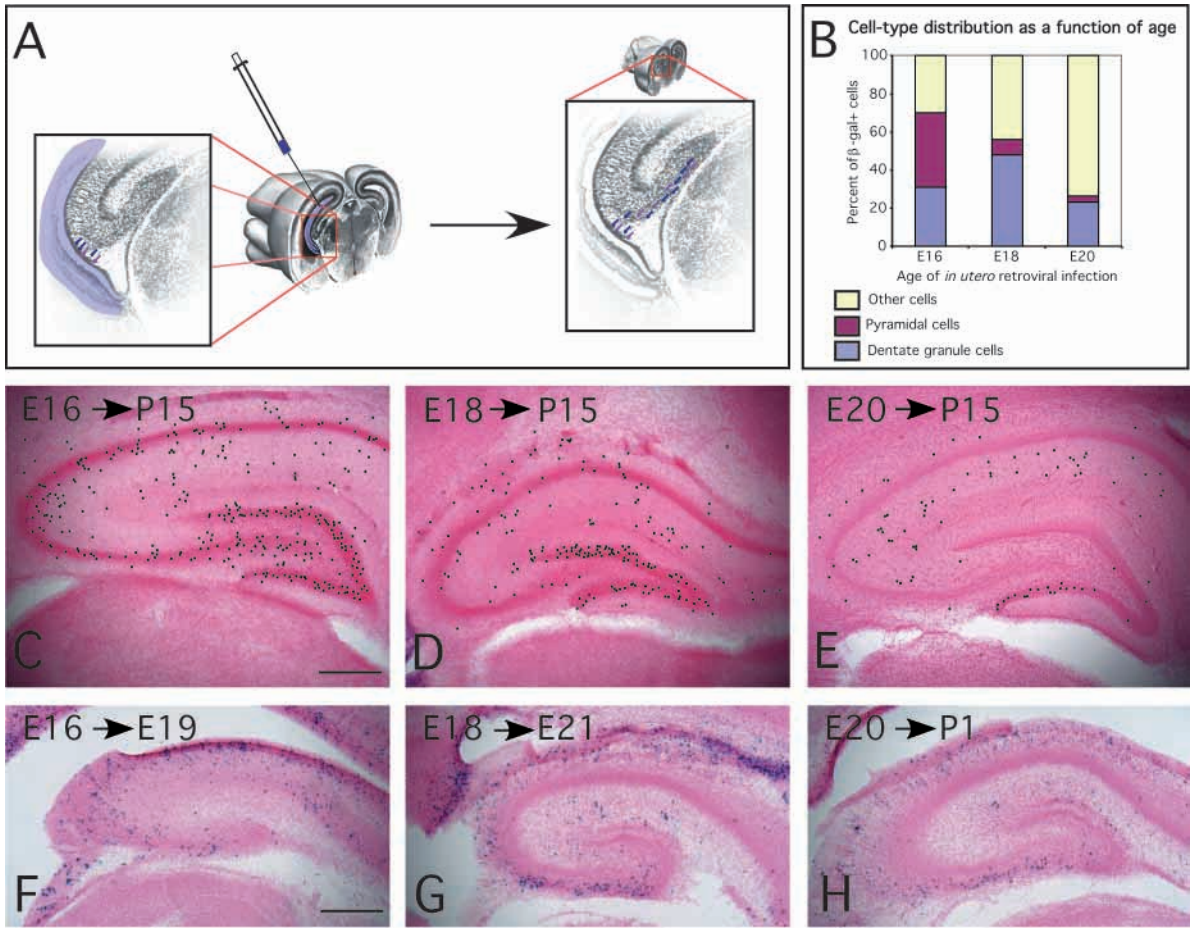

Fig. 1. In utero intraventricular injections of nls*lacZ* retrovirus at various ages labeled different populations of cells. Coronal sections of hippocampus were processed for X-gal histochemistry to detect β-gal activity and then counterstained with nuclear Fast Red. (A) The in utero intraventricular retroviral injection approach. Retrovirus was injected into the ventricle of embryonic rats in utero, allowing the virus to diffuse and infect cells in the ventricular zone (blue). During subsequent hippocampal development, the infected dentate granule cells and precursors (blue) migrated with uninfected cells (red) to the dentate gyrus. (B) Quantification of the percentage of cells positive for β-gal in the dentate granule cell layer (blue), pyramidal cell layer (red) or other regions of the hippocampus (yellow) at P15 after in utero injections were performed at different developmental ages. The graph depicts an average of five different animals at each age. (C-E) The distribution of cells at P15 when nls*lacZ* retrovirus injections were made at E16 (C), E18 (D) or E20 (E). As β-gal+ cells were difficult to resolve at this magnification and with the counterstain, black dots (indicating the position of each positive cell) were added to the images to facilitate their identification. Injections at E16 (C) result in the labeling of many dentate granule cells in addition to non-dentate granule cells. Injections at E18 (D) resulted in the labeling of a larger fraction of dentate granule cells, with a dramatic decrease in the labeling of pyramidal cells. Interestingly, injections at E20 (E) resulted in labeling of dentate granule cells primarily in the inferior blade of the granule cell layer. At this age, other cells comprised the largest cohort of labeled cells. (F-H) Injections of nls*lacZ* virus at E16 (F), E18 (G) and E20 (H) analyzed 72 hours after injection yielded a similar pattern of labeling in the dentate migratory stream, indicating that infected granule and precursor cells behaved in similar fashion, regardless of the age at which they were labeled. Scale bars: 250 μm in C-E; 500 μm in F-H.

were highly reproducible from animal to animal under the same conditions.

Timing of dentate gyrus migration

To determine how long various phases of dentate migration take to proceed in vivo, we used retroviral injections at E18 in rats and examined the brains at short time intervals after injection. This analysis was valid only in so far as we use it to examine primarily the leading edge of the wave of migrating cells in order to determine the minimum time of migration to the dentate gyrus. One day after retroviral injection, labeled cells were apparent in the hippocampal ventricular zone (Fig. 2A). The following day, there was extensive continued proliferation of cells in the dentate subventricular zone [the ‘secondary matrix’ according to Altman and Bayer (Altman and Bayer, 1990a; Altman and Bayer, 1990b)] and some cells entered the beginning portion of the migratory pathway (Fig. 2B). By 3 days after injection, there were many cells in the migratory stream toward the dentate and the first cells had reached the dentate hilus [the ‘tertiary matrix’ according to Altman and Bayer (Altman and Bayer, 1990a; Altman and Bayer, 1990b)] and a few isolated cells were seen in the dentate granule cell layer itself (Fig. 2C). Four days after injection, many cells had reached the dentate gyrus and cells were visible in the dentate granule cell layer (Fig. 2D). Thus, we conclude that cells originally in contact with the ventricle and their progeny

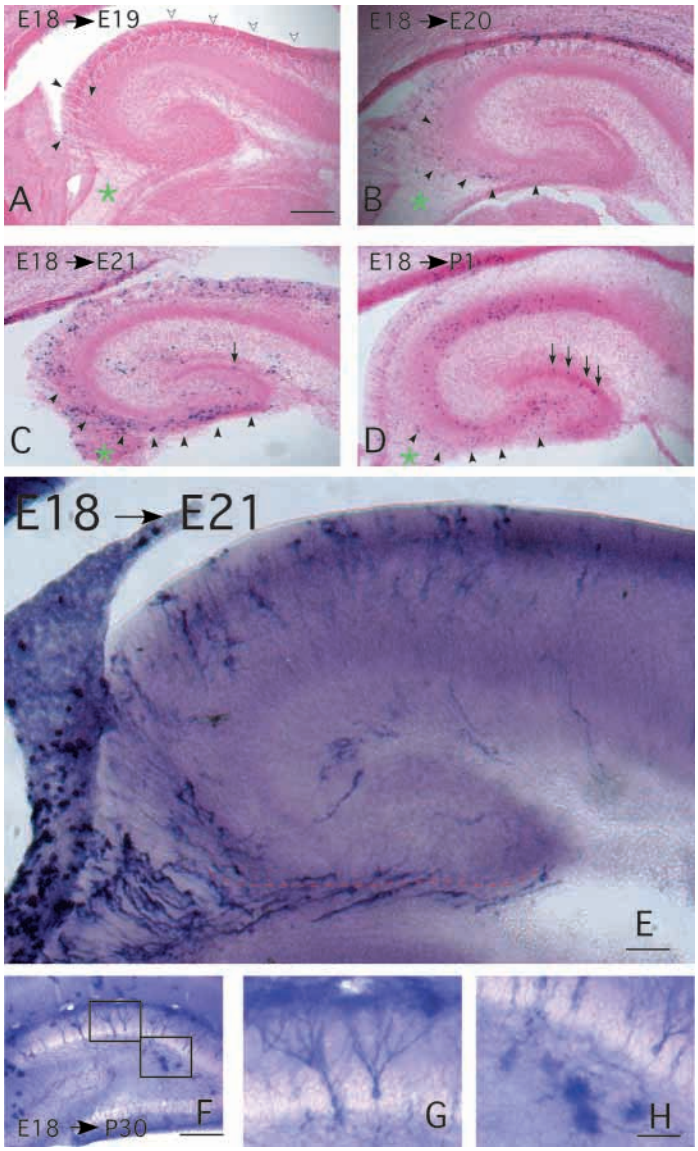

Fig. 2. Developmental time-course of migration from the ventricular zone to the dentate gyrus. Intraventricular injections of nls*lacZ* retrovirus were performed in E18 rats and the migratory pattern of labeled cells was analyzed after varying amounts of time. Coronal sections of hippocampus were processed for X-gal histochemistry to detect β-gal activity and then counterstained with nuclear Fast Red. For reference, the fimbria is marked with a green asterisk. Each experiment was performed on embryos from at least three different litters. (A) At E19, 24 hours after retroviral injection, labeled cells (blue) can be seen in the ventricular zone (open arrowhead). Additionally, a few cells can be seen to have left the ventricular zone (arrowhead). (B) Forty-eight hours after retroviral injection (E20), labeled cells are still seen to populate the ventricular zone. Additionally, cells can be seen above the fimbria in the dentate migratory stream (arrowheads), but have not yet entered the dentate gyrus. (C) 72 hours after retroviral injection (E21), labeled cells have migrated into the dentate gyrus and can be found in the germinal center in the dentate hilus. In addition, the first labeled cells migrate into the granule cell layer (arrow). (D) 96 hours after injection (P1), fewer labeled cells can be seen in the migratory stream; however, many labeled cells are now seen in the dentate hilus and populating the granule cell layer (arrows). (E) At E21, the morphology of labeled cells within the ventricular zone can be seen as they migrate above the fimbria and the migrating cells extended from the VZ into the dentate gyrus. The pattern and extent of migrating cells agreed with the pattern seen in the nls*lacZ* experiments. (F-H) By P30, cells had migrated to their final position and developed morphologies characteristic of mature differentiated cells. In the dentate granule cell layer (G, boxed area in F), many granule cells were labeled (G), and their highly branched dendritic trees and axonal arbors extended into the molecular layer and hilus, respectively. Additionally, in the hilus (H, boxed area in F), glia were also seen, confirming that cells labeled at the ventricular zone that migrate to the dentate gyrus do not have a homogenous fate. Scale bars: in A, 200 μm for A-D; in E, 75 μm; in F, 50 μm for F; in H, 50 μm for G,H.

accumulate in the dentate subventricular zone over the ensuing 2 days and then migrate to the dentate gyrus, taking about an additional 2 days to make this journey. To show that the timing of migration was generalizable to cells infected in the ventricular zone on different days, we also performed retroviral injections on E16 or E20 and found the same temporal parameters for the earliest cells to reach the dentate gyrus (Fig. 1F-H and data not shown). Again, because of significant animal-to-animal variability in the numbers of infected cells, we only used these data to determine the expected timing of the various migratory phases of cells to the dentate gyrus anlage.

Our experiments with nls*lacZ*-encoding retroviruses confirmed that this approach is effective for labeling cells migrating to the dentate gyrus, but it yielded little information about the morphology and cell type of the migrating cells. Although it is not a principal goal of this study, we wished to test whether this approach would be useful in the future for analyzing the morphology of cells migrating to the dentate. Therefore, we pursued experiments with a retrovirus expressing human placental alkaline phosphatase (PLAP), a glycophosphatidyl inositol linked extracellular heat stable phosphatase that covers the entire surface of the infected cell.

In brains from animals infected with PLAP retrovirus 2 or 3 days before (Fig. 2E), PLAP-expressing cells of several different morphology types were apparent. Many cells had the characteristic morphology of radial glial cells with long processes in contact with the ventricular zone and stretching into the dentate migratory pathway, even sometimes reaching all the way to the dentate gyrus (Fig. 2E). These cellular processes emanated from a wider region of ventricular zone above and below the dentate notch that all collected together into the migratory pathway. The ventricular and subventricular zone also contained clusters of round cells likely to be dividing precursor cells (Fig. 3E), as described previously (Altman and Bayer, 1990b). Last, in the dentate gyrus of older animals we saw numerous differentiated granule neurons (Fig. 2F-H). We also observed stellate cells in the SGZ of the more mature dentate gyrus, which are likely to be astrocyte-like cells that are the resident multipotential precursor that continue to produce granule cells in the dentate gyrus throughout life (Fig. 2H) (Seri et al., 2001). Future studies will more fully characterize the morphology and cell-type of migrating cells using cell-type-specific markers.

Granule cell migration is recapitulated in slice cultures

Using the descriptive data from our retroviral labeling studies

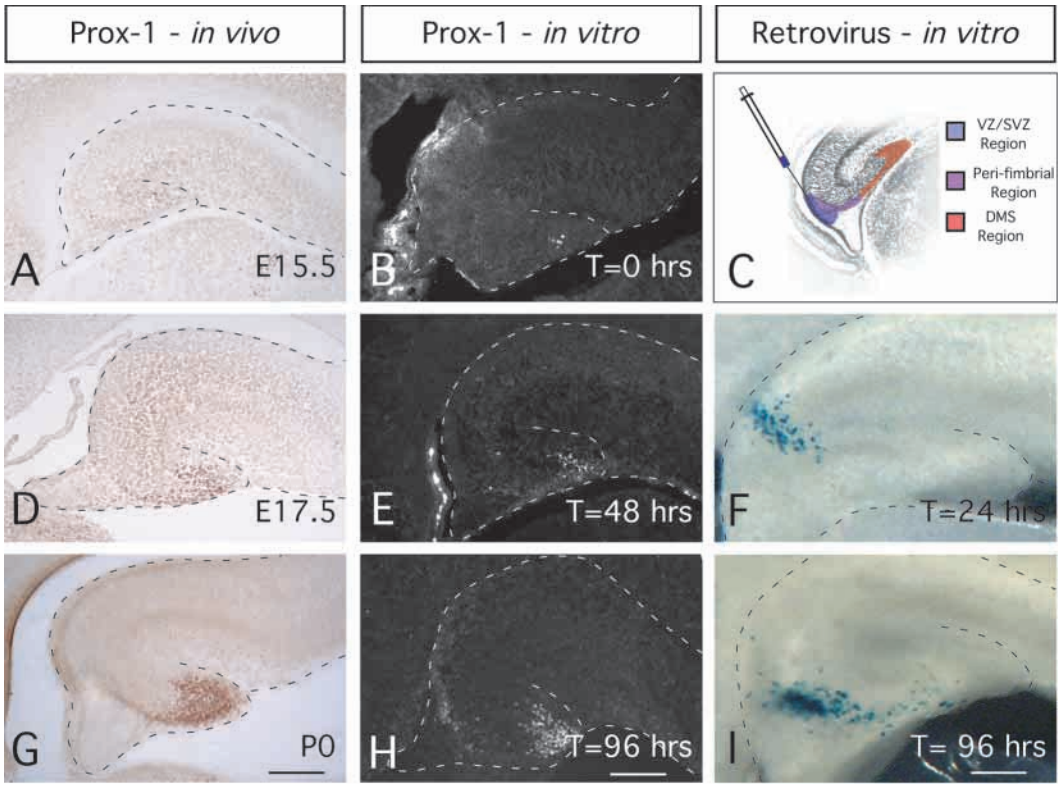

Fig. 3. In vivo and in vitro dentate granule cell migration/gyrus development occurred in a comparable manner. (A,D,G) Prox1 immunostaining labeled granule cells in coronal sections of E15.5 (A), E17.5 (D) and P0 (G) wild-type mice, and showed the accumulation of these cells in the developing dentate gyrus (broken outline). In addition to increasing numbers of Prox1-positive cells in the dentate, more weakly positive Prox1 cells can also be seen in the migratory stream at the E17.5 and P0 time-points. Additional Prox1-positive cells are seen in the thalamus and more weakly staining ones transiently in the cortex. (B,E,H) Prox1 immunostaining of E15.5 hippocampal slice cultures showed a comparable rate and pattern of dentate granule cell accumulation. Cultures fixed at *t*=0 days in vitro (B) had similar low levels of Prox1 immunoreactivity in the dentate when compared with E15.5 tissue (A). Cultures analyzed after 2 days of culturing (E) had an increased number of Prox1-positive cells in the dentate and few weakly positive cells were also seen in the migratory stream, similar to E17.5 tissue (D). This was also seen when 4 day cultures (H) were compared with P0 tissue (G). (C,F,I) The schematic (C) shows that nls*lacZ* retrovirus injections into the ventricular zone adjacent to the fimbria resulted in the labeling of many cells. Cultures fixed after 1 day of culturing (F) and processed for X-gal histochemistry showed that labeled cells (blue) were restricted to a region close to the site of injection. However, by 4 days in vitro (I), many labeled cells were seen in the migratory stream and within the dentate gyrus. Scale bars: 200 μm.

as a guide, we wanted to develop an in vitro assay that faithfully replicated features of dentate migration. We reasoned that such an assay should have robust migration and continued production of properly differentiated dentate granule cells throughout the period of time that the initial migration of dentate granule cells occurs in vivo; furthermore, we would like this assay system to recapitulate the normal timing of migration of cells to the dentate gyrus from the ventricular zone. because we have thus far been unable to assay direct migration of dentate granule cells in a dissociated culture or in collagen/matrigel explants and because, in any case, these types of assays are very divorced from the in vivo situation, we decided to try a different approach from that used to examine cerebellar granule cell migration.

Specifically, we adopted slice culture protocols previously shown to allow faithful long-range migration of interneurons from the basal ganglia to the cortex and hippocampus in developing mouse embryonic cortical slices (Anderson et al., 1997; Marin et al., 2001; Pleasure et al., 2000a). To make analysis of these slices straightforward and reproducible, we used an antibody to Prox1 [Prox1 is a divergent homeobox gene specifically expressed in differentiated granule cells (Chen et al., 2000; Cheng et al., 2001; Liu et al., 2000; Pleasure et al., 2000b)] because this assay does not depend on subjective dye or virus placement. Comparing the staining for Prox1 in brain sections taken from mouse brains at several embryonic ages (after making a conversion for shifting from the use of rats in the retroviral studies to mice for the remainder of the studies) showed good correspondence with the staining for Prox1 in the dentate gyrus that formed in slice explants (Fig. 3A-F). To be certain that this continued accumulation of Prox1 in the dentate gyrus represents continued migration of cells to the dentate rather than just continued proliferation or maturation of cells that had migrated to the dentate prior to explanting, we performed focal injections of nls*lacZ* retrovirus into the migratory stream in slice explants (Fig. 3G-I). These injected explants showed migration of cells from the dentate subventricular zone through the migratory pathway to the dentate gyrus with timing similar to that observed in the in vivo intraventricular experiments presented above. Thus, we conclude that slice explants are a viable and robust system for observing dentate migration in vitro.

The chemokine SDF1 is a candidate regulatory factor for granule cell migration

To find potential molecular cues that regulate dentate granule cell migration, we examined the expression of a number of known chemotactic molecules and their receptors in the developing dentate gyrus just before the onset of dentate migration in mice (E14.5) (Fig. 4A) and while it is well under way (P0) (Fig. 4B). We also thought that candidates for regulation of dentate migration came from previous studies

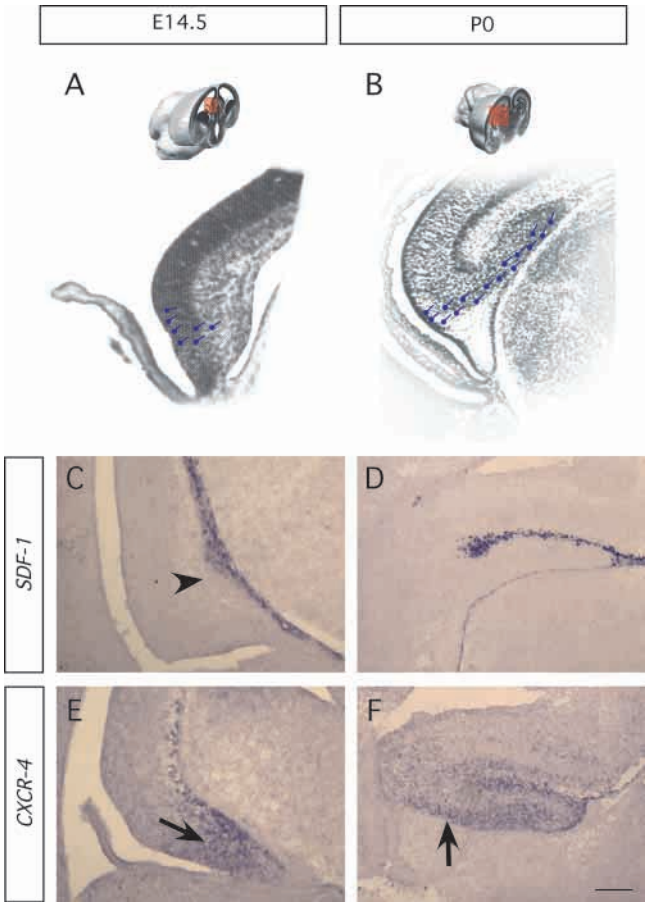

Fig. 4. The chemokine SDF1 and its receptor CXCR4 were expressed in complementary patterns in the developing hippocampus. Schematic representation of the migratory events ongoing at E14.5 and P0 (A,B). Red square shows enlarged area, migrating cells are in blue. Non-radioactive in situ hybridization for SDF1 (C,D) and CXCR4 (E,F) in E14.5 (C,E) and P0 (D,F) wild-type mouse tissue. (C,E) At E14.5, SDF1 (C) was expressed in the meninges, superficially in the cortex and within the developing dentate anlage (arrowhead). At the same age, CXCR-4 (E) was expressed in the dentate ventricular zone and in a continuum from the ventricular zone to the developing dentate (arrow). (D,F) At P0, SDF1 (D) was expressed primarily in the Cajal-Retzius cells that line the hippocampal fissure. CXCR4 (F) was expressed in the dentate migratory stream (arrow), starting above the fimbria and extending into the dentate gyrus. CXCR4 was also present in the forming granule cell layer. Scale bar in F: 250 μm for C-F.

showing that several axon-guidance molecules (including netrin 1, Slit1 and Slit2) are all expressed at varying levels in the neuroepithelium that generates the dentate gyrus (Barallobre et al., 2000; Nguyen Ba-Charvet et al., 1999; Shu and Richards, 2001; Tuttle et al., 1999). We reasoned that these molecules would then be candidate chemorepellants that might drive migration toward the dentate anlage; however, we were unable to find any defects in dentate gyrus formation in mouse mutants for netrin 1 (or its receptor – DCC) or Slit1/Slit2 double mutants (data not shown). Although this does not rule out potential functions of these molecules in the development of the dentate gyrus, it does imply that they are not required for the prenatal phases of granule cell migration.

We reasoned that other factors that guide migrating cells might play a role in regulating granule cell migration directly. An interesting set of candidate molecules in this group are chemokines, secreted factors that interact with G-protein-coupled receptors and increase the migration of leukocytes towards higher chemokine concentrations (Gale and McColl, 1999). In a broad survey of chemokine and chemokine receptor expression (see Materials and Methods for a list; none of the other chemokine ligands or receptors are expressed in a pattern that indicates any potential role in dentate migration – data not shown), we were intrigued to find expression of the chemokine SDF1 in the developing hippocampus by cells in the meninges and by large superficial layer I cells (probably Cajal-Retzius cells) throughout the period of dentate gyrus migration (Fig. 4C,D). The receptor for SDF1 is CXCR4 and previous studies in *Cxcr4* mutant mice showed a function for SDF1 in migration of cerebellar granule cells (Klein et al., 2001; Ma et al., 1998; Zou et al., 1998). In the developing hippocampus, CXCR4 was expressed at its highest level in the dentate region of the developing dentate subventricular zone at E14.5 and in cells within the migratory stream toward the dentate at P0 (Fig. 4E,F). At these times, the only cells known to be in the dentate subventricular zone and dentate migratory stream are immature dentate granule neurons and their precursors; it is thus very likely that the expression of CXCR4 is on one or both of these populations. Therefore, SDF1 is expressed in the target area for granule cell migration and its receptor is present in cells predicted to be among those migrating to the dentate gyrus, making this ligand/receptor combination a good candidate for regulating migration of cells to the dentate gyrus. Interestingly, in the cerebellum, unlike in the hippocampus, SDF1 is expressed at the origin of the migrating cells and is thought to delay migration away from the source of SDF1 until the appropriate developmental time (Klein et al., 2001; Ma et al., 1998; Zou et al., 1998), but SDF1 can also act as a chemotactic factor for dissociated neurons in vitro (Hesselgesser et al., 1997; Klein et al., 2001). To determine whether SDF1 has a chemotactic function in attracting cells to the dentate gyrus, we examined the phenotype of the dentate gyrus in CXCR-4 mutant mice.

Granule cell migration is disrupted in SDF1-receptor mutants

We examined the hippocampal region of *Cxcr4* mutants at E18.5 using the antibody to Prox1, as this is a time at which dentate granule cell migration is at its height from the ventricular zone. In these mutants (n=4/4), we observed the presence of numerous Prox1-expressing cells in ectopic locations near the ventricular zone, adjacent to the fimbria, or in the migratory stream towards the dentate anlage (Fig. 5A,B). This is consistent with a defect in migration of developing dentate granule neurons to the dentate gyrus. These cells would then differentiate and begin to express the dentate specific marker before arrival in the dentate gyrus. We cannot, however, from these data alone exclude a role for SDF1 in preventing the premature expression of Prox1 outside the dentate anlage, rather than affecting migration, but experiments using ectopic expression of SDF1 (discussed later) tend to argue against this possibility. It is also, in principle, possible that loss of SDF1 signaling leads to survival of Prox1-positive cells that would normally undergo

cell death outside the dentate anlage, although previous studies showing a very low level of cell death in the dentate gyrus at this developmental timepoint make this unlikely (Galceran et al., 2000). To determine whether dividing, migrating dentate precursor cells are also affected in the *Cxcr4* mutants we examined the distribution of Phosphohistone-H3, which reveals the distribution of cells in M-phase (Fig. 5C,D). In heterozygous animals, there was extensive labeling of cells in the dentate subventricular zone, migratory stream and within the hilar tertiary matrix, while in the mutants the number of labeled cells was dramatically decreased in all three of these areas. This finding, which is in agreement with others recently published findings (Lu et al., 2002), is consistent with SDF1 being a mitogen for dentate precursor cells. Interestingly, Phosphohistone-H3 staining is unchanged in other areas of the hippocampal ventricular zone (Fig. 5C,D), supporting the specificity of SDF1 action within the dentate region. In addition, the uniform decrease in the numbers of dividing cells within the subventricular zone, migratory stream and tertiary matrix without selective accumulation of dividing cells that are unable to reach the dentate gyrus implies that SDF1 is likely not a chemoattractant for precursor cells. This distinction between the actions of SDF1 on precursor cells (which will presumably generate both dentate granule neurons and glial cells) and newly differentiated granule neurons is quite interesting and worth further study in the future. Finally, we examined whether SDF1 was acting primarily on radial glial processes, with the granule cell migration defect arising secondarily. This seemed unlikely as CXCR4 does not appear to be expressed by radial glial cells whose cell bodies are in the ventricular zone. We indeed found that the distribution of radial glial fibers in the dentate, assessed using nestin antibodies, was normal (Fig. 5E-F), consistent with the effect of SDF1 being on granule cells rather than radial glial cells.

Interestingly, SDF1 was not absolutely necessary for all granule cells to reach the developing dentate gyrus, as many Prox1-expressing cells were present in the dentate anlage and did adopt the normal horseshoe-shaped structure (the dentate gyrus was somewhat smaller and disorganized in mutant animals, although this was not a consistent finding at all anatomic levels). This implies functional redundancy for migratory cues for granule cells and suggests that other perhaps novel chemotactic cues for granule cells must exist.

SDF1 directly regulates migration of dentate granule neurons

If SDF1 directly regulates migration of dentate granule cells, it could be operating to provide directional cues used for migration or as a non-directional motogenic (or chemokinetic) factor that stimulates the cells to migrate using other directional cues present in the tissue, as shown for HGF in interneuron migration (Powell et al., 2001)

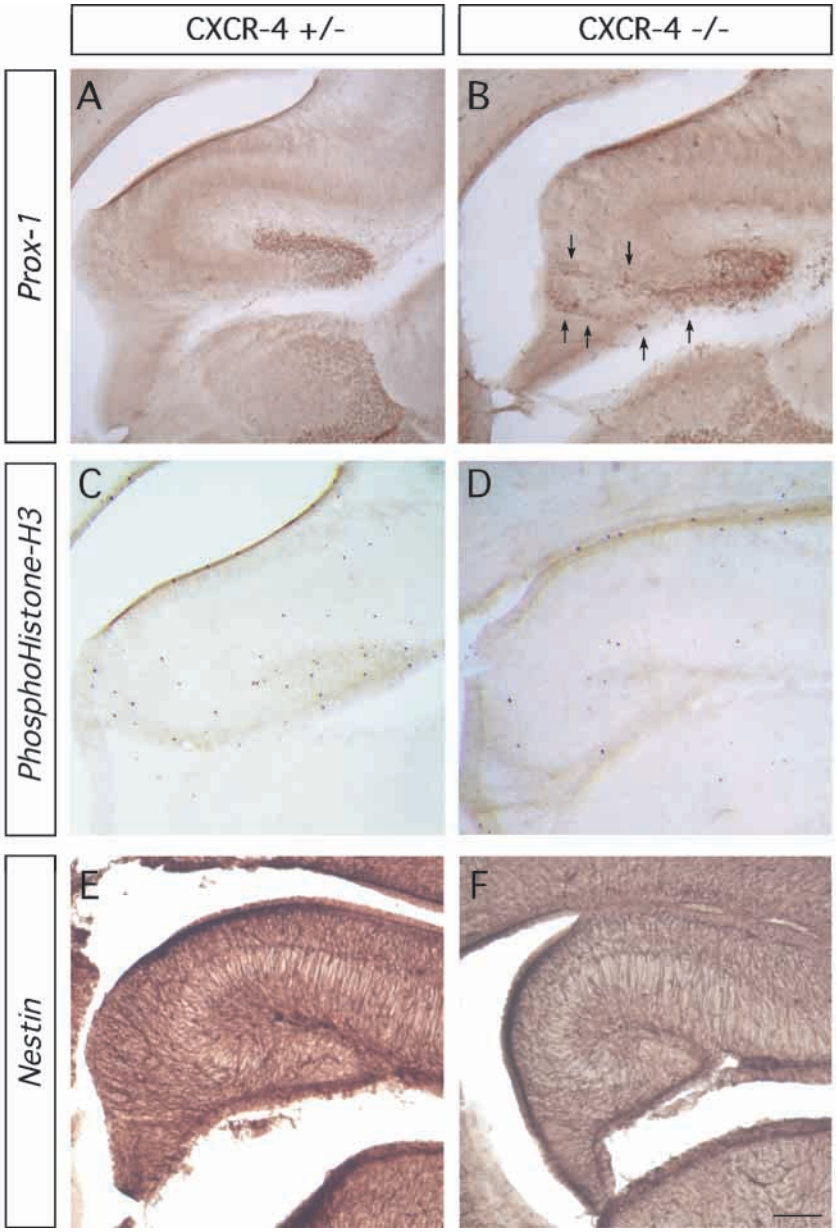

Fig. 5. *Cxcr4* mutant mice had ectopic dentate granule cells. Prox1 immunohistochemistry in the E18.5 hippocampus of $Cxcr4^{+/-}$ and $Cxcr4^{-/-}$ mice. Scale bar: 250 μm. (A,B) In $Cxcr4^{+/-}$ mice, Prox-1 positive granule cells were in the dentate gyrus. In $Cxcr4^{-/-}$ mice many Prox1-positive granule cells were in the dentate gyrus, but appeared disorganized. Additionally, many Prox1-positive cells were outside the dentate gyrus, near the ventricular zone, above the fimbria and in the dentate migratory stream (arrows). (C,D) In *Cxcr4*+/– mice Phosphohistone-H3 positive precursor cells were distributed in the dentate subventricular zone, migratory stream and in the tertiary matrix. In addition, note the scattered positive cells throughout the ventricular zone of the hippocampus. In $Cxcr4^{-/-}$ mice the number of dividing precursor cells labeled with Phosphohistone-H3 was dramatically decreased in all the regions but is preserved outside the dentate gyrus in the ventricular zone. (E,F) Nestin antibody staining in $Cxcr4^{+/-}$ and $Cxcr4^{-/-}$ mice showing that the overall distribution of the radial glial network is intact.

(Fig. 6A). We reasoned that if SDF1 is a motogen, then generalized ectopic expression of SDF1 in the hippocampus should not affect migration or should even speed up migration of granule cells to the dentate (Fig. 6A). However, if SDF1 is a chemattractant molecule, then generalized expression would destroy the normal gradient of SDF1 in the migratory stream and might lead to failed migration of some Prox1-expressing cells (Fig. 6A). As granule cells migrate on radial glial cells (Nowakowski and Rakic, 1979; Nowakowski and Rakic, 1981; Rickmann et al., 1987), we thought it possible that SDF1 might act as a polarity cue that determined the direction of migration on radial glial cells, but that it was unlikely that we would divert substantial numbers of cells from their normal migratory pathway to completely ectopic positions.

Using electroporation to misexpress SDF1 throughout the hippocampal field (Fig. 6B shows the general approach used), we found that Prox1-expressing cells were found in ectopic positions along the migratory pathways to the dentate gyrus, either at the origin of migration next to the fimbria or in midpoints of migration in the great majority of explants (11/13) (Fig. 6G-J), whereas control GFP electroporated explants rarely exhibited similar phenotypes (1/14) (Fig. 6C-F). As previous studies have demonstrated remarkable concordance (>95%) between the expression of two plasmids co-electroporated in slice explants using the same method (Stühmer et al., 2002), it is reasonable to use the pattern of GFP expression to infer the detailed localization of SDF1 expression. We also noted (but did not study systematically) that in many cases where SDF1 is expressed quite focally along the normal migratory route of the granule cells (e.g. Fig. 6G), the Prox-1-positive cells tended to cluster around this location and progress no further into the dentate gyrus. These observations tend to support the

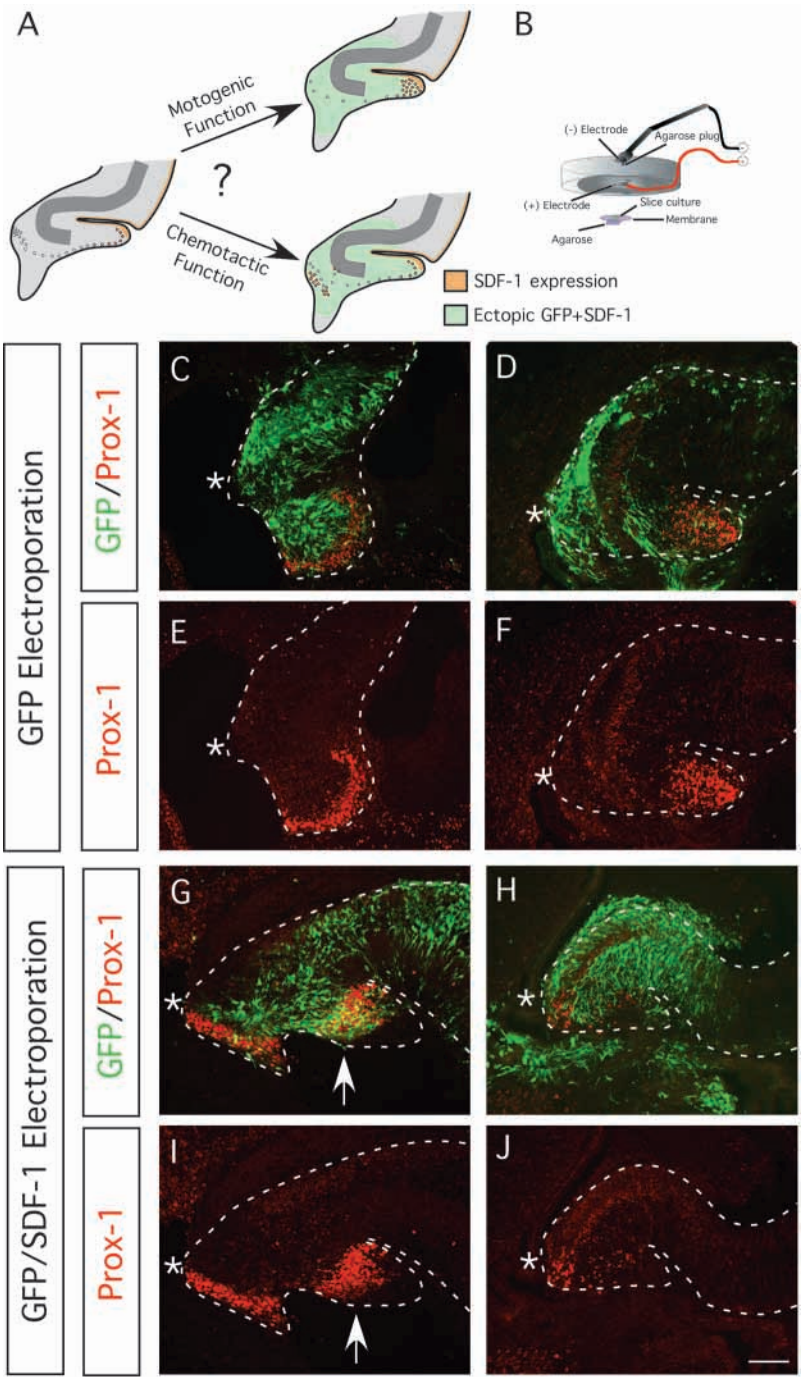

Fig. 6. Ectopic SDF1 expression disrupted migration of granule cells in vitro. GFP or SDF1 and GFP were electroporated into slice cultures and subsequently allowed to develop for 4 days. Cultures were then double immunostained for GFP and Prox1 to identify the extent of the electroporated tissue and location of granule cells, respectively, in the culture. (A) Schematic outlining the potential roles of SDF1 in dentate granule cell migration and the expected effect of ectopic expression (green shading) upon Prox1-positive cells (orange). (B) In order to express SDF1 ectopically in the hippocampus, we used focal electroporation. A schematic of the apparatus is shown. The slice culture on a membrane was placed on a 5% agarose section in contact with the positive electrode. Another agarose piece containing DNA was placed on the negative electrode and a current was applied. (C-F) Electroporation of GFP without SDF1 resulted in normal granule cell migration. Prox1 (red) is shown with GFP (green) staining to allow comparison of electroporation site with granule cell migration site. Additionally, to visualize clearly the location of all granule cells, Prox1 staining is shown alone. In both examples (C,E and D,F), granule cells migrate appropriately to the dentate gyrus despite GFP (green) expression throughout the hippocampus.
(G-J) Electroporation of GFP and SDF1 resulted in disruption of granule cell migration. Again, GFP staining was used to identify the location of electroporated tissue. We show two distinct examples here, one with electroporation throughout the hippocampal formation, including the forming dentate gyrus (G,I) and the other throughout the hippocampus but not including the forming dentate gyrus (H,J). In both examples, Prox1-positive cells can be seen in the fimbrial region. Additionally, when ectopic expression included the middle of the developing dentate (G,I), many cells migrated to the position of the ectopically expressed SDF1 but failed to migrate any further into the dentate gyrus (arrows). When SDF1/GFP is electroporated into the hippocampus, excluding the dentate (H,J), very few cells leave the vicinity of the fimbria. For reference, the fimbria is labeled with an asterisk. Scale bar: 150 μm.

idea that SDF1 is a chemoattractant for dentate granule neurons.

To ensure that the disruption of granule cell migration that we observed was both reliable and significant, we quantified the percentages of Prox1-positive granule cells that reached the dentate apex in GFP electroporated and GFP/SDF1 electroporated experimental conditions (we used percentages of cells rather than raw numbers of cells as the number of Prox1-positive cells present in a section varied based on anatomic level and exact section plane). For this analysis, we counted representative sections from five different slice experiments for each condition and found that following GFP electroporation 93% (±4%) of Prox1-positive cells reached the dentate anlage, while in the GFP/SDF1 electroporated slices and average of only 52% (±3%) of Prox1-positive cells reached the dentate (a total of 690 cells were counted from GFP slices and 752 from GFP/SDF1 slices, $P<0.00000002$).

We believe that these observations are most consistent with SDF1 providing a chemoattractive cue that is interpreted primarily as a migration polarity signal, presumably with migration also constrained by radial glial processes. These data also exclude a role for SDF1 in preventing ectopic differentiation of Prox1-positive cells outside the dentate gyrus and make it unlikely that SDF1 acts solely as a motogenic (or chemokinetic) factor.

DISCUSSION

The dentate gyrus plays a central role in controlling the normal function of the hippocampus by being the primary gatekeeper for the flow of excitatory inputs from the entorhinal cortex and contralateral hippocampus. Despite the importance of this structure and the recent focus on the role that ongoing neurogenesis of dentate granule cells may play in adult plasticity, little is known about the molecular control of dentate granule cell development. One of the crucial early events in its development is the migration of cells from the neuroepithelium to the dentate anlage. Migrating cells are likely to consist of a mixture of newly postmitotic neurons destined to differentiate into granule cells and precursor cells that continue to divide and produce new granule cells in situ in the dentate gyrus (Altman and Bayer, 1990a; Altman and Bayer, 1990b; Pleasure et al., 2000b). A role for radial glial cells as the scaffolding for granule cell migration has been suggested (Nowakowski and Rakic, 1979; Rickmann et al., 1987), and our study supports this idea. However, the relationship of radial glia to migrating dentate precursor cells must be revisited in the future, in light of studies that have shown a neurogenic role for radial glia in the neocortex and also the recent identification of the quiescent SGZ granule precursor cells as radial glial-like cells (Barres, 1999; Hartfuss et al., 2001; Malatesta et al., 2000; Noctor et al., 2001; Parnavelas and Nadarajah, 2001; Seri et al., 2001). Future experiments may reveal molecular changes occurring during the transition from radial glial cell to migrating granule cell precursor, and perhaps the acquisition of SDF1 responsiveness will prove to be one such event.

The relationship of granule cell migration to radial migration in other cortical regions is supported by the phenotype of mice with defects in the reelin pathway. Reelin has been proposed to be a stop signal for radially migrating cells that, when absent, results in severe neocortical abnormalities with inversion of the normal laminar pattern (Frotscher, 1998). In the dentate gyrus, reelin-expressing Cajal-Retzius cells are perfectly positioned in the upper dentate blade to be supplying such a stop signal to halt the further migration of granule cells along radial glial fibers (Alcantara et al., 1998). Mice with defective reelin signaling have ectopic granule cells within the dentate gyrus and an ill-formed dentate granule cell layer but do not have ectopic granule cells outside the immediate region of the dentate gyrus (Stanfield and Cowan, 1979a; Stanfield and Cowan, 1979b; Trommsdorff et al., 1999). These findings are most consistent with a short-range function for reelin as the proposed stop signal.

Until now, there have been no studies elucidating the identity of chemoattractant or repellent cues for granule cell migration. For this reason, we felt the need to develop a robust in vitro assay that could be used to determine the molecular underpinnings of this process. As we have show in this study, dentate granule cell migration is a process quite amenable to the use of assays developed to analyze the migration of other cell types in the developing cortex and hippocampus. To validate this assay, we first showed that a molecular marker of dentate granule neurons, Prox1, is initially expressed at the protein level while granule cells are en route to the dentate anlage. This marker may then be used as a robust indicator of the efficacy of granule cell migration. Using retroviruses that infect granule cell precursors in contact with the ventricular zone, we determined the embryonic time frame during which granule cell migration is at its maximal level, in order to decide the best embryonic age to develop our in vitro assay. Coronal brain slices taken at this time support continued proliferation and migration of dentate granule cells and are amenable to manipulation by focal electroporation-mediated gene transfer.

Analysis of the expression of several guidance molecules known to regulate neuronal migration revealed several plausible candidates for regulatory cues for dentate migration, but analysis of mutants for all of these failed to show any evidence of migration defects. The chemokine SDF1 and its receptor CXCR4 are also expressed in domains, suggesting a possible role in granule cell migration, but in this case we did observe evidence of a defect in granule cell migration in mice mutant in the receptor. Furthermore, using our slice explant assay, we showed that SDF1 is likely to be acting as a chemoattractant for the migrating neurons.

Chemokines in neuronal migration

The chemokines are a large family of peptide ligands that act through G-protein coupled seven-transmembrane spanning receptors (Gale and McColl, 1999). Most of these factors were discovered and studied most intensively because of their role in regulating the chemotaxis of hematopoetic lineage cells (Gale and McColl, 1999). There have been a few studies that have implicated specific chemokines in regulating the migration of cells of neural origin (Hesselgesser et al., 1997; Klein et al., 2001; Ma et al., 1998; Zou et al., 1998). The most frequently studied of these has been SDF1, because of the realization after production of SDF1 mutants and mutants of its only known receptor (CXCR4) that these mice have cerebellar abnormalities, consistent with a migration regulatory role for SDF1 (Ma et al., 1998; Zou et al., 1998). In the cerebellum, SDF1 is expressed in the meninges and keeps the

dividing cerebellar granule cell precursors in the external granule cell layer (EGL) from leaving the EGL prematurely (Klein et al., 2001; Lu et al., 2001; Ma et al., 1998; Zou et al., 1998). At the appropriate developmental time, these cells lose their responsiveness to SDF1, leave the EGL and radially migrate through the Purkinje cell layer to the internal granule cell layer (IGL) (Klein et al., 2001; Lu et al., 2001; Ma et al., 1998; Zou et al., 1998). Thus, in the cerebellum, SDF1 actively prevents cerebellar granule neurons from migrating too early by acting as a chemoattractant, whereas in the hippocampus, SDF1 appears to act as a diffusible cue to attract granule cells to the dentate gyrus. In both cases SDF1 acts molecularly as an attractant but the difference in biological response elicited is regulated by the distribution of SDF1.

It is unclear at this point whether SDF1 is involved in regulating migration in other groups of radially migrating cells. Although SDF1 is expressed in Cajal-Retzius cells in the neocortex (data not shown), its receptor is not expressed at high levels in the ventricular zone in cortical regions outside the hippocampus at the time of active radial migration (data not shown); therefore we do not foresee a major role of SDF1 in regulating radial migration of other cortical neurons. The function of SDF1 in the remainder of the cortex is therefore not yet clear, but CXCR4 does begin to appear in the intermediate zone later in embryogenesis, perhaps indicating a role in other phases of cortical development. It is interesting and perhaps not coincidental that the migration of cerebellar granule cells and dentate granule cells are both regulated by SDF1. Other than their similar morphology these cells have been thought to share certain aspects of their developmental program and have been observed to express some shared markers (Hatten et al., 1997; Yang et al., 1996). In addition, both cerebellar and dentate granule cells express large amounts of astrotactin, a molecule that plays a contact-dependent regulatory role in radial migration (Zheng et al., 1996), and both types of granule cells migrate along radial glial fibers that have the unusual feature of early expression of glial fibrillary acidic protein compared to radial glia elsewhere in the cortex (Dahl et al., 1985; Liu et al., 2000). Thus, although derived from distinct CNS regions, cerebellar and dentate granule cells may share a number of developmentally important characteristics, including their responsiveness to SDF1.

Recent observations regarding the function of SDF1 in the cerebellum and in leukocyte chemotaxis may also have important implications for future studies of migration in the developing hippocampus. In the cerebellum, despite the continued presence of SDF1 in the meninges adjacent to the EGL, granule cells still migrate radially to the IGL at the appropriate developmental stage. Why do these cells choose to leave a chemoattractive source? Flanagan and colleagues have recently shown that an ephrin ligand-binding protein expressed in migrating cerebellar granule neurons acts to silence CXCR4 signaling (Lu et al., 2001). In addition, signaling via the Slit family of ligands has been shown to regulate responsiveness of leukocytes to SDF1 (Wu et al., 2001). As Eph receptors, Ephrin ligands, Slits and Slit receptors are all expressed in the developing hippocampus, future studies will focus on whether SDF1-CXCR4 signaling is also modulated by these factors in dentate granule cell migration.

Despite the defect in dentate granule cell migration in the *Cxcr4* mutant mice, many Prox1-expressing cells do reach the dentate anlage and form a dentate granule cell layer. Thus, SDF1 may be but one of several directional cues directing granule cell migration. Such cues might include Slits, Ephrins, other chemokines or other positive factors known to regulate radial type migration, such as reelin and integrins. It is also possible that there are multiple pools of granule cells: those that require CXCR4 signaling and others that do not. Future studies will help determine whether there is heterogenity of responses of different dentate cell populations (e.g. the differences between the mitogenic response of precursor cells versus the chemoattractant response of the differentiated neurons), and what other factors collaborate with SDF1 to effect accurate guidance.

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