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Cell Migration from the Ganglionic Eminences Is Required for the Development of Hippocampal GABAergic Interneurons

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Summary

GABAergic interneurons have major roles in hippocampal function and dysfunction. Here we provide evidence that, in mice, virtually all of these cells originate from progenitors in the basal telencephalon. Immature interneurons tangentially migrate from the basal telencephalon through the neocortex to take up their final positions in the hippocampus. Disrupting differentiation in the embryonic basal telencephalon (lateral and medial ganglionic eminences) through loss of *Dlx1/2* homeobox function blocks the migration of virtually all GABAergic interneurons to the hippocampus. On the other hand, disrupting specification of the medial ganglionic eminence through loss of *Nkx2.1* homeobox function depletes the hippocampus of a distinct subset of hippocampal interneurons. Loss of hippocampal interneurons does not appear to have major effects on the early development of hippocampal projection neurons nor on the pathfinding of afferrent tracts.

Introduction

The hippocampal formation is critical for learning and memory. The classic hippocampal circuit is a trisynaptic circuit utilizing glutamatergic neurotransmission. At each of these steps the excitatory tone is modulated by a diverse group of inhibitory and excitatory interneurons (Freund and Buzsáki, 1996). In fact, in some regions of the hippocampal formation the principal excitatory cells make more synapses with inhibitory interneurons than with the next link in the excitatory circuit (Acsády et al., 1998). The modulation of the excitability of principal neurons within the hippocampus by interneurons has been proposed to underlie many cases of temporal lobe epilepsy and various forms of learning disabilities (Keverne 1999; Swann et al., 1999).

γ-aminobutyric acid producing (GABAergic) neurons found in the hippocampus are born before the principal glutamatergic neuron populations (Soriano et al., 1986, 1989a, and 1989b). It has been postulated that interneurons, present from the early stages of hippocampal formation, may play a role in organizing the development of the hippocampus in a manner analogous to Cajal-Retzius cells (Super et al., 1998). Cajal-Retzius cells direct the migration of the principal neurons of the hippocampus and are also required for the proper termination of entorhinal afferents on the dendrites of both the pyramidal cells and the dentate granule cells (Del Rio et al., 1997). Soriano and colleagues recently presented evidence that the hippocampal commissural axons terminate transiently on GABAergic interneurons in the stratum radiatum before transferring their synapses to the inner two thirds of the dendrites of pyramidal neurons (Super et al., 1998). They proposed that the interneurons might be a crucial intermediate target for these afferents and necessary for their proper laminar termination.

Because interneurons play many pivotal roles in the normal and abnormal function of the hippocampus, their physiology and anatomy have been studied extensively. Many subtypes of GABAergic interneurons have been identified based on their location, morphology, physiology, and expression of specific secondary neurotransmitters (Freund and Buzsáki, 1996). Despite the relatively large amount known about the anatomical and functional properties of GABAergic interneurons in the adult, our understanding of the factors controlling development of these cells in the hippocampus is limited.

In recent years, studies in rats and mice have shown that many of the GABAergic interneurons present in the neocortex and olfactory bulb are derived from subcortical sources (reviewed in Pearlman et al., 1998; Anderson et al., 1999). Tangential migrations from the basal telencephalon to the cortex have been established through vital dye labeling experiments (de Carlos et al., 1996; Anderson et al., 1997a; Tamamaki et al., 1997; Lavdas et al., 1999; Wichterle et al., 1999). Also, analysis of mutant mice with reduced numbers of neocortical GABAergic interneurons revealed that the lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE) contribute substantially to the GABAergic interneuron population of the neocortex and the olfactory bulb (Anderson et al., 1997a; Casarosa et al., 1999; Sussel et al., 1999). The existence of mice with mutations in genes required for LGE and MGE development have proven particularly useful in understanding this phenomenon. Mice with mutations of both *Dlx1* and *Dlx2* homeobox genes have defects in the development of the MGE and LGE (Anderson et al., 1997b; Marin et al., 2000). These mice have a great reduction of GABAergic and tyrosine hydroxylase–expressing neurons in the olfactory bulb, as well as an ~75% reduction in the number of neocortical GABAergic neurons on the day of birth (Anderson et al., 1997a). The phenotype of the *Dlx1/2* mutant mice

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is due to a defect in differentiation of later born neurons that affects their ability to migrate in both the LGE and MGE (Anderson et al., 1997a, 1997b; Marin et al., 2000). Mice with mutations in the *Nkx2.1* gene, a homolog of *Drosophila NK2*, have abnormal development of the MGE with an apparent conversion of the MGE to an LGE-like phenotype (Sussel et al., 1999). These mice have a smaller reduction (50%) of GABAergic interneurons in the neocortex and a normal appearing olfactory bulb (Sussel et al., 1999). This suggests that the MGE contributes at least 50% of the GABAergic interneurons to the developing neocortex. Combined with studies analyzing interneuron migration from subcortical sources, these findings suggest that both the LGE and MGE contribute GABAergic interneurons to the neocortex, while only the LGE contributes interneurons to the olfactory bulb (Anderson et al., 1999).

One interesting implication of these studies is that pyramidal projection neurons in the cortex and locally projecting inhibitory interneurons would be derived from distinct precursor cells. A variety of techniques for lineage analysis support this prediction. Retroviral lineage studies demonstrated that some clones tend to be radially arranged, whereas others are more dispersed (Walsh and Cepko, 1988, 1992, 1993; Parnavelas et al., 1991; Luskin et al., 1993; Mione et al., 1994, 1997; Lavdas et al., 1996). Clonal populations of interneurons marked by retroviral infection tend to be more widely distributed through the cortex (Parnavelas et al., 1991; Luskin et al., 1993; Mione et al., 1994, 1997; Reid et al., 1995; Lavdas et al., 1996). Furthermore, analyses of genetically distinct clones within mouse chimeras show that pyramidal cell clones tend to be arranged radially, whereas interneuron clones are tangentially dispersed within the cortex (Tan and Breen, 1993; Tan et al., 1998). While retroviral lineage studies have been used to study hippocampal pyramidal and granule cells (Grove et al., 1992; Grove and Tole, 1999), we are unaware of studies that compare the origins of hippocampal glutamatergic and GABAergic cells.

In this study, we sought to determine whether the GABAergic interneurons of the hippocampus originate subcortically, and whether these cells are required for organization of some of the main axon pathways in the developing hippocampus. To answer these questions, we directly analyzed the migration of neurons to the hippocampus from subcortical germinative zones, examined the development of hippocampal interneurons in *Dlx1/Dlx2* and *Nkx2.1* mutants, and used anterograde and retrograde labeling of hippocampal afferents with lipophilic tracers. Our results suggest that virtually all hippocampal GABAergic interneurons originate from the ganglionic eminences and migrate tangentially to the hippocampus. Furthermore, their presence is not required for the targeting of entorhinal and commissural afferent pathways into the hippocampal formation.

Results

DLX2-Expressing Cells Are First Apparent in the Hippocampus by E15.5

Because recent work (Anderson et al., 1997a) established the subcortical origin of a large proportion of neocortical GABAergic neurons, we wished to determine whether any hippocampal neurons are also derived subcortically. Previously, we provided evidence that immature neocortical interneurons express DLX homeodomain proteins (Porteus et al., 1994; Anderson et al., 1997a). Thus, we studied the expression of DLX2 at stages of hippocampal development when many interneurons are known to be born (Soriano et al., 1986). By E15.5, but not at earlier stages, DLX2 was detectable in the hippocampal primordium but only in the stratum radiatum (Figures 1A and 1B). Scattered DLX2-positive cells are apparent throughout the cerebral cortex. By E16.5, the pattern of expression of DLX2 (Figure 1C) included cells in the developing stratum oriens and stratum radiatum and resembled the pattern seen at P0 (see Figure 6).

Cells from the Ganglionic Eminences Migrate to the Hippocampal Anlage

The DLX2 immunofluorescence results are consistent with the hypothesis that subcortical cells can tangentially migrate from the basal ganglia to the hippocampus. To test this directly, we used a slice culture system that allows the visualization of cells migrating from the LGE and MGE to the neocortex (Anderson et al., 1997a). When DiI crystals were placed in the basal telencephalon of coronal sections from E13.5 wild-type mice, we were able to detect fluorescent cells throughout the cortex, including the hippocampal anlage after 72 hours of incubation (Figures 2A and 2B). This is consistent with tangential migration of cells from the ganglionic eminences through the cortex. Higher magnification views of these cells reveal that they have the typical appearance of migrating immature neurons with leading and trailing processes (Figure 2C).

The DiI-labeling experiments show that cells migrate from the ganglionic eminences to the hippocampus, but they do not define their identity (i.e., whether they are GABAergic cells). To determine whether the cells that tangentially migrated into the hippocampus have a GABAergic phenotype, we used a transplantation paradigm in which a donor slice was labeled with BrdU, and the MGE from the donor slice was transplanted into the MGE of a host slice and cultured for 72 hours. E12.5 embryos were chosen because this is the peak of hippocampal GABAergic interneuron birth (Soriano et al., 1986). The distribution of BrdU-labeled cells was determined by sectioning the slices and identifying donor MGE-derived cells using BrdU immunohistochemistry. We found that BrdU-labeled cells migrated tangentially from the MGE to the neocortex and hippocampus in 3/3 explants (Figures 2D–2F). We counted a total of 104 BrdU-labeled cells that migrated to the hippocampus in the three explants when the entire series of 12 μm sections from all three explants were examined. To determine if the BrdU-labeled cells that migrated to the hippocampus expressed GABA, we used double-label fluorescent immunocytochemistry and antibodies directed against BrdU and GABA. Of the 104 BrdU-labeled cells in the hippocampus, 56 were also labeled with GABA. These double-labeled cells frequently had the characteristic morphology of migrating neurons with either a leading or both a leading and lagging process (Figures 2G–2I). Although this analysis is not strictly quantitative, it shows many GABAergic neurons born on

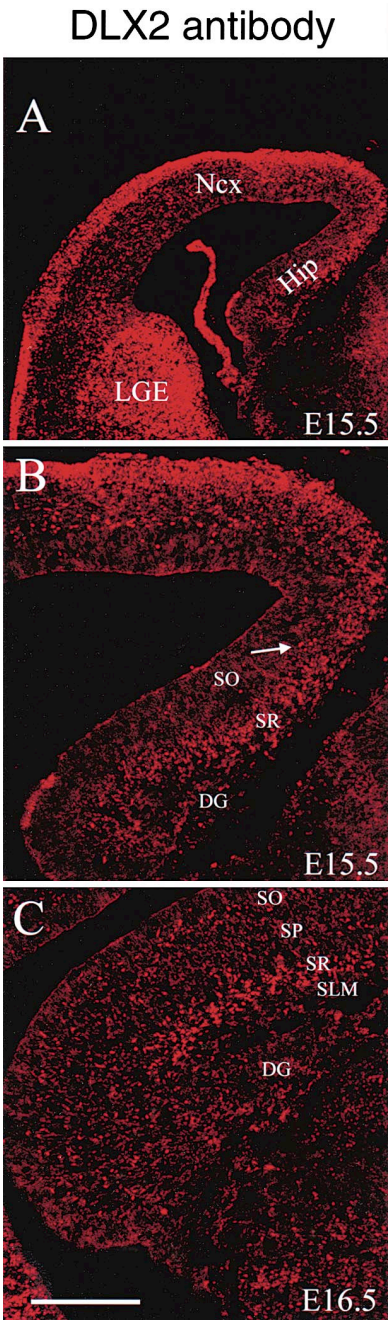

Figure 1. DLX2-Expressing Cells Arrive in the Stratum Radiatum by E15.5 and the Stratum Oriens by E16.5

(A) Low power view of coronal section of an E15.5 wild-type mouse brain stained with an antibody to DLX2. Note the area of intense staining in the LGE.

(B) Higher magnification view of the medial cortex including the hippocampal anlage. Note that there are DLX2-stained cells in the forming stratum radiatum but not the stratum oriens. Note that the $DLX2^+$ cells in the deeper layers of the neocortex (white arrow), which are continuous with stratum oriens, have not yet reached the hippocampus.

(C) Coronal section of an E16.5 wild-type mouse brain stained with the DLX2 antibody. By this time, DLX2-expressing cells have reached the stratum oriens. Bar = 1 mm for (A), 500 μm for (B), 250 mm for (C). DG, dentate gyrus; Hip, hippocampal anlage; LGE, lateral ganglionic eminence; Ncx, neocortical anlage; SLM, stratum lacunosum moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.

E12.5 in the mouse migrate to the hippocampal anlage. Cells labeled with BrdU but not GABA are likely to be either cells of a different type or cells that have not yet begun to express their GABAergic phenotype. The fact that BrdU-labeled cells make up only a minority of the GABA-labeled population is expected because we made no effort to remove the entire host ganglionic eminences and the 2 hr BrdU labeling period is likely to only label a small fraction of cells born on that day.

Dlx1/2 Mutant Mice Lack GABAergic Cells in the Hippocampus

The analysis shown above indicates that some GABAergic neurons migrate from the MGE to the hippocampus, but it does not address whether there are other sources for GABAergic interneurons or what fraction of interneurons depend on migration from subcortical sources. *Dlx1/2* mutants have a defect in migration to the neocortex and striatum from LGE and MGE slice explants (Anderson et al., 1997a; Marin et al., 2000; S. Anderson et al., submitted). Because of this defect and the 4-fold reduction of GABAergic interneurons in the neocortex of these mice, we elected to use these mice to determine the extent to which the ganglionic eminences contribute to the hippocampal interneuron pool.

We compared the immunohistochemical staining for GABA in the lateral neocortex and hippocampus of postnatal day 0 (P0) control and mutant mice (Figures 3A–3F). In wild-type or heterozygous mice (n = 6/6) there were GABAergic cells in all laminae of the hippocampal formation, but with the greatest numbers in the stratum oriens, stratum radiatum, and hilus (Figure 3C), consistent with the findings of earlier studies (Freund and Buzsáki, 1996). In the *Dlx1/2* mutant mice, as shown previously, there was roughly a 4-fold reduction in the number of cells stained for GABA in the lateral cortex (Figures 3E and 3F). In contrast, no $GABA^+$ cells were detectable in the hippocampus of mutant mice (n = 6/6 mutant mice) (Figure 3D). It is clear that the lack of GABA staining in the hippocampus of mutant animals was not due to a technical problem with immunodetection in the mutant mice because there was abundant GABA staining in the abnormally formed striatum of the mutant animals (Figure 3B). Likewise, no RNA encoding glutamic acid decarboxylase-67 (GAD67), the biosynthetic enzyme for GABA, was detectable in the hippocampal formation (Figures 3G and 3H). As reported previously (Sloviter et al., 1996), there was some expression of GAD67 mRNA in dentate granule cells in the mutant, heterozygote, and wild-type animals. These findings demonstrate that DLX1/2 function is required for the generation of GABA-ergic interneurons in the hippocampus and are consistent with a subcortical origin of these cells.

The Loss of Hippocampal GABAergic Staining Reflects a Loss of Cells Rather Than a Defect in GABA Production or Calbindin Expression

We next sought to determine if the lack of GABAergic cells in *Dlx1/2* mutant mice reflected the absence of these cells or simply a failure to express their appropriate phenotype. Using histochemical stains for DNA, we labeled all the cells in the hippocampi of mutant and heterozygote mice and saw a decrease in the number

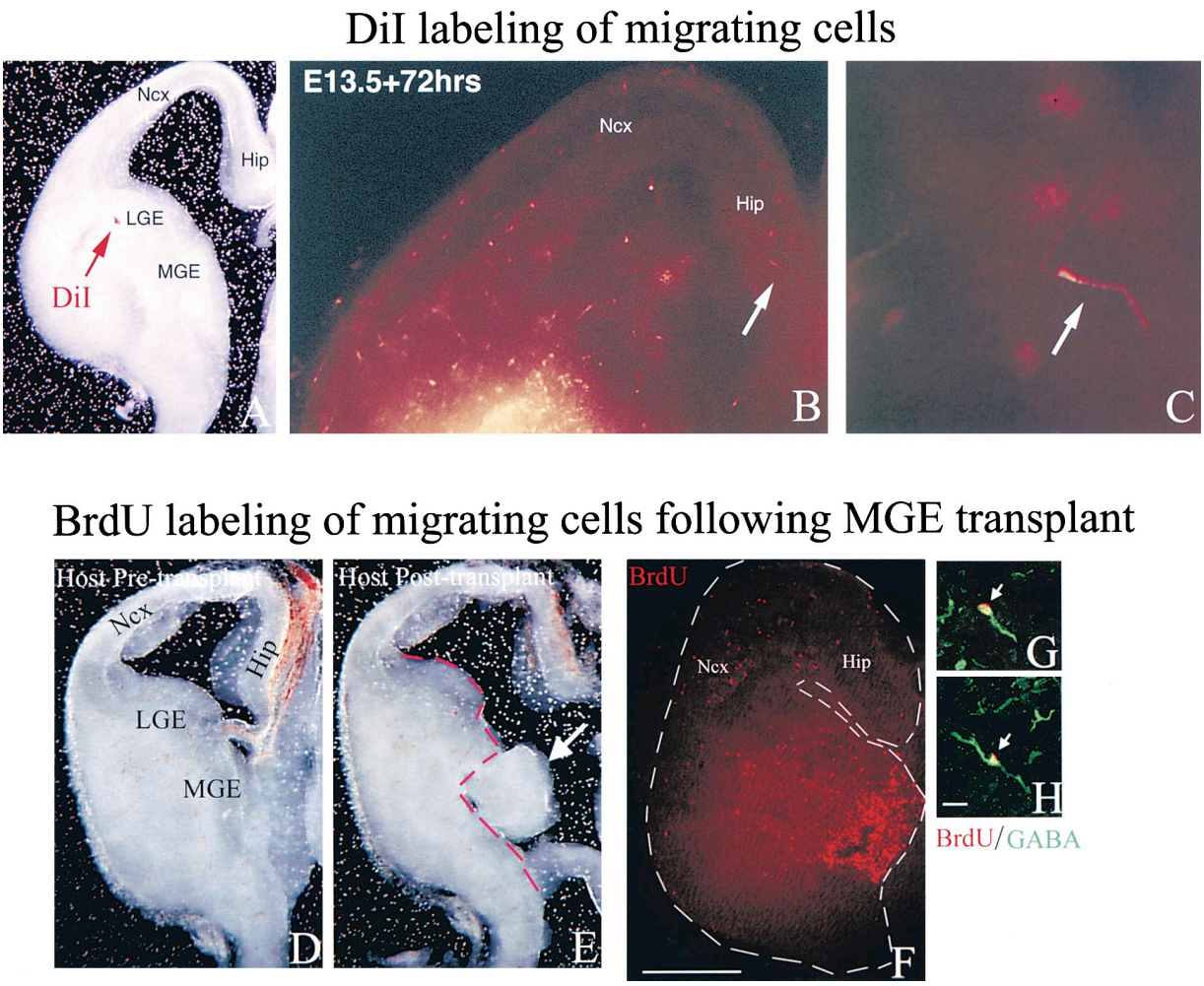

Figure 2. Tangential Migration of GABAergic Neurons from the Ganglionic Eminences to the Hippocampal Anlage Using DiI Labeling and Transplantation of Labeled MGE

(A) Bright-field image of a 250 μm thick explant from an E13.5 wild-type mouse implanted with a DiI crystal in the LGE.

(B) Flourescent image of DiI-labeled cells 72 hr after culturing the explant. The arrow indicates a cell in the hippocampus shown at higher magnification in (C).

(D) Bright-field image of a 250 μm thick explant from an E12.5 wild-type mouse prior to receiving a BrdU-labeled donor MGE.

(E) The same explant following the transplantation of a BrdU-labeled donor MGE (the red dashed line indicates the outline of the host explant and the arrow indicates the site of the donor tissue).

(F) Low power view of BrdU-labeled cells in a 12 μm section from the same explant shown in (D) and (E) revealing migration of individual cells to the neocortex and hippocampal anlage.

(G and H) Two examples of BrdU/GABA double-labeled cells from the hippocampal anlage. Bar in (F) = 500 μm and the bar in (H) = 20 μm for (G) and (H). Hip, hippocampal anlage; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Ncx, neocortical anlage.

of nuclei in the stratum radiatum of *Dlx1/2* mutant mice (Figures 4A and 4B). To quantify this difference, a blinded observer counted the nuclei from heterozygous (n = 3) and mutant mice (n = 3). The results showed an ~2-fold decrease in the density of nuclei in the stratum radiatum of mutant mice (*Dlx1/2*$^{+/-}$: 16.0 [SD = 1.5] nuclei per 15,000 μm^2 [p = 0.01]; *Dlx1/2*$^{-/-}$: 7.3 [SD = 0.2] nuclei per 15,000 μm^2 [p = 0.01]). These data support the hypothesis that GABAergic interneurons within the stratum radiatum are missing from the hippocampus of *Dlx1/2* mutant mice.

To further characterize the cells that are missing from the hippocampus of *Dlx1/2* mutants, we compared the expression of GABA with other markers of interneurons (calbindin) and markers of other subtypes of hippocampal neurons. In addition, GABA staining combined with nuclear labeling (Figures 5A and 4B) allowed us to roughly determine the proportion of cells in the stratum radiatum that were GABAergic. We counted cells in the stratum radiatum of three hippocampal sections from *Dlx1/2* heterozygote mice and found that 137 of 254 cells (or 54%) counted were GABAergic. These data, combined with the fact that about half the number of cells in the stratum radiatum remain in *Dlx1/2* mutant mice (see above), show that virtually all of expected number of GABAergic are missing in the *Dlx1/2* mutant mice. The identity and origin of the non-GABAergic cells in the stratum radiatum is currently unknown.

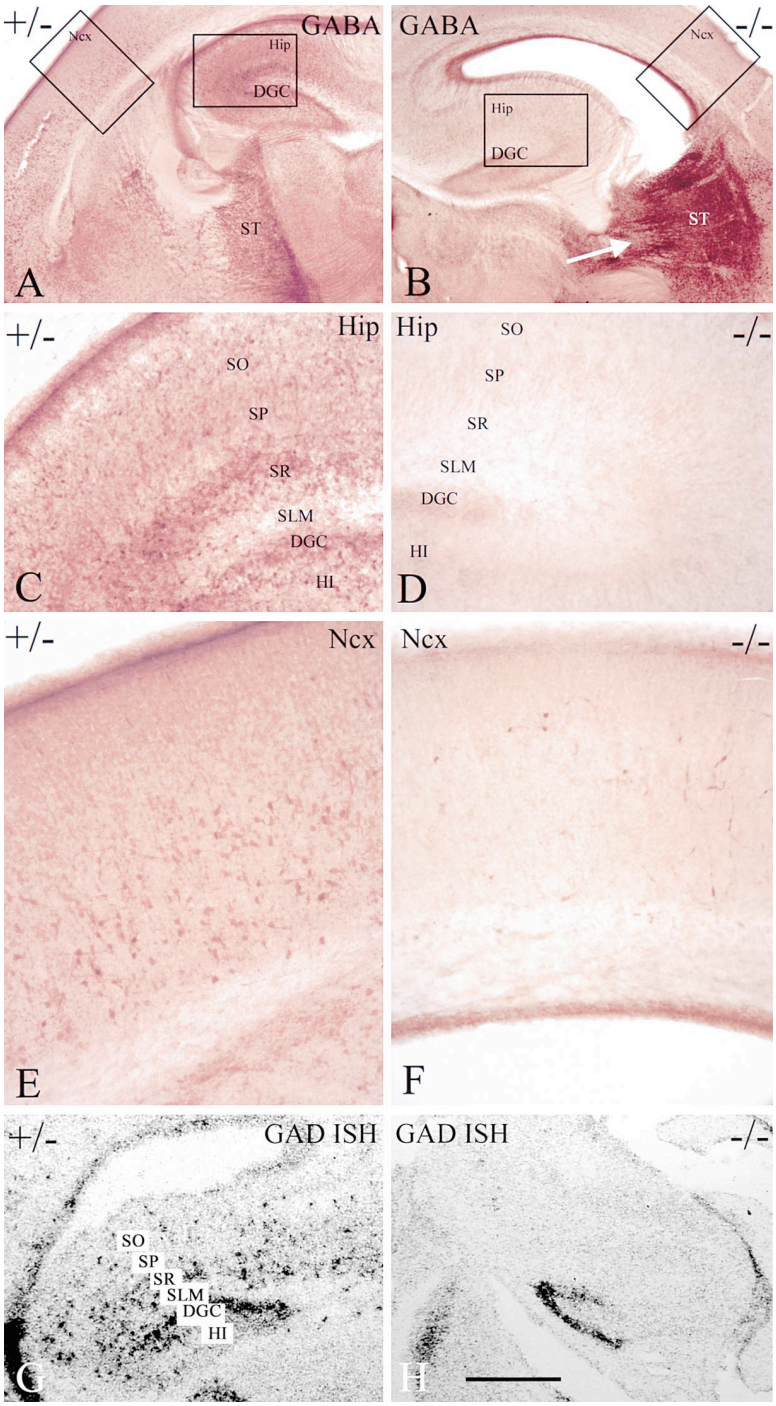

Figure 3. *Dlx1/2*$^{-/-}$ Mutant Mice Lack GABA Immunoreactivity in the Hippocampus at P0

(A and B) Low power views of GABA staining in coronal sections of P0 *Dlx1/2* mutant mice and littermates. Note the strong GABA staining in the striatum of the mutant mouse that demonstrates the activity of the antibody in this section (arrow). The immunohistochemical reactions were always allowed to proceed for equal or greater time periods in the mutant mice. The boxed areas marked with Hip or Ncx are shown below at higher magnification.

(C and D) Higher magnification views of the hippocampus. Note the absence of GABA staining in the hippocampus of the *Dlx1/2*$^{-/-}$ mouse.

(E and F) Higher magnification views of the neocortex. Note the decreased number of GABA stained cells in the neocortex of the *Dlx1/2*$^{-/-}$ mouse.

(G and H) The reduction in the in situ hybridization signal for GAD67 in the *Dlx1/2* mutant is consistent with the findings using the GABA antibody. The ability to detect GAD67 but not GABA in dentate granule cells in both the heterozygote and homozygote mutant mice likely represents an issue of relative sensitivities of the two techniques. Bar = 750 μm for (A) and (B), 190 μm for (C) and (F), 250 μm for (G) and (H). DGC, dentate granule cell layer; HI, hilus; Ncx, neocortical anlage; Hip, hippocampus; SLM, stratum lacunosum moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; ST, striatum.

During development, calbindin is expressed in the majority of GABAergic interneurons, although its expression in the cerebral cortex is not limited to interneurons (Alcantara et al., 1996). In the hippocampus of wild-type and heterozygous mice (n = 3/3) calbindin had a distribution similar to GABA with the highest number of cells in the stratum radiatum and stratum oriens (Figures 5A and 5B, 5D and 5E). In the mutant mice (n = 3/3), calbindin cell body staining was markedly reduced (Figures 5D and 5E). Unlike the pattern seen with the GABA antibody (Figure 5B), there were a few residual calbindin-expressing cells primarily seen in the stratum lacunosum moleculare and pyramidal layer (Figure 5E). Interestingly, there was extensive neuropil staining with the calbindin antibody in the stratum radiatum of the mutant mice (Figures 5D and 5E). At this time, we are uncertain of the origin of these fibers although they may be derived from one of the hippocampal afferent pathways from the septum, entorhinal cortex, or contralateral hippocampus.

Next we examined whether the *Dlx1/2* mutants had defects in other classes of hippocampal cells. There

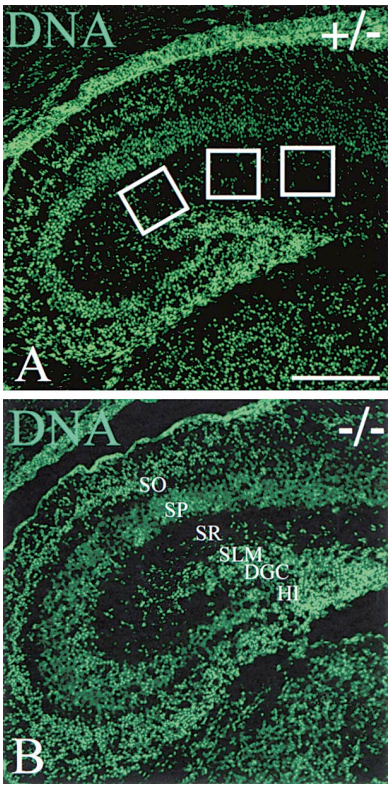

Figure 4. Decreased Cell Density in the Stratum Radiatum of P0 *Dlx1/2*Mutant Mice

(A and B) Sytox Green nuclear staining of hippocampal sections from a mutant and control littermate. The white squares in (A) show the position and size of the circles that were used for cell counting. Each square is 15,000 μm^2. Bar = 220 μm. DGC, dentate granule cell layer; HI, hilus; SLM, stratum lacunosum moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.

appeared to be no defect in the organization and number of cells in the granule cell layer or the pyramidal cell layers at P0. For instance, general neuronal markers, such as microtubule-associated protein (MAP2) and a neuron-specific tubulin isoform (β3-tubulin) appeared normal in the *Dlx1/2* mutants (data not shown). In addition, birthdating analysis following a single BrdU injection at either E14.5 or E16.5 showed normal genesis and migration of pyramidal and granule cells, respectively (data not shown). The continued expression of GAD67 (Figures 3G and 3H) in the granule cells also implies that these cells have differentiated normally.

We also analyzed the *Dlx1/2* mutant mice for the presence of Cajal-Retzius cells. These neurons, which express Reelin and calretinin, are critical in the radial migration of neurons and the appropriate laminar termination of afferents from the entorhinal cortex and contralateral hippocampus into the hippocampus (Del Rio et al., 1997; Super et al., 1998; Borrell et al., 1999a and 1999b; Deller et al., 1999). In the mutant mice, reelin and calretinin staining appeared unchanged in the hippocampus (n = 3/3 pairs of mice) (Figures 5F and 5I). Thus, as in the neocortex, the *Dlx1/2* mutation affects the development of GABAergic neurons and does not affect the development of glutamatergic cells. Furthermore, the *Dlx1/2* mutation affects the hippocampus more severely than the neocortex.

These findings suggest that hippocampal glutamatergic and GABAergic neurons have distinct developmental histories. This is consistent with birthdating studies that demonstrated that hippocampal interneurons have their last mitotic division before hippocampal projection neurons (Soriano et al., 1986). We repeated these birthdating studies using single injections of BrdU at E12.5, E14.5, and E16.5, followed by analysis on P0. While most hippocampal pyramidal cells were heavily labeled with BrdU (i.e., became postmitotic) by BrdU injections on E14.5 and E16.5 (data not shown), very few were heavily labeled following E12.5 injections. On the other hand, we found that a substantial portion of the GABAergic cells were double-labeled for BrdU after a single injection of BrdU at E12.5 (Figure 5C) and virtually no double labeling was observed in embryos receiving BrdU injections at E14.5 and E16.5 (data not shown).

Nkx2.1 Mutant Mice Lack Distinct Subpopulations of Hippocampal GABAergic Interneurons

Basal telencephalic progenitor cells in mice lacking the *Nkx2.1* homeobox gene exhibit a ventral-to-dorsal transformation in their molecular properties that leads to loss of cell types produced by the MGE and an expansion of cell types produced by the LGE (Sussel et al., 1999). Thus, these mutant mice can be used to assess which subtypes of hippocampal interneurons are derived from the MGE. A previous study found that the neocortex of these animals has ~50% decrease in the number of GABAergic neurons, while the number of GABAergic neurons in the olfactory bulb appears unaffected (Sussel et al., 1999). This is in contrast to the *Dlx1/2* mutants that have ~80% loss of neocortical GABAergic neurons and the nearly 100% loss of olfactory bulb GABAergic and tyrosine hydroxylase neurons (Anderson et al., 1997a, 1997b; Bulfone et al., 1998). The hippocampus of *Nkx2.1* mutant mice shows a clear decrease in the numbers of cells expressing DLX2 and calbindin (n = 3/3 pairs of mice) (Figures 6A–6F), but this defect is not as severe as in the *Dlx1/2* mutant mice. Quantification of the DLX2-expressing cells per 15,000 μm^2 in the stratum radiatum and stratum oriens showed a ~2-fold decrease (stratum radiatum: $Nkx2.1^{+/-}$ 69.4 [SD = 2.4]; $Nkx2.1^{-/-}$ 25.2 [SD = 1.50] [P = 0.002]; stratum oriens: $Nkx2.1^{+/-}$ 27.4 [SD = 2.3]; $Nkx2.1^{-/-}$ 11.9 [SD = 1.3] [P = 0.001]).

NPY and somatostatin-expressing cells are a major subpopulation of hippocampal GABAergic interneurons. *Nkx2.1* mutants have almost no expression of RNA encoding these neuropeptides in their hippocampus (Figures 6H, 6I, 6K and 6L). The lack of expression of NPY and somatostatin was also confirmed by immunohistochemistry (data not shown). All other specific markers of subsets of GABAergic interneurons (e.g., parvalbumin, vasoactive intestinal peptide, corticotrophin releasing hormone, enkephalin, cholecystikinin, and cortistatin) begin to be expressed in the first postnatal week (Gall et al., 1984; Hill et al., 1994; de Lecea et al., 1995, 1997; Avishai-Eliner et al., 1996; Freund and Buzsáki, 1996; Takeda et al., 1996), so we were unable to examine these due to early postnatal lethality of the *Nkx2.1* mutants.

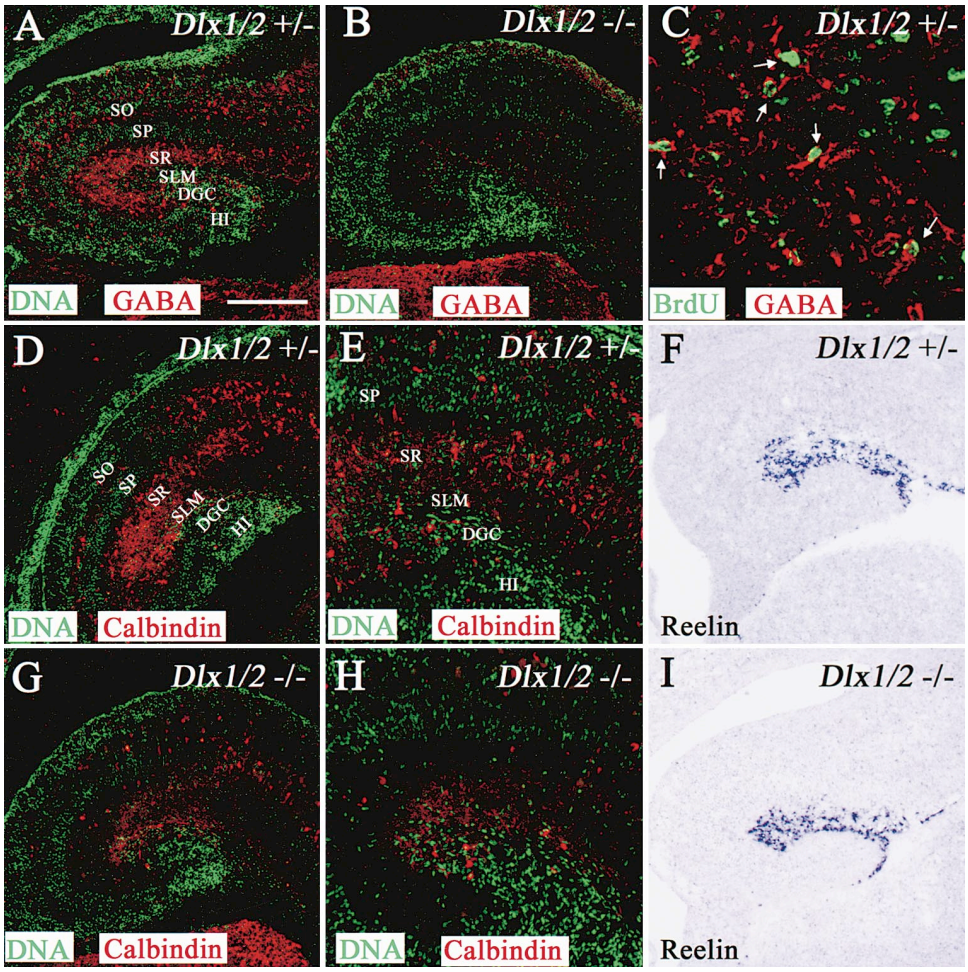

Figure 5. The Cells Missing in P0 *Dlx1/2* Mutant Mice Are an Early Born Group of GABAergic, Calbindin-Expressing Neurons

(A, B, D, and I) Labeling for cellular markers of interneurons or Cajal-Retzius cells in *Dlx1/2* mutant mice.

(A, B, D, and G) Note the absence of GABA and great reduction of calbindin staining in the *Dlx1/2*$^{-/-}$ mice.

(E and H) Higher magnification views of the calbindin staining are presented to show that the remaining labeled cells are largely in the SLM and DGC.

(F and I) In situ hybridization for *reelin* shows that the density of Cajal-Retzius cells is unchanged in the mutant mice.

(C) BrdU and GABA double labeling at P0 in a heterozygote *Dlx1/2* mouse after an injection of BrdU at E12.5. The arrows mark GABAergic cells in the stratum radiatum born on E12.5. Bar = 220 μm for (A), (B), (D), and (G), 110 μm for (E), (F) (H), and (I), and 35 μm for (C). DGC, dentate granule cell layer; HI, hilus; SLM, stratum lacunosum moleculare; SP, stratum pyramidale; SO, stratum oriens; SR, stratum radiatum.

We conclude that many GABAergic interneurons of the stratum oriens and stratum radiatum, particularly those expressing NPY and somatostatin, are likely to originate in the MGE, while the remainder are derived from progenitors cells in other regions. Cajal-Retzius cell markers (Reelin and calretinin) are unchanged in the *Nkx2.1* mutant mice (data not shown) implying that the origin of these cells is not dependent on the function of *Nkx2.1*.

Laminar Termination of Entorhinal and Commissural Fibers Is Normal in *Dlx1/2* Mutant Mice

On the basis of their location in stratum radiatum and detailed anatomical analysis, it has been suggested that hippocampal interneurons may guide hippocampal commissural axons into stratum radiatum (Super et al., 1998). To determine if commissural axon targeting is altered in the absence of hippocampal interneurons in *Dlx1/2* mutants, we used fluorescent lipophilic dyes (DiI, DiA, and DiQ) to trace axonal projections at P0. In mutants and controls, tracer injections into the hippocampal commissure labeled neurons and processes in the CA1 and CA3 regions (Figures 7A–7D). As shown in other studies, neurons in stratum pyramidale were labeled retrogradely; these pyramidal cells extended apical dendrites into stratum radiatum but not into stratum lacunosum-moleculare (Figures 7C and 7D) (see also Super et al., 1998). Commissural axons (labeled anterogradely) were likewise excluded from stratum lacunosum moleculare (Figures 7C and 7D).

Because it is also possible that interneurons in the stratum radiatum might be involved in regulating axon pathfinding (perhaps by excluding fibers normally destined to terminate in neighboring laminae), we studied the entorhinal afferents that project to the stratum lacunosum moleculare. Entorhinal tracer injections labeled

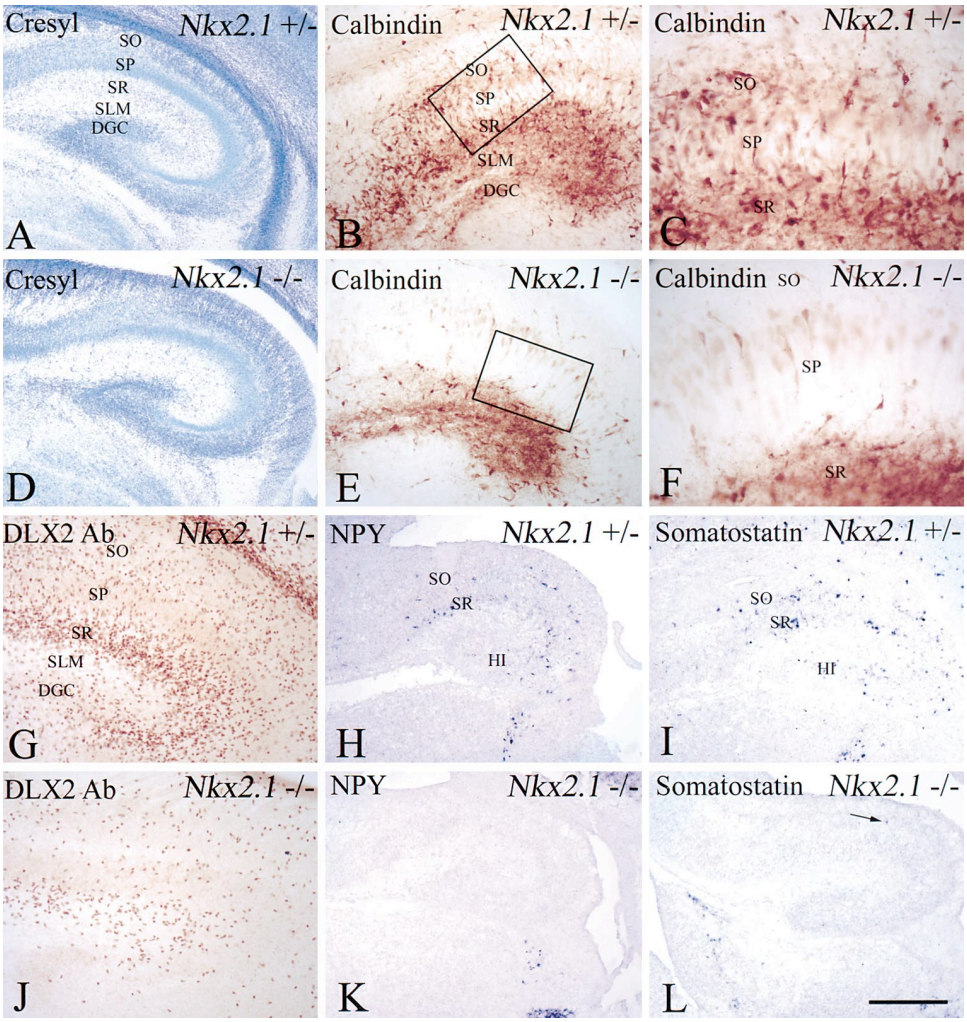

Figure 6. E18.5 *Nkx2.1*$^{-/-}$ Mutant Mice Have a Selective Loss of NPY and Somatostatin-Expressing Interneurons in the Hippocampus

Coronal sections of *Nkx2.1* mutant and control brains.

(A and D) Cresyl violet stain of *Nkx2.1* mutant mice showing that the laminar architecture of the hippocampus is preserved.

(B, C, E, and F) Calbindin antibody staining demonstrates that a substantial fraction of the cells are missing (these cells also express GABA, data not shown). There is extensive neuropil staining for calbindin in the stratum radiatum of both control and mutant mice. We do not know the origin of these fibers.

(G and J) DLX2 antibody staining confirms the reduction of GABA- and calbindin-expressing cells. The nuclear staining with DLX2 was utilized for quantitative assessment of the cell number because of the unambiguous nature of the staining.

(H, I, K, and L) In situ hybridization for NPY and somatostatin shows the severe reduction in the expression of these mRNAs in the HI, SR, and SO of *Nkx2.1* mutant mice. An arrow shows one remaining somatostatin-expressing cell. Bar = 325 μm for (A), (D), (H), (I), (K), and (L), 165 μm for (B), (E), (G), and (J) and 80 μm for (C) and (F). DGC, dentate granule cell layer; SLM, stratum lacunosum moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; HI, hilus.

two major projections into the hippocampus: the alvear pathway (fibers from the entorhinal cortex before they perforate to their final target) and the perforant pathway, whose fibers crossed the subiculum to enter stratum lacunosum moleculare (Figures 7E–7H). The laminar specificity of these projections was unaffected in *Dlx1/2* mutants (Figures 7F and 7H). Commissural and entorhinal pathways were directly compared by injecting tracers with different fluorescence spectra (DiQ and DiA) into the hippocampal commissure and entorhinal cortex of the same specimen (Figures 7I–7L). These experiments confirmed that commissural and entorhinal axons did not overlap in heterozygotes or in homozygous mutants. Importantly, the stratum radiatum received commissural but not entorhinal projections, while stratum lacunosum moleculare received entorhinal but not commissural projections (Figures 7K and 7L).

Discussion

In this report, we provide evidence that GABAergic hippocampal interneurons are derived from the ganglionic eminences and specific hippocampal interneuron subtypes are derived from distinct subcortical progenitor zones. We show that the organization of the glutamatergic neurons of the hippocampal CA-fields and the

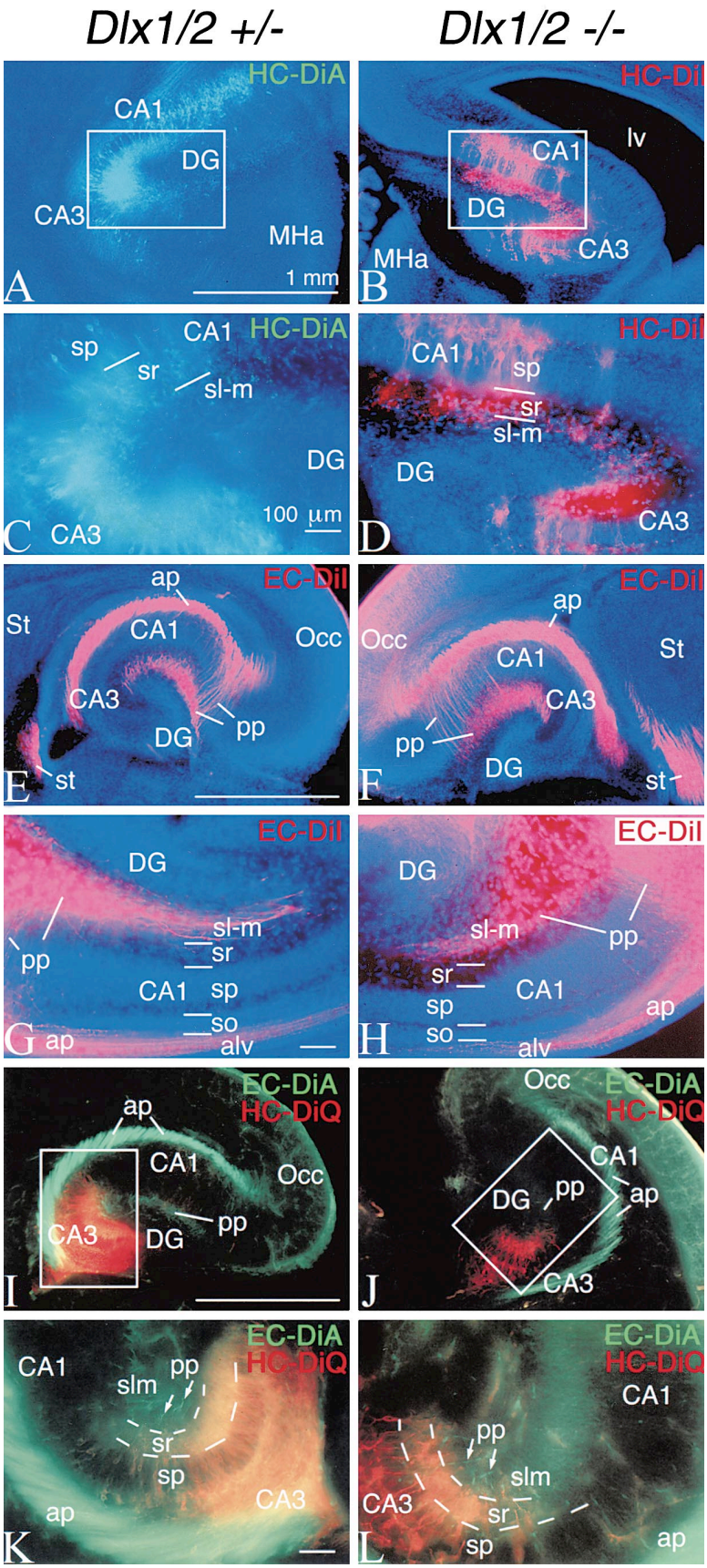

Figure 7. Targeting of Hippocampal Connections Is Preserved in *Dlx1/2*$^{-/-}$ Mutants at P0

(A–D) Connections from the hippocampal commissure (hc). In controls (A and C), the tracer (DiA, in this example) retrogradely filled neurons in CA1 and CA3 and anterogradely labeled commissural axons. Commissural axons and apical dendrites of pyramidal cells were found mainly in the stratum radiatum (sr) and only rarely entered stratum lacunosum-moleculare (sl-m). The dark blue DAPI counterstain binds to DNA. Boxed area in (A) is enlarged in (C). In *Dlx-1/2*$^{-/-}$ mutants (B and D), retrograde and anterograde pathways were intact, and labeled processes were correctly targeted to the sr. Boxed area in (B) is enlarged in (D).

(E–H) Connections from the entorhinal cortex (EC). In controls (E and G) and mutants (F and H), two major projections entered the hippocampus from the EC: the alvear pathway (ap) and the perforant pathway (pp). The ap axons traveled within the alveus (alv), while the pp axons traveled within the sl-m. This laminar specificity was unchanged in mutants.

(I–L) Double labeling of connections from the entorhinal cortex (EC) traced with DiA (green) and from the hippocampal commissure (hc) traced with DiQ (orange/red). In controls (I and K) as well as mutants (J and L), entorhinal-hippocampal pathways were segregated into separate layers from commissural pathways. Note the sharp boundary between DiQ-labeled hippocampal dendrites in sr and perforant pathway axons in sl-m (arrows in [K]–[L]). Boxed areas in (I) and (J) are enlarged in (K) and (L), respectively. Alv, alveus; ap, alvear pathway; DG, dentate gyrus; lv, lateral ventricle; Mha, medial habenula; Occ, occipital cortex; pp, perforant pathway; sl-m, stratum lacunosum-moleculare; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; St, striatum; st, stria terminalis.

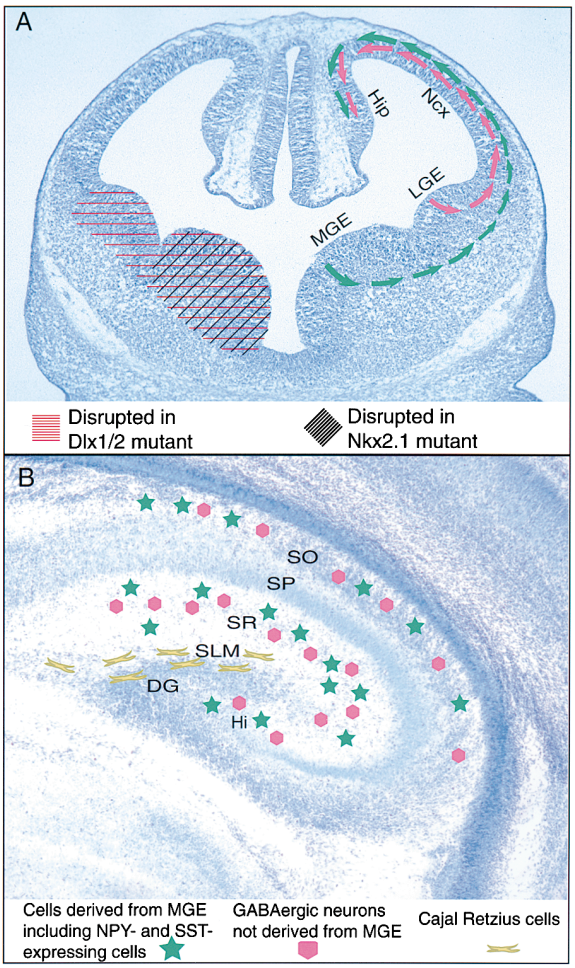

Figure 8. Schematic Model of the Origin of Hippocampal GABAergic Interneurons

(Top) E12.5 mouse coronal section stained with Cresyl violet and overlayed with arrows on the right indicating the migratory pathway of cells from the LGE and MGE to the hippocampal anlage. On the left, the regions disrupted by either the *Dlx1/2* or *Nkx2.1* mutations are indicated by red or black crosshatching, respectively.

(Bottom) E19 mouse coronal section stained with Cresyl violet and overlayed with cells of different colors representing two distinct groups of GABAergic interneurons and Cajal-Retzius cells. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Ncx, neocortex; Hip, hippocampus; Hi, hilus; DGC, dentate granule cell layer; SLM, stratum lacunosum moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.

dentate gyrus is not dependent on the presence of hippocampal interneurons. Finally, we took advantage of the well-described laminar nature of the hippocampal afferent systems to analyze the role of GABAergic interneurons in targeting events. We found that, contrary to previous hypotheses (Super et al., 1998), we have shown that these interneurons are dispensable for the laminar termination of the hippocampal commissural afferents.

Loss of *Dlx* Function Eliminates Hippocampal GABAergic Neurons

Hippocampal GABAergic interneurons depend on *Dlx1/2* function in the developing basal ganglia (Figure 8). These homeobox genes are required for later waves of neurogenesis in the LGE and MGE (Anderson et al., 1997b; Marin et al., 2000). The lack of GABA-immunoreactive cells in the hippocampus of *Dlx1/2* mutant mice contrasts with the neocortex where roughly 25% of GABAergic neurons remain. It is unclear why the hippocampus is more severely affected than the neocortex. Perhaps the neocortex has a distinct subtype of GABAergic interneuron, not present in the hippocampus and not dependent on *Dlx1/2* function for its development. Such cells would then be the GABAergic neurons remaining in the neocortex of *Dlx1/2* mutant mice. Alternatively, *Dlx1/2* may not have a fully penetrant migration defect (Anderson et al., 1997a) and the longer distance to the hippocampus may lead to a more severe defect on the basis of distance alone.

In addition to regulating migration, there is evidence that *Dlx* genes control differentiation of the GABAergic phenotype. Most DLX$^+$ cells are GABAergic, (Anderson et al., 1997a, 1999; Stuehmer and Rubenstein, unpublished data), and there is evidence that most, if not all, forebrain GABAergic neurons expressed the *Dlx* genes at some point in their development (Stuehmer and Rubenstein, unpublished data). Gain of function studies show that ectopic expression of DLX2 in cortical cells can induce them to produce GABA (Anderson et al., 1999; Stuehmer, Anderson, and Rubenstein, unpublished data). In addition, ectopic expression of the *Mash1* bHLH transcription factor in the cortex induces DLX1 and GABA expression (Fode et al., 2000). These data support the possibility that some of the residual cells in the stratum radiatum in the *Dlx1/2* mutant mice might be cells derived from the MGE or LGE but unable to adopt a GABAergic fate because they lack the necessary transcriptional effectors. However, our data comparing the numbers of cells lost from the stratum radiatum with the fraction that are normally implies that this theory, if true, could only account for a small number of residual cells in the stratum radiatum.

The MGE Produces a Distinct Subtype of Hippocampal Interneuron that Is Known to Be Critical for Controlling Abnormal Excitability in Epilepsy

One unique feature of the hippocampus is the well-studied relationship between network excitability and epileptogenesis. The role of interneurons in the regulation of abnormal network excitability is well established (Freund and Buzsáki, 1996), especially those expressing NPY and somatostatin (Vezzani et al., 1996, 1999; Baraban et al., 1997). The *Nkx2.1* mutant mice are helpful in delineating the origin of subtypes of hippocampal interneurons, because their MGE does not produce its normal set of neurons (Figure 8). These mutants appear to have a ventral-to-dorsal transformation that respecifies neuroepithelial progenitors in the MGE to produce LGE-derived cells. *Nkx2.1* mutants have about a 3-fold reduction in the number of hippocampal interneurons, implying that at least 60%–70% of the hippocampal interneurons are derived from the MGE. Additional sources for hippocampal interneurons are not known for certain but are likely to include other basal telencephalic regions that express *Dlx1* and *Dlx2*. These include the LGE, septum, telencephalic stalk, and parts of the caudal ganglionic eminence.

There are numerous subtypes of interneurons in each region of the hippocampus (Freund and Buzsáki, 1996). The *Nkx2.1* mutation eliminates a specific neuronal subtype, that expressing NPY and somatostatin, suggesting that this class of neuron is specifically derived from the MGE. Indeed, *Nkx2.1* mutants lack NPY and somatostatin in the striatum as well (Marin et al., 2000). Interestingly, *Dlx1/2* mutation does not eliminate all NPY$^+$ and somatostatin$^+$ interneurons in the striatum and neocortex (Marin et al., 2000; S. Anderson et al., submitted). We suggest that *Dlx1* and *2* are required in both the LGE and MGE to regulate production of interneurons and that the *Dlx1/2* mutation partially affects development of most/all types of tangentially migrating interneurons. On the other hand, we suggest that *Nkx2.1* regulates development of interneurons derived from the basal-most telencephalon (e.g., MGE) such as NPY$^+$ and somatostatin$^+$ telencephalic interneurons.

The interneurons expressing NPY and somatostatin are especially important in regulating the response of the hippocampus to abnormal excitability (Vezzani et al., 1996, 1999). In mice lacking NPY, brief seizures, which are well tolerated in normal mice, produce status epilepticus and death (Baraban et al., 1997). NPY and somatostatin are both potent antiepileptic agents when infused locally in the hippocampus (Vezzani et al., 1999). This has led to the idea that interneurons expressing these neuropeptides have a critical role in maintaining the balance of excitatory and inhibitory influences within the hippocampus. Unfortunately, *Nkx2.1* and *Dlx1/2* mutants die the day of birth and therefore one cannot determine whether they would suffer from epilepsy. Nonetheless, these results raise the possibility that some forms of human epilepsy may be due to developmental abnormalities in hippocampal interneurons that result from dysfunction in neurogenesis or tangential migration from the basal ganglia.

Development of Hippocampal Glutamatergic and GABAergic Neurons Are under Distinct Genetic Controls

While genes that control basal telencephalon development are essential for producing hippocampal interneurons, genes that are expressed in the ventricular zone of the hippocampal anlage are now known to regulate formation of glutamatergic pyramidal neurons of the CA fields and granule neurons of the dentate gyrus. Thus, loss of function mutations in either *Lef1* (an HMG-box transcription factor) or *NeuroD* (a basic helix-loop-helix transcription factor) eliminate the dentate gyrus, while not appearing to affect the production of GABAergic neurons in this region (Galceran et al., 2000; Liu et al., 2000). A neomorphic allele of *Lef1*, which can inhibit β-catenin-mediated Wnt signaling, eliminates the entire hippocampal field (Galceran et al., 2000), as does a mutation in *Wnt3a* (Lee et al., 2000). Mutations in *Lhx2*, *Lhx5*, and *Emx2* also disrupt development of hippocampal glutamatergic neurons to varying degrees although the development of GABAergic interneurons was not carefully analyzed in these mutants (Pellegrini et al., 1996; Yoshida et al., 1997; Porter et al., 1997; Zhao et al., 1999; Mallamacci et al., 2000; Tole et al., 2000).

The absence of any obvious defect in Cajal-Retzius cells in either the *Dlx1/2* or *Nkx2.1* mutants is interesting in light of recently published reports that these cells may originate in the MGE (Lavdas et al., 1999). These authors show direct evidence of tangential migration from the MGE of cells with some features of Cajal-Retzius cells to the neocortex. The failure of the *Dlx1/2* or *Nkx2.1* mutations to affect these cells implies one of several possibilities. First, the Cajal-Retzius cells might originate in the MGE but not require *Nkx2.1* or *Dlx1/2* function for their origin or migration. In this case, they may be related in some way to the subset of about 20% of neocortical interneurons that do not depend on *Dlx1/2* function. Second, the Cajal-Retzius cells may not originate in the MGE but instead migrate from some other origin by a pathway that allows them to be labeled by DiI crystals placed in the MGE. Third, there may be confusion due to the imprecise nature of previous criteria for identifying Cajal-Retzius cells. This confusion has led to recent attempts to rigorously define criteria that need to be fulfilled to define a cell as a Cajal-Retzius cell (Meyer et al., 1999). In any case, we suggest that Cajal-Retzius cells have a cortical origin based on their expression of genes, (e.g., *Tbr1*) that are largely restricted to the cerebral cortex (R. F. Hevner, L. Shi, R. LeWinter, J. L. R. R. Rubenstein, 1999, Soc. Neurosci., abstract *25*, 502). In any case, further analysis of the origin of Cajal-Retzius cells will be critical to our understanding of cortical development and migration because of their central role in these processes.

GABAergic Interneurons Are Not Required for the Appropriate Laminar Termination of Hippocampal Afferents

The normal organization of hippocampal afferents is dependent on Cajal-Retzius cells (Borrell et al., 1999a, and 1999b; Deller et al., 1999; Super et al., 1998). These are early-born neurons that regulate the migration of hippocampal neurons to their appropriate locations and the laminar termination of entorhinal afferents (Super et al., 1998; Borrell et al., 1999b) and by repelling commissural afferents (Borrell et al., 1999b; Deller et al., 1999). In vivo and in vitro analyses provide evidence that Cajal-Retzius cells serve as intermediate targets for axons projecting from the entorhinal cortex (Del Rio et al., 1997; Super et al., 1998). These axons arrive in the stratum lacunosum moleculare where the Cajal-Retzius cells are concentrated, and later form synapses with the dendrites of CA pyramidal cells.

Using tracing techniques and electron microscopy, Soriano and colleagues have proposed that a similar function is served by GABAergic cells found in the stratum radiatum prior to birth (Super et al., 1998). In this case, they proposed that the hippocampal commissural afferents require these interneurons as intermediate targets to generate their appropriate lamina specific synaptic connections. Once the final targets of the afferents are present, i.e., the pyramidal cell dendrites, there is a transfer of the afferent axon terminals from the interneurons to the pyramidal cells. The *Dlx1/2* mutant mice provided us with a means to directly test this hypothesis by studying the terminations of hippocampal commissural fibers, because these animals lack the hippocampal GABAergic cell population. In addition, we analyzed

the termination of the entorhinal afferents because the GABAergic interneurons might be required either to exclude entorhinal afferents from the stratum radiatum. However, we found no defects in the terminations of either of these afferent tracts suggesting that the GABAergic interneurons of the stratum radiatum are dispensable for generating normal hippocampal afferent connections.

Experimental Procedures

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. Litters from matings between two *Dlx1/2*$^{+/-}$ animals were allowed to reach term and the P0 animals were anesthetized by cooling, perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), and postfixed in PFA for 2–4 hr. Neonatal *Dlx1/2* mutant animals were easily recognized by cleft palate and survive for several hours. Additional genotyping was performed as described previously (Anderson et al., 1997b). Litters expected to have *Nkx2.1* mutant mice were obtained by Caesarian section on the expected morning of birth. The animals were anesthetized by cooling, perfused with 4% PFA in PBS, and postfixed in PFA for 2–4 hr. Mutant animals were recognized by their lack of lungs, and additional genotyping was performed as previously described (Sussel et al., 1999). Following postfixation brains were cryoprotected in 30% sucrose and either frozen in embedding medium and cut using a cryostat or frozen and cut with a sliding microtome.

Anitbodies and Immunohistochemistry

Free-floating sections were preincubated in 5% normal serum of the species in which the secondary antibody was raised, 1% BSA and 0.3% TX in PBS for 1 hr at room temperature, and subsequently incubated with the primary antisera for 36 hr at 4°C in 2% normal serum and 0.3% TX in PBS. The following antibodies were used: rabbit anti-GABA (Sigma; diluted 1:2000), rabbit anti-calbindin (Swant, Bellinzona, Switzerland; diluted 1:5000), rabbit anti-calretinin (Chemicon, Temecula, CA; diluted 1:5000), rabbit anti-NPY (Incstar, Stillwater, MN; diluted 1:3000) and rat anti-SOM (Chemicon; diluted 1:250). Sections were then incubated in biotinylated secondary antibodies (Vector, Burlingame, CA; diluted 1:200) and processed by the ABC histochemical method (Vector). The sections were then mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), dried, dehydrated, and coverslipped with Permount (Fisher Scientific). In each experiment, sections from homozygous mutants and their wild-type or heterozygous littermates were processed together. Primary antiserum omission controls and normal mouse, rabbit, and goat serum controls were used to further confirm the specificity of the immunohistochemical labeling. For immunofluorescent double-labeling the protocol was essentially the same, except that 12 μm cryostat sections were used instead of free floating sections and Streptavidin-Alexa594 (Molecular Probes, Eugene, OR; diluted 1:400) was used instead of the ABC complex. For BrdU labeling, following primary antibody incubation, the tissue was postfixed, treated with 2N HCl, and washed with PBS prior to incubation with biotinylated secondary antibody and rat anti-BrdU (Harlan Laboratories; diluted 1:250). DNA counterstaining was performed with Sytox Green (Molecular Probes; diluted 1:25,000), and the slides were mounted with Prolong Antifade (Molecular Probes).

In Situ Hybridization and Probes

Nonradioactive in situ hybridization was performed essentially as described using a protocol obtained from Dr. David J. Anderson (California Institute of Technology), which was modified from published protocols (Schaeren-Wiemers and Gerfin-Moser, 1993). Briefly, sections were pretreated with Proteinase K then prehybridized for 3 hr at 65°C in a solution containing yeast tRNA, 50% formamide, and Denhardt's solution. Following prehybridization, sections were hybridized overnight at 65°C with digoxigenin-labeled probes at a final concentration of 1 μg/ml. The next day, the slides were washed at high stringency and incubated with sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (diluted 1:2000) for 2–3 hr at room temperature. Following washes, the slides were incubated in buffer containing nitrobluetetrazolium and BCIP until developed. The probes for Reelin, NPY, and somatostatin were derived from I.M.A.G.E. clones (734262, 482891, and 476246, respectively). Radioactive in situ hybridization for GAD67 was performed as described previously (Anderson et al., 1997).

Cell Counting

For cell counting in the stratum radiatum of *Dlx1/2* mutant mice, sytox green stained 12 μm sections were used. Optical sections (0.5 μM thick) from six different hippocampal sections per animal were obtained using a Biorad 1024 confocal microscope. Using these electronic images, three standardized boxes (15,000 μm^2) were placed in the stratum radiatum of the hippocampus. In each section, one box was placed in the stratum radiatum above the apex of the dentate granule cell layer, one above the midportion of the upper blade and one above the end of the upper blade. Cells were counted in each box and because they did not differ substantially were used to generate a value for the number of cells per unit area for each animal using six distinct hippocampal sections for each animal. The same technique was used for cell counting of DLX2 stained cells in the stratum radiatum and stratum oriens except that the counting was performed on 40 μm stained sections and the images were acquired using a conventional microscope and a Spot2 CCD camera.

Slice Migration Assay

The slice migration assay was performed as described previously (Anderson et al., 1997a). Briefly, brains from E13.5 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. DiI crystals (C-16 DiI; Molecular Probes) were placed into the tissue with an insect pin and slices were returned to the incubator for the appropriate time, then fixed with 4% PFA and mounted on slides.

For the MGE transplantation experiments, donor explants (E12.5) were incubated in 10 μg/ml of BrdU for two hours. These slices were then rinsed three times in medium to remove excess BrdU and small pieces of MGE were excised and placed into host slices (E12.5). The host slices were manipulated in order to try to increase the physical separation of the hippocampal anlage and the ganglionic eminences to decrease the possibility of artifactual migration of cells across the ventricular space. These slice explants were then cultured for 72 hr, fixed, embedded in OCT and cut at 12 μm on a cryostat. These sections were then used for double-labeling experiments to detect GABA and BrdU incorporated into the DNA of cells derived from the donor MGE. In other experiments (Anderson et al., submitted), we have shown that this method yields visualization of specific migration of cells from the ganglionic eminences to the cortex because transplants of irrelevant pieces of donor neuroepithelium yield no evidence of BrdU-labeled cells in the neocortex or hippocampus.

Axon Tracing

Axon tracing was performed on P0 mouse brains following perfusion. The brains were postfixed by immersion for 2–4 weeks in cold (4°C) fixative solution containing 4% paraformaldehyde, 4% sucrose, and 0.1 M sodium phosphate (pH 7.3). The brains were bisected into hemispheres, each of which was used for a separate experiment. Small crystals of DiI, DiA, or DiQ (all from Molecular Probes) were injected into the hippocampal commissure or entorhinal cortex with an insect pin. For double-labeling experiments, DiA was injected into one structure and DiQ into the other. The brains were then incubated in fixative solution for 4–8 weeks, rinsed in 0.1 M sodium phosphate (pH 7.3), and embedded in 4% low-melt agarose in PBS. Sections were cut on a vibrating microtome in the horizontal or coronal plane, or in a plane tilted 45° between the horizontal and coronal, such that the mid-portion of the hippocampus was cut perpendicular to its long axis. Sections were counterstained with DAPI (Molecular Probes), cover slipped in 30% sucrose in PBS, and examined with a fluorescence microscope.

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