

Early specification and autonomous development of cortical fields in the mouse hippocampus

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SUMMARY

Studies of the specification of distinct areas in the developing cerebral cortex have until now focused mainly on neocortex. We demonstrate that the hippocampus, an archicortical structure, offers an elegant, alternative system in which to explore cortical area specification. Individual hippocampal areas, called CA fields, display striking molecular differences in maturity. We use these distinct patterns of gene expression as markers of CA field identity, and show that the two major hippocampal fields, CA1 and CA3, are specified early in hippocampal development, during the period of neurogenesis. Two field-specific markers display consistent patterns of expression from the embryo to the adult. Presumptive CA1 and CA3 fields (Pca1, Pca3) can therefore be identified between embryonic days 14.5 and 15.5 in the mouse, a week before the fields are morphologically distinct. No other individual cortical areas have been detected by gene expression as early in development. Indeed, other features that distinguish between the CA fields appear after birth, indicating that mature CA field identity is acquired over at least 3 weeks. To determine if Pca1 and Pca3 are already specified to acquire mature CA field identities, the embryonic fields

were isolated from further potential specification cues by maintaining them in slice culture. CA field development proceeds in slices of the entire embryonic hippocampus. More strikingly, slices restricted to Pca1 or Pca3 alone also develop appropriate mature features of CA1 or CA3. Pca1 and Pca3 are therefore able to develop complex characteristics of mature CA field identity autonomously, that is, without contact or innervation from other fields or other parts of the brain. Because Pca1 and Pca3 can be identified before major afferents grow into the hippocampus, innervation may also be unnecessary for the initial division of the hippocampus into separate fields. Providing a clue to the source of the true specifying signals, the earliest field markers appear first at the poles of the hippocampus, then progress inwards. General hippocampal development does not follow this pronounced pattern. We suggest that the sources of signals that specify hippocampal field identity lie close to the hippocampal poles, and that the signals operate first on cells at the poles, then move inwards.

Key words: mouse, cerebral cortex, hippocampus, cortical area, CA field, specification

INTRODUCTION

The mammalian cerebral cortex is divided into many functionally specialized areas that also differ anatomically. Many recent studies have addressed the questions of when and how the developing rodent cerebral cortex is patterned into different areas, but most have focused on only one part of the cerebral cortex, the neocortex (reviewed in Grove, 1992; Levitt et al., 1993; McConnell, 1995; O'Leary et al., 1994). In the present study, we focus on another major component of the cerebral cortex, the hippocampus, and ask when the area divisions of the hippocampus, the CA fields, are specified.

The hippocampal CA fields are anatomically and neurochemically distinct, and appear to make different contributions to the role of the hippocampus in learning and memory (Blackstad, 1956; Hess et al., 1995; Lorente de No, 1934; Swanson and Cowan, 1977; Swanson et al., 1978; Woodhams et al., 1993; Zimmer and Haug, 1978). Information flows

through the CA fields in a largely unidirectional cascade (Nauta and Feirtag, 1986) and, in consequence, long-term memory is dependent upon the integrity of a single field (Tsien et al., 1996; Zola-Morgan et al., 1986). Accordingly, the development of normal hippocampal circuitry and function also depends on the correct division of the hippocampal anlage into its component CA fields. In the first step of this process, distinct populations of hippocampal cells must be specified to follow a particular developmental program, and thereby take on a particular CA field identity.

A previous study indicates that the field identity of hippocampal neurons, like the area identity of neocortical cells, is not determined by cell lineage (Grove et al., 1992; Walsh and Cepko, 1992). That is, the CA fields are not generated by precursor populations that are permanently specified, or committed, to produce neurons for a single field. Rather, field identity is likely to be specified by interactions between hippocampal cells and their environment. The cellular nature of

these specification signals, and when they may act has not been previously investigated.

The hippocampus is a promising system for this type of investigation for two reasons. First, the hippocampus is anatomically simpler than most other parts of the cortex, containing only a single principal cell layer. Previous studies (Blackstad, 1956; Lorente de No, 1934; Swanson and Cowan, 1977; Swanson et al., 1978; Woodhams et al., 1993; Zimmer and Haug, 1978), supported by the present study, indicate that this cell layer is divided into only two major fields, CA1 and CA3, each of which contains a distinct pyramidal cell type. The two classes of pyramidal neuron mingle in the small transitional field, CA2. Thus, in contrast with neocortex, in which area boundaries are marked by changes in several cell layers containing many cell types, the transition from CA1 to CA3 is marked by a simple shift from one major pyramidal cell type to another. Second, mature CA1 and CA3 pyramidal cells can be distinguished not only by their morphology, connections and physiological properties, but also by their expression of several macromolecules (see Table 1). Consequently, a cell's development of CA1 or CA3 identity can be readily determined *in vivo*, and in a variety of experimental settings, by the cell's expression of an appropriate set of molecular markers.

In the present study in the mouse, we ask when hippocampal cells are specified to acquire either a CA1 or CA3 field identity, using a panel of molecular markers, as well as morphological features, to define that identity. We find that two regions that we term presumptive CA1 and CA3 (Pca1 and Pca3) can be distinguished in the mouse hippocampus several days before birth by a complementary pattern of gene expression. However, this initial distinction between two embryonic hippocampal cell populations does not immediately indicate that the mature *field identity* of the cells is now specified. Many features make up a field identity, and these features may be specified at different times in development. Consistent with this, we find that new molecular and morphological field-specific features continue to appear in the developing hippocampus for at least 2–3 weeks after birth. Moreover, in another part of the cortical mantle, the neocortex, area identity appears to be specified gradually (Grove, 1992; Levitt et al., 1993; McConnell, 1995). Some features of neocortical area identity are specified during the peak of neurogenesis; others are specified much later, perhaps only just before the particular features appear postnatally (Cohen-Tannoudji et al., 1994; Levitt et al., 1993; O'Leary et al., 1994; O'Leary and Stanfield, 1989; Schlaggar and O'Leary, 1991; Stanfield and O'Leary, 1985).

To address the question of whether the presumptive hippocampal fields identifiable in the embryo are already specified to develop complex, mature CA field identities, the embryonic hippocampus was removed from its normal environment to one in which it would receive no further extrinsic specification cues: the developing hippocampal slice culture (Gahwiler et al., 1991; Stoppini et al., 1991). To attempt to identify candidate sources of specification cues, we first removed only extrahippocampal sources by culturing slices of the entire hippocampus. Next, the slices were subdivided into individual presumptive CA fields, which were then maintained separately in culture. In each case, we asked if hippocampal cells in these reduced environments could nonetheless develop a range of

molecular and morphological features of mature CA field identity.

MATERIALS AND METHODS

Tissue preparation

Outbred CD-1 mouse pups and timed-pregnant females were obtained from the University of Chicago Cancer Research Center Transgenic Facility. The day on which a vaginal plug was discovered is termed E0.5, and the day of birth P0. To harvest embryo brains, timed-pregnant dams were killed by CO₂ inhalation, embryos were removed, and brains dissected out and immersed overnight in 4% paraformaldehyde in PBS. To harvest older brains, mouse pups were deeply anaesthetized by hypothermia (pups only, P0–P7), or nembutal (P8 on), and perfused transcardially with the same fixative. After cryoprotection, each brain was sectioned into 40 µm coronal sections using a sledge microtome (Leica).

Slice cultures

Slice cultures were prepared according to the method described by Stoppini et al. (1991) with modifications. Timed-pregnant dams were killed by CO₂ inhalation, embryos were removed, and the telencephalon dissected out into Hepes-buffered ACSF. The telencephalon was sliced at a thickness of 200–400 µm on a McIlwain tissue chopper, slices were floated in Hepes-ACSF, and the hippocampus cut away from the rest of the telencephalon with a surgical microblade. The hippocampus proper was separated from the adjacent subiculum using a notch at the crest of the dentate gyrus as a landmark. A line drawn from this notch through and orthogonal to the cortical plate roughly marks the boundary between presumptive subiculum and CA1 (Paxinos et al., 1991). To generate slices of individual presumptive CA fields, each hippocampal slice was subdivided into four pieces: presumptive dentate gyrus (discarded), presumptive CA3, presumptive CA1, and a middle portion between the presumptive CA1 and CA3 pieces (discarded). Slices were placed on Millicell-CM inserts (Millipore) in 6-well culture plates, and maintained in B-27-supplemented NeuroBasal Medium (Gibco). For *in situ* hybridization or immunohistochemistry, cultures were fixed in 4% paraformaldehyde.

In situ hybridization

Riboprobes were transcribed from five cDNA clones used previously for *in situ* hybridization experiments, and tested for specificity of hybridization: pTy3IS.236 (*Tyro3*), O46.2B (*SCIP*), pKA1405 (*KA1*), pJDM33 (*NGFI-A*) and pmβ6T-3utr (class III β-tubulin) (Frantz et al., 1994; Lai et al., 1994; Wisden and Seeburg, 1993). *In situ* hybridization was performed using a non-radioactive method described previously (Tole and Patterson, 1995). For two-color *in situ* hybridization, one riboprobe was labeled with digoxigenin-UTP and the second with fluorescein-UTP (Boehringer Mannheim). Probes were detected with anti-digoxigenin or anti-fluorescein antibodies conjugated to alkaline phosphatase (Boehringer Mannheim). Color reactions were carried out using different substrates for alkaline phosphatase: nitro blue tetrazolium (NBT, Boehringer-Mannheim), 5-bromo-6-chloro-3-indolyl phosphate (magenta-phos, Molecular Probes), or a novel chromagen (T. Sanders and C. W. Ragsdale, personal communication).

Immunohistochemistry

Fixed cultures or tissue sections were incubated overnight with the Py antibody or with an antibody against calbindin-D-28K (Sigma), then incubated with secondary antibodies coupled to fluorescein or rhodamine (Tago, Boehringer Mannheim).

Morphological analysis

To examine differences in cell size between CA1 and CA3, cultures

were maintained for 12 days in vitro (DIV) to allow cell morphology to mature, then fixed and stained for Nissl substance. From each of three culture batches, a whole hippocampal slice and a pair of Pca1 and Pca3 slices were selected at low magnification, at random, from cultures that displayed clear Nissl staining. For each Pca slice, or field within a whole hippocampal slice, the outlines of 25–30 cell bodies were drawn, and perimeters and cross-sectional areas determined using a computer reconstruction system (Neurolucida, MicroBright-Field). Samples of CA1 and CA3 cells from whole hippocampal slice cultures were compared using a one-tail, paired two-sample *t*-test. Samples of cells from Pca1 and Pca3 slices were compared in the same way.

Cell death assay

Dying cells in embryonic hippocampus were identified using the TUNEL procedure (Gavrieli et al., 1992). Hippocampii were processed from 3–8 mice at each of the following ages: E15.5, 16.5 and 17.5. TUNEL staining was carried out using a kit (Boehringer Mannheim, In Situ Cell Death Detection Kit, Alkaline Phosphatase) according to the manufacturer's instructions. Intense TUNEL labeling of cells appeared in a variety of positive control tissues, including embryonic dorsal root ganglia, head mesenchyme and brain meninges. In hippocampus, TUNEL-stained cells were counted in the developing pyramidal cell layer – the hippocampal cortical plate plus the intermediate zone. To analyze possible regional differences in cell death, the developing pyramidal cell layer in each coronal section was divided into three sectors of equal length, one at the subicular end of the hippocampus, one at the dentate end, and a third intervening sector. These sectors are thus referred to as the subicular, dentate and 'gap' sectors (see Results).

RESULTS

Expression of several macromolecules provides a panel of CA1 and CA3 cell markers

The first step was to identify a panel of CA1 and CA3 pyramidal cell markers from among the many macromolecules reported to be differentially expressed in hippocampus (Table 1). Such markers should distinguish unambiguously between the two cell classes and label all, or almost all, cells in a class. In situ hybridization and immunohistochemistry were used to examine systematically in adult mouse hippocampus the expression of several macromolecules listed in Table 1. Expression patterns were compared in serial sections from the same brains, or in single sections labeled with a two-color in situ hybridization procedure. To compare marker-defined boundaries with CA field boundaries defined by classical morphological criteria, one series of sections from each brain was processed for Nissl substance. Nissl staining reveals differences in cell size and cell packing that clearly distinguish CA field boundaries (Blackstad, 1956; Lorente de No, 1934; Woodhams et al., 1993; Zimmer and Haug, 1978).

Four robust, field-specific markers were selected for the present study: Py-immunoreactivity, and the expression of *KAI*, *SCIP* and *Tyro3* mRNA. CA1 pyramidal cells strongly express *SCIP* and *Tyro3* (Figs 1G, 3A). CA3 pyramidal cells express *KAI* (Fig. 1H) and are Py-immunoreactive (Py-IR) (Fig. 3B). All four markers label all, or virtually all, cells in the pyramidal layer of either CA1 or CA3 (Figs 1G,H, 3A,B). Only two of the four markers label non-pyramidal cell types in the hippocampus: *KAI* is also expressed by dentate granule cells, and Py-immunoreactivity is detected in interneurons

Table 1. A diversity of molecular markers reported* to distinguish between CA1 and CA3 in adult mouse and rat

Feature	Function	CA1	CA3	Detection
<i>SCIP</i>	POU-domain transcription factor	+	–	ISH
<i>Brn-1</i>	POU-domain transcription factor	+	–	ISH
<i>Brn-2</i>	POU-domain transcription factor	+	–	ISH
<i>NGFIA</i>	Immediate early gene	+	–	ISH
<i>Tyro-3</i>	Receptor tyrosine kinase	+	–	ISH, ICC
<i>KAI</i>	Glutamate receptor subunit	–	+	ISH
<i>COX2</i>	Cyclooxygenase isoform	–	+	ICC
<i>Py</i>	Undefined antigen	–	+	ICC
<i>neurexin Ib, IIIa</i>	Brain-specific cell surface proteins	–	+	ISH
<i>neuronal pentraxin</i>	Taipoxin (snake venom) binding protein	±	+	ISH

*He et al. (1989); Woodhams et al. (1989); Bettler et al. (1990); Wisden and Seeburg (1993); Frantz et al. (1994); Herms et al. (1994); Lai et al. (1994); Alvarez-Bolado et al. (1995); Breder et al. (1995); Schlingens et al. (1995); Ullrich et al. (1995).

ISH, in situ hybridization; ICC, immunocytochemistry. The symbol ± indicates low expression of a marker.

scattered throughout the hippocampus (Wisden and Seeburg, 1993; Woodhams et al., 1989). As previously reported, all the markers additionally label cell types outside the hippocampus (Alvarez-Bolado et al., 1995; Frantz et al., 1994; He et al., 1989; Lai et al., 1994; Wisden and Seeburg, 1993; Woodhams et al., 1989).

Although the hippocampal pyramidal cell layer is classically divided into four CA fields, CA1–CA4 (Lorente de No, 1934), expression patterns of Py, *KAI*, *SCIP* and *Tyro3* indicate that there are only two major classes of pyramidal neuron in this cell layer. Each marker labels a subpopulation of cells in CA2 (Figs 1G,H, 2H), consistent with the mixing of CA1 and CA3 cell types in this field (Woodhams et al., 1993). Further, CA3 marker expression continues into the hilus of the dentate gyrus (Fig. 1H, Woodhams et al., 1993, and data not shown), indicating that the CA3 pyramidal cell type also continues into this region – the part of the pyramidal cell layer classically defined as CA4. Thus, the entire pyramidal cell layer appears to be divided between two major cell types, CA1 and CA3 cells. When do these cell types first show a difference in development?

Complementary expression of *SCIP* and *KAI* defines two presumptive CA fields

By E15.5, 4 days before the mouse is born, strong *SCIP* mRNA expression appears in presumptive CA1 (Pca1), as well as an adjacent cortical field, the presumptive subiculum (Fig. 1A). At the same age, strong *KAI* expression appears in presumptive CA3 (Pca3) and the dentate gyrus (Fig. 1B). A few cells in Pca3 express *KAI* still earlier, at E14.5, an age at which *SCIP* is detected in neocortex, but not in hippocampus (data not shown). Both *SCIP* and *KAI* are expressed by cells at different stages of their maturation. Migrating cells in the intermediate zone and settled cells in the hippocampal cortical plate express *SCIP* and *KAI* (Fig. 1A,B,E). *SCIP*, but not *KAI*, is also expressed by scattered cells close to the ventricular surface. The latter *SCIP*-expressing cells may be postmitotic cells at the start of migration, or precursor cells that are still dividing.

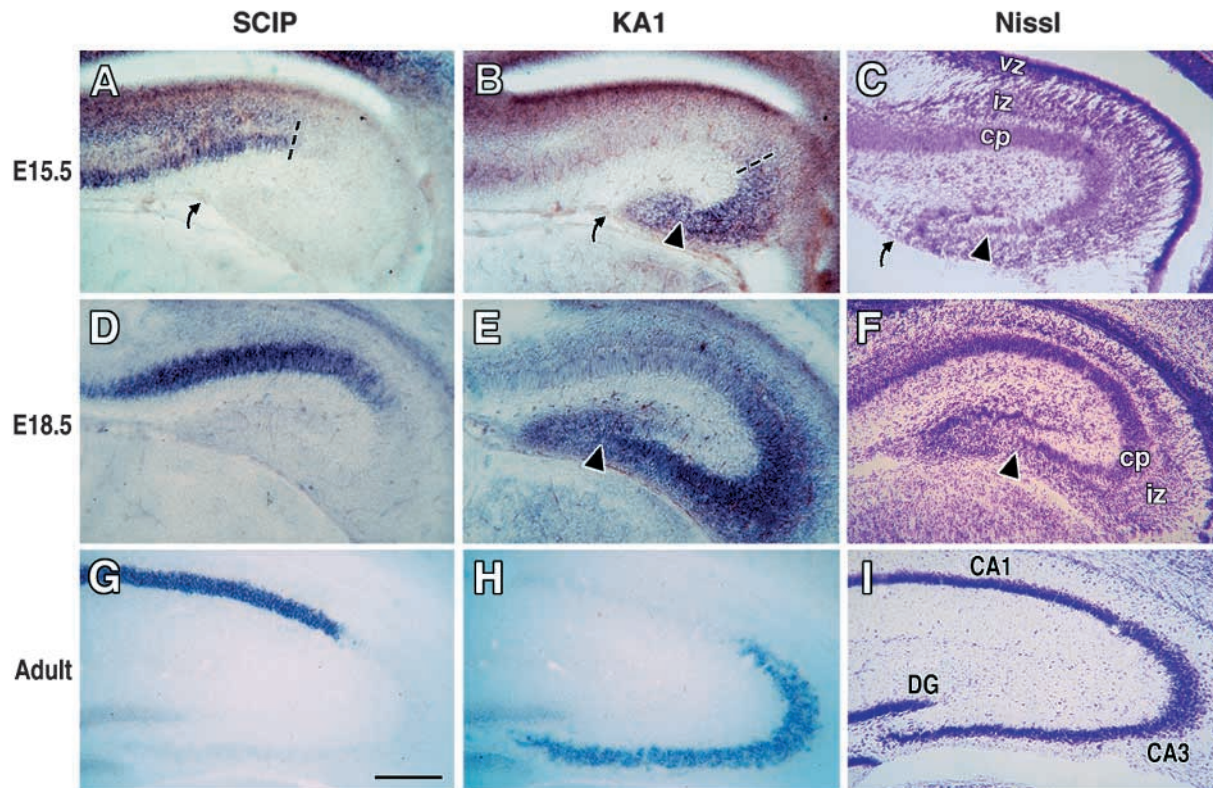


Fig. 1. *SCIP* and *KA1* expression distinguishes CA1 and CA3 in adult hippocampus, and presumptive CA1 and CA3 in embryonic hippocampus. (A–F) In embryonic hippocampus, *SCIP* is expressed in presumptive CA1 (A,D); *KA1* in presumptive CA3 (B,E). Both the cortical plate (cp) (A,B,D,E), and intermediate zone (iz) (A,B,E) contain *SCIP*- and *KA1*-expressing cells. Arrowheads (in B,C,E,F) indicate the tip of presumptive CA3; *KA1* expression medial to the tip of presumptive CA3 is in the developing dentate gyrus (B,E). At E15.5, the fronts of *SCIP* and *KA1* expression (dotted lines, A,B) do not yet meet, and a region in between expresses neither marker. By E18.5, the two fronts of expression have advanced inwards (D,E). (G–I) In adult hippocampus, *SCIP* is expressed in CA1 (G), *KA1* in CA3 (H) and *SCIP*- and *KA1*-expressing cells mix in CA2 (G,H). Arrows in A–C indicate a hippocampal landmark, a notch at the crest of the dentate gyrus. A line drawn from this notch through and orthogonal to the cortical plate roughly marks the boundary between presumptive subiculum and CA1 (Paxinos et al., 1991). Scale bar, 250 μ m (A–I).

Early field-specific differentiation begins at the hippocampal poles and moves inward

Regionally restricted expression of *SCIP* in the embryonic rat hippocampus has been reported previously (Alvarez-Bolado et al., 1995; Frantz et al., 1994; He et al., 1989), but these studies did not identify a complementary marker for developing CA3 cells. Consequently, the presumptive fields Pca1 and Pca3 could not be defined with respect to one another, and a further striking feature of early hippocampal development was missed. The hippocampal pyramidal cell layer has two ends, or 'poles', one at the dentate gyrus, and the other at the boundary between CA1 and the subiculum (see Figs 1, 2). At E15.5, cells near the dentate and subicular poles of the hippocampus express *KA1* and *SCIP*, respectively, but a broad stretch of the hippocampus in between expresses neither regional marker (compare positions of dotted lines in Fig. 1A,B). Over the next 3–4 days, however, the two fronts of *SCIP* and *KA1* expression advance gradually inwards from the poles (Fig. 1D,E).

The progression of early marker expression in the developing hippocampus is best seen in sections processed with a two-color in situ hybridization procedure (Fig. 2, Fig. 5A). At E16.5 a substantial gap is apparent between the fronts of *SCIP* and *KA1* expression (Fig. 2A–C) that is particularly prominent at mid and caudal levels through the hippocampus. By the next day, E17.5,

the gap between the fronts of *SCIP* and *KA1* expression has narrowed (Fig. 2D–F). Closing of the gap between the two fronts of gene expression is complicated by the simultaneous expansion of the embryonic hippocampus, but as hippocampal expansion slows, progression of the two markers catches up. No gap remains, even in the most caudal hippocampus, at birth (E19.5/P0, data not shown). In early postnatal hippocampus, expression of *SCIP* and *KA1* overlaps in field CA2 (Fig. 2G,H), showing the mature, adult pattern (Fig. 1G,H).

Does the poles-inward progression of *SCIP* and *KA1* expression reflect a more general pattern of hippocampal development? If the hippocampal cortical plate forms in this way, the gap between the fronts of early *SCIP* and *KA1* expression might simply reflect a lower cell density in that part of the embryonic hippocampus. However, no such region of low cell density is seen in Nissl stain (Fig. 1C). Further, hippocampal neuronal differentiation, in general, does not follow a poles-inward pattern. At E17.5, when there is still a gap between the fronts of *SCIP* and *KA1* expression (Fig. 5A), class III β -tubulin mRNA, a general marker of differentiating neurons, is expressed evenly throughout the developing hippocampal cortical plate and intermediate zone (Fig. 5B). Similarly, even expression of class III β -tubulin is seen as the cortical plate is forming (E14.5–E16.5) and, still

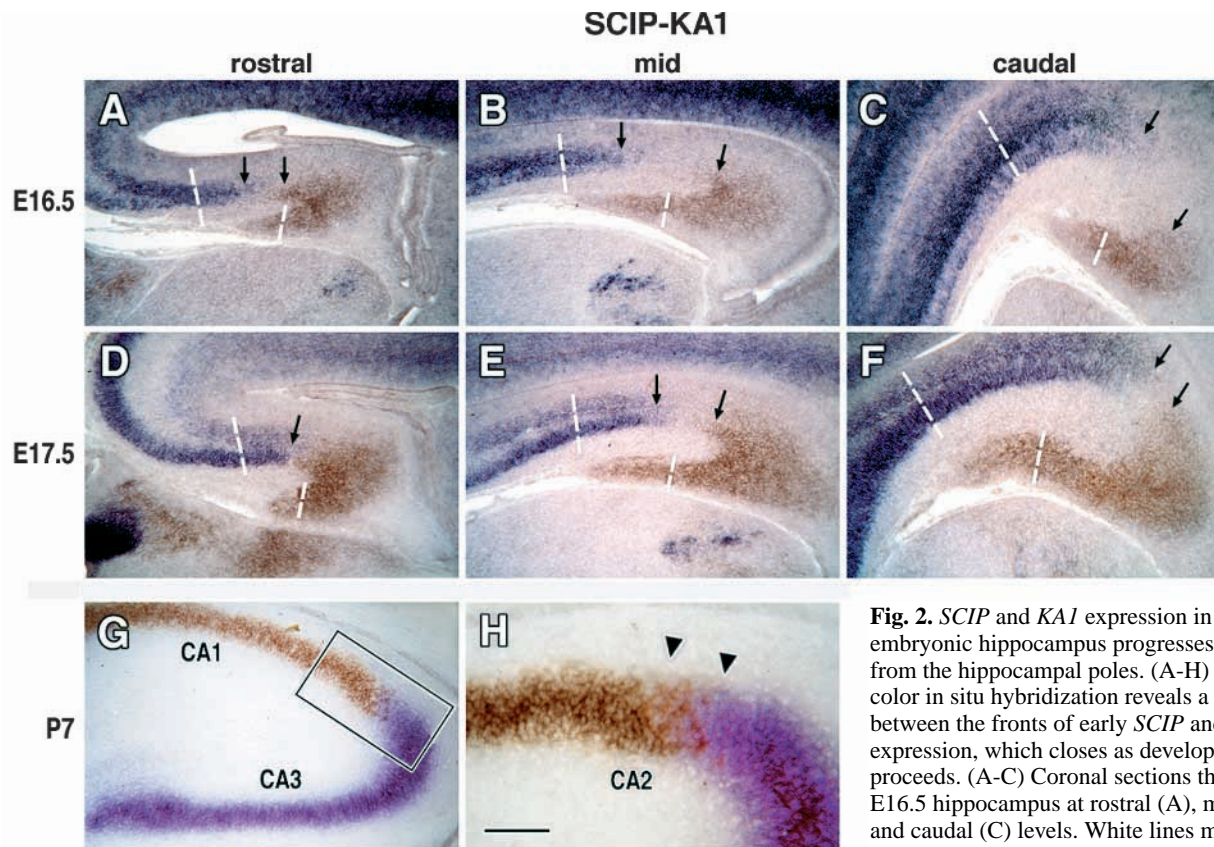


Fig. 2. *SCIP* and *KA1* expression in embryonic hippocampus progresses inwards from the hippocampal poles. (A-H) Two-color in situ hybridization reveals a gap between the fronts of early *SCIP* and *KA1* expression, which closes as development proceeds. (A-C) Coronal sections through an E16.5 hippocampus at rostral (A), mid (B) and caudal (C) levels. White lines mark the two ends of the developing hippocampal

pyramidal cell layer, determined as for Fig. 1. About one third of this layer (between black arrows) lacks *SCIP* (purple) or *KA1* (brown) expression. (D-F) Corresponding rostral to caudal levels through an E17.5 hippocampus. The gap between the fronts of *SCIP* and *KA1* expression has narrowed (D-F), and at the most rostral level (D) almost closed. (G,H) In P7 hippocampus, expression of *SCIP* (brown) and *KA1* (magenta) overlaps in field CA2. (H) is a higher magnification of boxed area in (G), arrowheads indicate intermingled *SCIP*- or *KA1*-expressing cells. Scale bars, 200 μ m (A-F); 250 μ m (G); 100 μ m (H).

earlier, in the hippocampal preplate (E10.5-E13.5) (data not shown). The poles-inward progression appears characteristic of the development of early field-specific neuronal markers.

A final, formal possibility is that the gap between the fronts of *SCIP* and *KA1* expression closes, not because cells between the two fronts upregulate marker expression, but because cells in this region selectively die. We used the TUNEL procedure (Gavrieli et al., 1992) to identify dying (TUNEL+) cells in the hippocampus at E15.5, E16.5 and E17.5, the period of 'gap closing'. Results were inconsistent with a role for cell death in closing the gap. In agreement with previous reports (Ferrer et al., 1994; Stanfield and Cowan, 1988), only low levels of cell death were evident in the embryonic hippocampus. TUNEL+ cells were sparse throughout the developing pyramidal cell layer, and TUNEL+ cells in the 'gap' region of this layer were no more numerous than in neighboring regions. At E17.5, for example, mean numbers of TUNEL+ cells were 1.0, 0.5 and 0.5 per section, respectively, for three equal-sized subicular, 'gap' and dentate sectors of the pyramidal cell layer (s.e.=0.6, 0.1, 0.4; TUNEL+ cells counted in 40 sections through four hippocampi; see Materials and methods for details of the division of the hippocampus into three sectors).

Mature CA1 or CA3 field identity is acquired over at least 3 weeks

Additional field-specific features distinguish CA1 and CA3 only

after birth. Consistent with previous observations in rat (Zimmer and Haug, 1978), CA3 cells in mice are not detectably larger than CA1 cells until P3 (Table 2). Selective expression of *Tyro3* and *NGFIA* in CA1, and Py-immunoreactivity in the pyramidal cells of CA3, is detectable still later, 1-2 weeks after birth, and shows an adult pattern by 3 weeks (Herms et al., 1994; Lai et al., 1994; Woodhams et al., 1989) (Table 2). In contrast with early CA field differentiation, none of these later features appears to follow a poles-inward pattern of development.

CA1 and CA3 field development proceeds without specification cues from outside the hippocampus

Are the presumptive fields, Pca1 and Pca3, already specified to

Table 2. Features that distinguish CA1 and CA3 continue to appear over at least three weeks

Feature	First field-specific appearance	Expression at E17.5	Expression in the adult
<i>KA1</i>	E14.5	CA3	CA3
<i>SCIP</i>	E15.5	CA1	CA1
Pyramidal cell size difference	P3	No difference	CA3 larger than CA1
Py	P5*	No expression	CA3
<i>Tyro3</i>	P10	No expression	CA1
<i>NGFIA</i>	P14	No expression	CA1

*In rat, Woodhams et al. (1989).

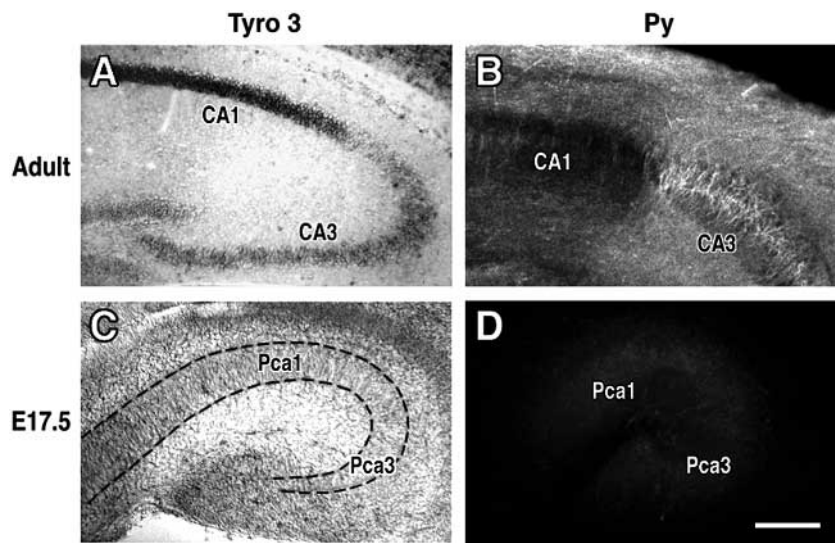


Fig. 3. Late-appearing molecular markers of CA1 and CA3. In adult hippocampus, *Tyro3* mRNA is strongly expressed in CA1 pyramidal cells (A), whereas CA3 pyramidal cells are strongly Py-immunofluorescent (B). Neither *Tyro3* expression nor Py-immunofluorescence is detectable in the hippocampus at E17.5 (C,D). Scale bars, 250 μ m (A,C,D); 140 μ m (B).

develop mature CA1 and CA3 identities, or do they require further specification signals from other hippocampal fields, or other parts of the brain? To address this question, E17.5 hippocampus was isolated from further potential extrinsic specification cues by maintaining hippocampal slices in organotypic culture. At E17.5, *Pca1* and *Pca3* can be detected by *SCIP* and *KA1* expression, but other field-specific features, such as *Tyro3* expression and Py-immunoreactivity, have not appeared (Fig. 3C,D). After 5–21 DIV, slice cultures were examined for expression of the early field markers *SCIP* and *KA1*, and for the development of three late-appearing features: *Tyro3* expression, Py-immunoreactivity and the characteristic difference in cell body size between CA1 and CA3 pyramidal neurons.

Slices prepared from E17.5 hippocampus maintain expression of *SCIP* and *KA1*, and develop all three new features of field identity in an appropriate, field-specific manner (Figs 4, 7). In many slices, the pyramidal cell layer is much broader than in vivo, probably reflecting the eventual breakdown of normal cell migration in cortical slice cultures (Gotz and Boltz, 1992), as well as spreading of the embryonic cortical plate. Nonetheless, slices retain a recognizable hippocampal morphology (Fig. 4), and although not confined to a compact pyramidal cell layer, the expression of each field marker is confined to a single region of each slice, either proximal (CA3) or distal (CA1) to the dentate gyrus (Fig. 4).

Appropriate expression of field-specific markers was highly

consistent (Table 3). Most cultures displayed field-specific staining patterns, and only a single culture showed a pattern of marker expression that was the reverse of that seen in vivo (Table 3). For each marker, however, a few slices displayed staining in both CA1 and CA3, due either to non-specific staining, or genuine upregulation of expression in both fields.

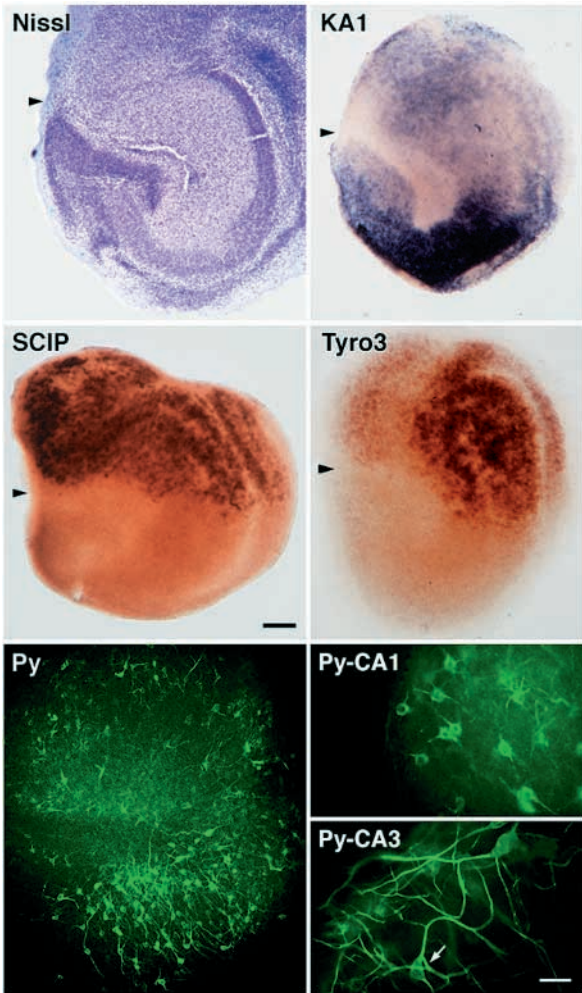


Fig. 4. E17.5 hippocampus develops new CA1 and CA3 field differences in vitro. (Nissl, KA1, SCIP, Tyro3, Py) A hippocampal slice (12 DIV) with adjoining cortex attached shows a well-defined pyramidal cell layer and dentate gyrus (Nissl). Other slices shown, containing the hippocampus only, display a much broader pyramidal cell layer, but can be oriented by reference to the dentate gyrus (arrowheads indicate landmark notch at the crest of the dentate gyrus). After 9–21 DIV, slices of E17.5 hippocampus maintain field-specific expression of *KA1* and *SCIP*, and upregulate expression of new field-specific markers, *Tyro3* and Py. (Py-CA1, Py-CA3) CA3 contains many large Py-IR neurons, with pyramidal-shaped cell bodies (Py-CA3, arrow), and thick, brightly stained processes. CA1 contains only scattered small, multifiform Py-IR cells, likely to be Py-IR interneurons. Scale bars, 150 μ m (Nissl, SCIP, KA1, Tyro3); 125 μ m (Py); 40 μ m (Py-CA1, Py-CA3).

Table 3. CA field identity develops in slice cultures of E17.5 hippocampus

Feature	Field-appropriate expression	Expression in inappropriate field only	Expression in both fields	No expression	Total*
<i>SCIP</i>	30 (94%)	0	2 (6%)	0	32
<i>Tyro3</i>	34 (67%)	1 (2%)	10 (19%)	6 (12%)	51
<i>KAI</i>	38 (85%)	0	1 (2%)	6 (13%)	45
Py	28 (78%)	0	4 (11%)	4 (11%)	36

*Total for each marker includes cultures prepared in at least 4 separate experiments.

The difference in size that developed between CA1 and CA3 cells in slice culture was strongly reminiscent of the difference these cell types develop in vivo (Fig. 7). In vivo, embryonic pyramidal cells appear roughly equal in size, but by adulthood in the rat or mouse, CA3 cells are about 50% larger than CA1 cells with respect to cell body diameter, and twice as large as CA1 cells with respect to cross-sectional area (Blackstad, 1956; Lorente de No, 1934; Zimmer and Haug, 1978; present study). Similarly, in E17.5 hippocampal slices maintained for 12 DIV, the mean cross-sectional area of CA3 cells ($138 \mu\text{m}^2$, s.e.=6) was roughly twice that of CA1 cells ($68 \mu\text{m}^2$, s.e.=4), and the difference was highly significant (one-tail, paired two-sample *t*-test, $P<0.001$).

At E17.5, a stretch of the hippocampal cortical plate still does not express *SCIP* or *KAI* (Figs 2D-F, 5A). Can cells express *SCIP* or *KAI* de novo in slice culture without extrinsic signals? After 12 DIV, the fronts of *KAI* and *SCIP* expression overlap in slice cultures (Fig. 5C,D) as they do in vivo (Fig. 2G,H), indicating that the poles-inward progression of *KAI* and *SCIP* expression has continued in vitro, and that a CA2-like field has developed. For this to occur, cells that were initially *SCIP/KAI*-negative have upregulated expression of these genes in culture.

Pca1 and Pca3 can develop a mature CA1 or CA3 identity autonomously

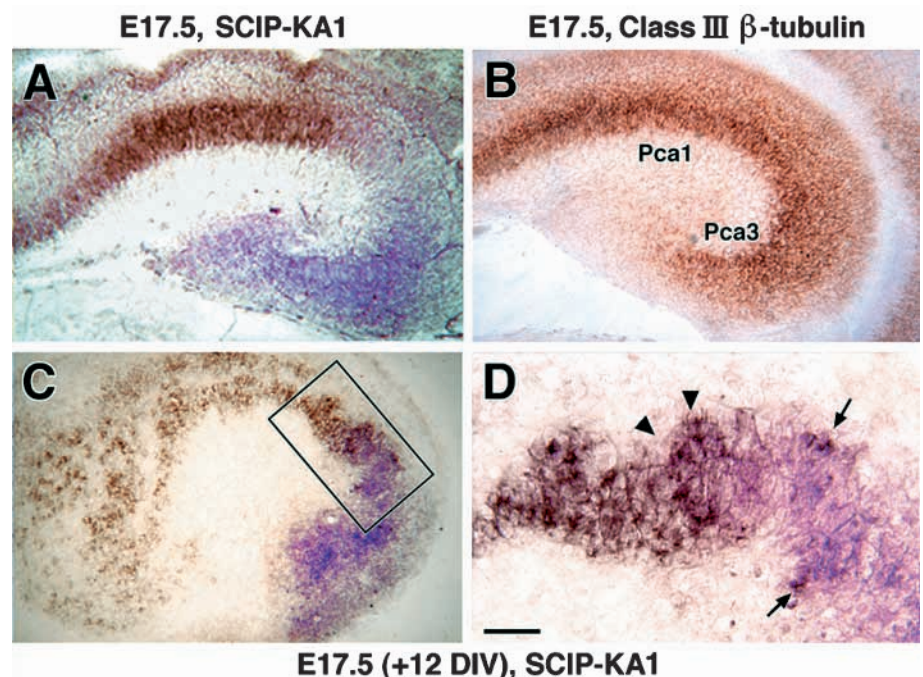
In cultures of whole embryonic hippocampal slices, therefore,

cells develop both early and late-appearing field-specific features without ongoing cues from outside the hippocampus. The next step was to remove potential specification cues from within the hippocampus itself, asking if the cells that remained could direct their own field-specific development. Hippocampal slices were again prepared from E17.5 mouse embryos, but this time, the presumptive CA fields, Pca1 and Pca3, were subdissected out and cultured separately for 5-21 DIV.

Strikingly, Pca1 and Pca3 slices not only maintain their expression of *SCIP* and *KAI*, but also develop, appropriately, all three late-appearing features of field identity: Py-immunoreactivity, *Tyro3* expression and cell size differences (Figs 6, 7). Moreover, appropriate expression of field-specific markers was highly consistent (Table 4). Even in the reduced environment of Pca slices, hippocampal cells develop morphological characteristics that are reminiscent of CA1 and CA3 cells in vivo (Figs 6, 7). After 12 DIV, the mean cross-sectional area of cells in Pca3 slices was about twice that of cells in Pca1 slices ($206 \mu\text{m}^2$, s.e.=21; $106 \mu\text{m}^2$, s.e.=14), and the difference was highly significant (one-tail, paired two-sample *t*-test, $P<0.005$).

Could cells from other hippocampal fields have contaminated the Pca slices, due to incomplete subdissection, and played a role in their development? To ensure that Pca1 and Pca3 were separated completely, a portion of the hippocampus at the transition between the two presumptive fields was discarded during subdissection. To check that Pca3 slices contained few or no

Fig. 5. The fronts of *SCIP* and *KAI* expression progress towards each other in hippocampal slice culture. (A,B) At E17.5, two-color in situ hybridization shows a gap between the fronts of expression of *SCIP* (brown) and *KAI* (purple); section taken from a mid level along the rostral-caudal axis of the hippocampus (A). In contrast, expression of class III β -tubulin mRNA marks a uniform layer of neurons with no corresponding gap (B). (C,D) In a slice of E17.5 hippocampus, taken from the same mid level of the hippocampus shown in A, and maintained for 12 DIV, the fronts of expression of *SCIP* (brown) and *KAI* (purple) have progressed towards each other, and now overlap. (D) High magnification of boxed area in C. Arrowheads indicate region of overlap of *SCIP* and *KAI* expression; arrows point to individual *SCIP*-expressing cells in a field of *KAI*-expressing cells. Scale bars, 140 μm (A,B), 175 μm (C), 60 μm (D).



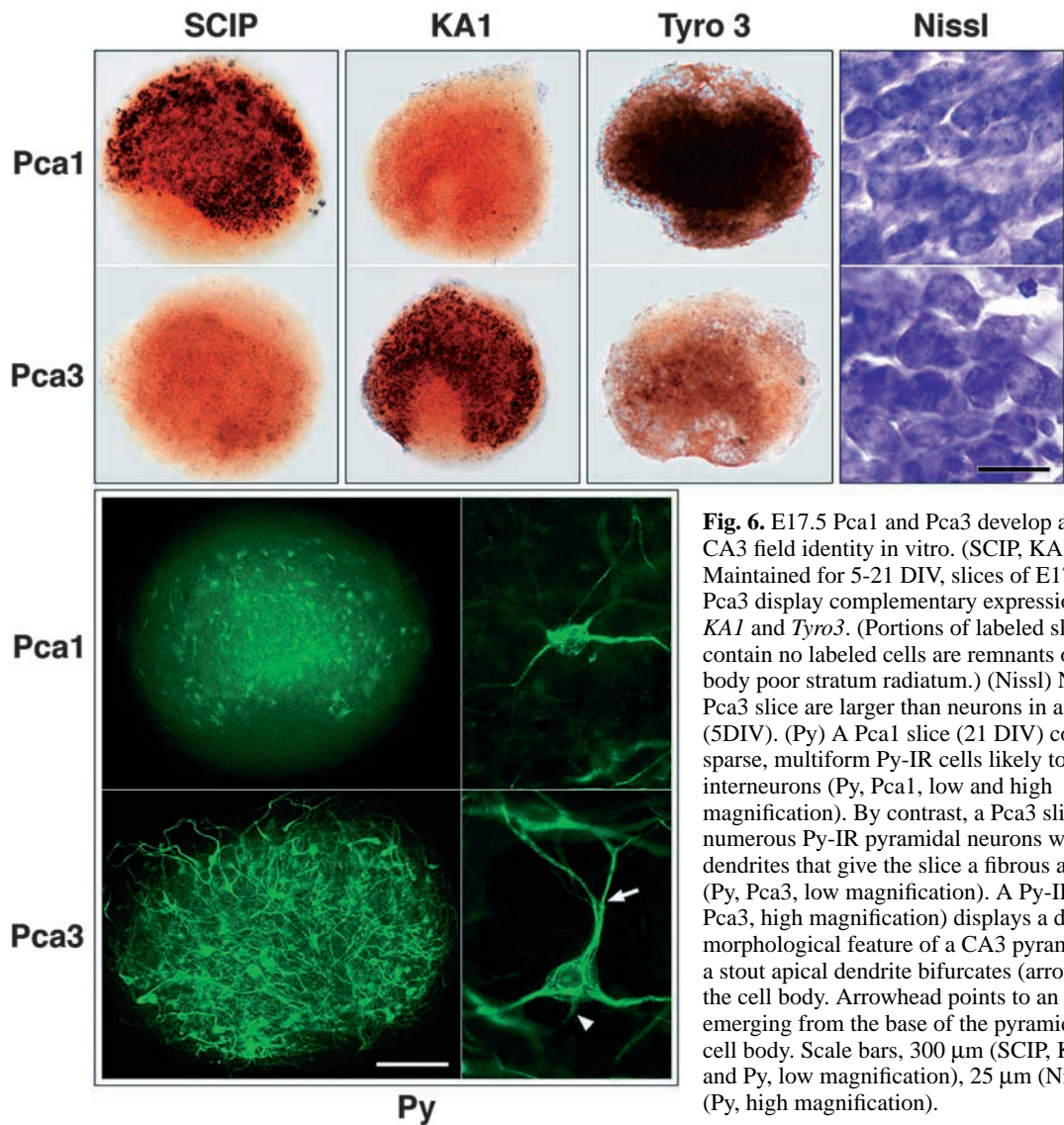


Fig. 6. E17.5 Pca1 and Pca3 develop a CA1 and CA3 field identity in vitro. (SCIP, KA1, Tyro3) Maintained for 5–21 DIV, slices of E17.5 Pca1 and Pca3 display complementary expression of *SCIP*, *KA1* and *Tyro3*. (Portions of labeled slices that contain no labeled cells are remnants of the cell-body poor stratum radiatum.) (Nissl) Neurons in a Pca3 slice are larger than neurons in a Pca1 slice (5DIV). (Py) A Pca1 slice (21 DIV) contains sparse, multi-form Py-IR cells likely to be interneurons (Py, Pca1, low and high magnification). By contrast, a Pca3 slice contains numerous Py-IR pyramidal neurons with long, thick dendrites that give the slice a fibrous appearance (Py, Pca3, low magnification). A Py-IR cell (Py, Pca3, high magnification) displays a defining morphological feature of a CA3 pyramidal neuron: a stout apical dendrite bifurcates (arrow) close to the cell body. Arrowhead points to an axon emerging from the base of the pyramidal-shaped cell body. Scale bars, 300 μ m (SCIP, KA1, Tyro3 and Py, low magnification), 25 μ m (Nissl), 40 μ m (Py, high magnification).

dentate gyrus cells, some slices were stained for calbindin-D-28K (calbindin) immunoreactivity, a marker of dentate granule cells (Sloviter, 1989) (Fig. 8A). Consistent with accurate subdissection, few calbindin-IR cells were found in Pca3 slices (Fig. 8C). As a direct test of the influence of dentate granule cells on Pca3 development, slices were prepared of Pca3 only, or of Pca3 with part or all of the DG still attached (Pca3+DG). No difference was evident in either the density of Py-IR cell bodies and processes, or the intensity of Py staining, between slices of Pca3 only ($n=13$), and Pca3+DG slices in which numerous calbindin-IR DG cells were identified ($n=15$) (Fig. 8B,C). Complementary differences in gene expression between Pca1 and Pca3 are detected by E15.5. Slice cultures were prepared slightly later, at E17.5, because at this age the mouse hippocampus is large enough not only to be dissected cleanly away from adjacent cortical fields, but also to be subdissected into separate Pca fields. Accurate subdissection is impracticable at E15.5, when the hippocampus is smaller, and its morphology less distinct. Whole hippocampal slices, however, can be prepared at E15.5, and bisected into regions that consist mainly of either Pca1 or Pca3. Experiments in which both whole and

bisected E15.5 hippocampal slices were cultured indicate that similar development of field-specific features occurs in cultures prepared at E17.5 (Figs 4–8), or E15.5 (data not shown).

DISCUSSION

Embryonic hippocampus is subdivided into presumptive CA fields

We have shown that the developing hippocampus is divided into two presumptive fields that prefigure the mature CA1 and CA3 fields. These presumptive fields are able to develop a range of mature CA field-specific features autonomously, without ongoing cues from outside the hippocampus, or even from other hippocampal fields. Thus, the embryonic hippocampus contains two distinct cell populations, each of which is already specified to develop a complex CA1 or CA3 field identity.

Initial CA field division occurs early in the overall development of the hippocampus. In the mouse, hippocampal pyramidal neurons are born beginning at E10.5 (Angevine, 1965; Stanfield and Cowan, 1988), and by E14.5 enough cells have migrated to their final position to form a detectable cortical

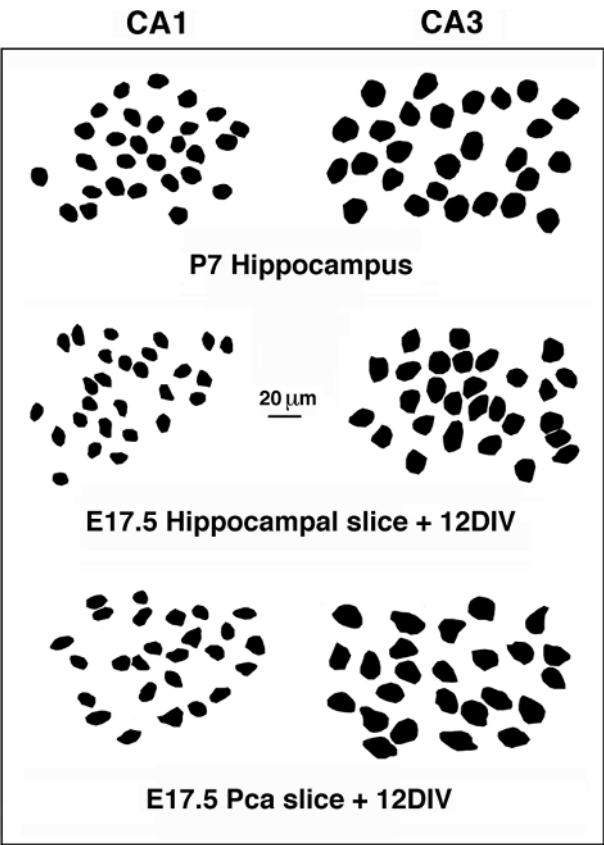


Fig. 7. A classic morphological difference between CA1 and CA3 cells develops in vitro. Filled cell body outlines of CA1 and CA3 pyramidal cells in three conditions: P7 hippocampus in vivo; slice cultures of whole E17.5 hippocampus after 12 DIV; and slice cultures of single E17.5 Pca fields after 12 DIV. In vivo, the cross-sectional area of a mature CA3 cell is about twice that of a CA1 cell; the same size difference develops in culture. Absolute sizes of CA1 and CA3 cells in the three conditions also appear comparable.

plate (our unpublished observations; Super and Soriano, 1994). By E14.5, *KAI* expression appears in Pca3, and a day later, at E15.5, *SCIP* expression is detected in Pca1. The two presump-

Table 4. CA field identity develops in slice cultures of isolated E17.5 Pca1 or Pca3

Feature	Expression or staining	No expression or staining	Total*
Pca1			
<i>SCIP</i>	35 (88%)	5 (12%)	40
<i>Tyro3</i>	40 (83%)	8 (17%)	48
<i>KAI</i>	0	17 (100%)	17
Py	6 (23%)	20 (77%)	26
Pca3			
<i>SCIP</i>	4 (16%)	21 (84%)	25
<i>Tyro3</i>	13 (32%)	27 (68%)	40
<i>KAI</i>	16 (100%)	0	16
Py	26 (90%)	3 (10%)	29

*Total for each marker includes cultures prepared in at least 3 separate experiments.
Field-appropriate expression is shown in bold.

tive fields are therefore distinct almost as soon as the first cohort of pyramidal cells populates the cortical plate.

A previous study has shown that a molecular marker for a large region of cortex that includes many areas is expressed comparably early in rodent cortex (Pimenta et al., 1996). Expression of *lamp* mRNA distinguishes limbic from non-limbic cortex by E16 in the rat, an age comparable to E14.5 in the mouse, and *lamp* expression is detectable at low levels at even earlier ages (Pimenta et al., 1996). In the present study, still finer divisions are distinguished in embryonic cortex, using complementary markers for individual areas within the hippocampus, and thus within the large cortical sector distinguished by *lamp* expression. As gene expression studies of the developing cortex continue, it seems likely that additional subdivisions in the embryonic cortex will be revealed.

By contrast with the initial subdivision of the hippocampus, the acquisition by hippocampal cells of a complex CA field identity, made up of many molecular and morphological features, is not complete until a few weeks after birth (present study; Zimmer et al., 1978; Woodhams et al., 1989; Lai et al., 1994). Thus, early in the overall development of the hippocampus, two populations of cells have already been specified to differ – that is, to express either *SCIP* or *KAI* – but are these

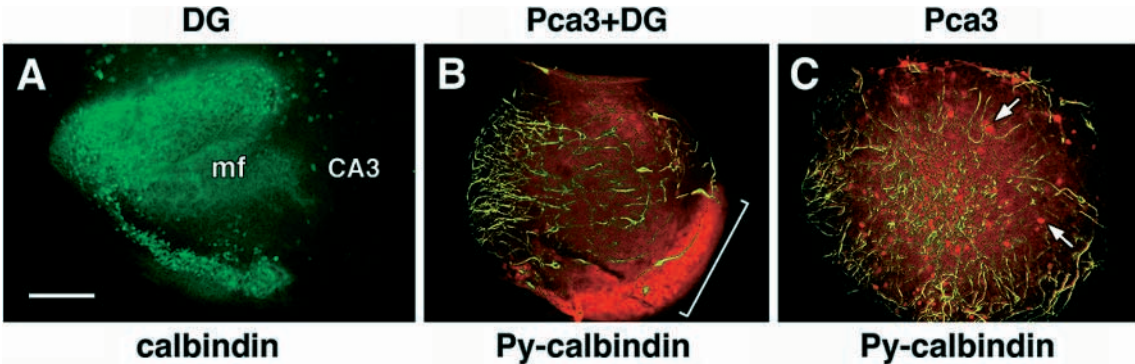


Fig. 8. Pca3 elaborates a CA3 field identity in the absence of the dentate gyrus. (A) A slice of E17.5 hippocampus (18 DIV) contains numerous calbindin-IR granule cells (green) that form a v-shaped dentate gyrus. Mossy fibers (mf) within the hilus of the dentate gyrus are also calbindin-IR. Calbindin-IR cells are sparse in CA3. (B,C) Slices of E17.5 Pca3 (18 DIV), prepared either with (B) or without (C) part of the dentate gyrus attached, display numerous Py-IR cell bodies and processes (yellow). Bracket in B indicates part of a dense band of calbindin-positive dentate granule cells, corresponding to an entire blade of the dentate gyrus (red). Arrows in C point to two of the sparse calbindin-positive cells in this slice. Scale bar, 200 μm (A,B,C).

cell populations also already specified to elaborate their mature CA field identities? One possibility is that the cells that make up Pca1 and Pca3 are already specified to develop a range of field-specific features several weeks later. A second is that the features that develop later are also specified later, so that specification of field identity is a gradual process that parallels differentiation. Findings from the present study support the first alternative. Embryonic hippocampal cells were isolated in slice culture from further potential extrinsic specification cues at a time when Pca1 and Pca3 could be distinguished by *SCIP* and *KAI* expression, but when none of the other field markers we examined had yet appeared. We found that slices through the whole embryonic hippocampus regulate both early and late markers of field identity in culture in an appropriate field-specific manner. More striking still, slices restricted to Pca1 or Pca3 alone maintain their expression of early field markers in vitro, and go on to regulate expression of CA1 or CA3 markers that do not appear in vivo until 1-2 weeks after birth.

Previous studies of cortical area specification have focused on the development of single region-specific features (Arimatsu, 1994; Arimatsu et al., 1992; Barbe and Levitt, 1991; Cohen-Tannoudji et al., 1994; Ferri and Levitt, 1993; O'Leary and Stanfield, 1989; Schlaggar and O'Leary, 1991; Stanfield and O'Leary, 1985), showing when these individual features are specified. The findings of several of these studies are consistent with those reported here, indicating that region-specific features are specified early in embryonic cortical development (Arimatsu et al., 1992; Barbe and Levitt, 1991; Cohen-Tannoudji et al., 1994; Ferri and Levitt, 1993). The findings of Arimatsu and colleagues are particularly similar to those reported here, in that expression of latexin, a marker of a small region of lateral cortex, is specified several weeks before it actually appears (Arimatsu, 1994; Arimatsu et al., 1992). However, in the present study, we have used a panel of several area-specific markers to ask not merely when individual features are specified, but rather when area identity – as defined by a set of characteristic features – is specified. Our findings indicate that complex CA field identities are conferred on hippocampal cells during the embryonic period.

Timing of CA field specification excludes some candidate mechanisms

The embryonic Pca fields continue to develop a complex CA field identity even when deprived of innervation from other parts of the brain. A possible remaining role for extrinsic innervation, however, could be to provide the patterning cues that initially subdivide the embryonic hippocampus into the Pca fields. In developing neocortex, for example, it has been proposed that afferent axons from the thalamus interact with subplate cells to impart regional identity to the overlying cortical plate (Allendoerfer and Shatz, 1994; McConnell, 1995; O'Leary et al., 1994). However, several observations throw doubt on an equivalent mechanism for the hippocampus. First, thalamic afferents to the hippocampus appear unlikely to play a similar role. Whereas each neocortical area is connected with several thalamic nuclei, few thalamic nuclei project to hippocampus, and some hippocampal fields may receive no thalamic input (Herkenham, 1978). Perhaps consistent with a sparse thalamic input, the embryonic hippocampus lacks a conspicuous subplate (Paxinos et al., 1991; Super and Soriano, 1994). Second, major afferents to the hippocampus, which might be

more likely to play a patterning role, reach their target *after* Pca1 and Pca3 are distinct. Afferents from the septum arrive in the mouse hippocampus at E17.5, and afferents from entorhinal cortex reach the dentate gyrus at E19.5 (Super and Soriano, 1994). Although some entorhinal afferents reach CA1 earlier, at E16.5 (Super and Soriano, 1994), this is still 1 day after Pca1 cells begin to express *SCIP*. Finally, interactions between afferent axons and cortical cells are proposed to take place in the subplate or marginal layers of the developing cortex (O'Leary et al., 1994; Super and Soriano, 1994), inducing pattern in the adjacent cortical plate (O'Leary et al., 1994). Such interactions would occur too late in the life history of many hippocampal cells to specify their field identity. Many hippocampal cells express the field-specific markers *SCIP* and *KAI* as they migrate through the intermediate zone. These cells are still distant from the subplate, cortical plate and marginal zone, and from putative patterning interactions within these layers.

Previous studies indicate that certain other region-specific features of the cortex are also unlikely to be specified by extrinsic innervation. Both LAMP protein expression in limbic cortex, and latexin expression in lateral cortex, appear to be specified before major subcortical afferents grow into the embryonic rat cortex (Arimatsu et al., 1992; Eagleson et al., 1997; Ferri and Levitt, 1993, 1995; Pimenta et al., 1996). Thus, although some features of cortical area differentiation may be induced by cues from ingrowing afferents, some aspects of the basic subdivision of the embryonic cortex appear likely to depend on other patterning signals.

The poles-inward differentiation of the CA fields suggests candidate sources of specifying signals

A striking observation could provide a clue to the signals that pattern the hippocampus. The early field-specific differentiation of CA1 and CA3 progresses from the poles of the hippocampus inwards. This progression does not appear to reflect a general pattern of hippocampal development in which cells at the poles of the hippocampus are born first, and then continue to differentiate in advance of cells in the middle. Previous studies of rodent hippocampal neurogenesis indicate only shallow birthdate gradients within the hippocampus, with no pronounced poles-inward pattern in the CA fields (Bayer, 1980; Stanfield and Cowan, 1988). The present study indicates that the general differentiation of the CA fields does not follow a poles-inward pattern with respect to the development of the hippocampal cortical plate, the expression of a general marker of early neuronal differentiation, class III β -tubulin, or the expression of late-appearing markers of field identity. Rather, the poles-inward pattern appears uniquely characteristic of early field-specific differentiation, encouraging a search for field specification signals that also originate at the poles of the hippocampus.

Patterning in spinal cord and brainstem is regulated by the diffusion of growth factors from adjacent structures (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). The position of the hippocampus at the extreme medial edge of the cortical mantle suggests the hippocampus could receive patterning signals from tissue near this edge. Candidate structures include midline head mesenchyme, the lamina terminalis (near the anterior end of the hippocampus), and a zone of neuroepithelial tissue between the developing hippocampus and the choroid plexus. Interestingly, this last tissue expresses multiple

Wnt genes during the period of hippocampal morphogenesis (Grove et al., 1996). Elsewhere in the embryo, *Wnt* gene expression marks territories, such as the midbrain-hindbrain junction, that provide patterning signals to neighboring tissue (Lumsden and Krumlauf, 1996).

Within the hippocampus, the dentate gyrus could provide signals that specify the identity of the adjacent CA3 field. Removing the dentate gyrus does not prevent Pca3 from developing mature CA3 features. However, given that the first dentate granule cells are generated at the same time as the first CA3 neurons (Stanfield and Cowan, 1988), the dentate gyrus might play an earlier patterning role. Intriguingly, in mutant mice lacking functional expression of *Emx2*, a mouse cognate of the *Drosophila* head gap gene *empty spiracles*, the dentate gyrus is missing, and the hippocampus is reduced in size (Pellegri et al., 1997; Yoshida et al., 1997). It will be informative to examine the development of CA field division in *Emx2* mutant mice.

A model of CA field specification

Previous studies suggest that cortical cells are specified to develop particular aspects of their laminar and regional identity when the cells withdraw from the cell cycle (Eagleson et al., 1997; Levitt et al., 1993; McConnell, 1995). It remains to be determined when in their maturation hippocampal cells are specified to a CA field fate. Observations from the present study suggest, however, that specification might not occur at a single key time for all hippocampal cells. Cells first express the early markers of field identity, *SCIP* or *KAI*, at various stages of maturation. Many cells already express these markers while migrating through the intermediate zone, but cells between the early fronts of *SCIP* and *KAI* expression reach their final position in the cortical plate without expressing either marker. The latter cells presumably go on to express *SCIP* or *KAI*, since the alternative – a wavefront of cell death in the embryonic hippocampus – was not observed (present study; Stanfield and Cowan, 1988). Thus, older, settled cells in the cortical plate can lag behind younger, migrating cells in the intermediate zone with respect to their expression of field-specific markers, a delay that is difficult to reconcile with a simple model in which all hippocampal cells are specified at the same stage of maturation.

When a hippocampal cell begins to express an early marker of CA field identity appears to depend not on the cell's maturation stage, but rather on its position relative to the hippocampal poles. Our observations suggest that the sources of signals that specify CA field identity lie close to the poles of the hippocampus, and that the signals operate first on cells close by, then move inwards. In one possible scenario, in which specifying signals influence postmitotic cells, those cells close to the poles of the hippocampus might be caught just as they begin to migrate; other cells, closer to the middle of the hippocampus, not until they have almost completed their migration. The former cells might begin to express markers of field-specific differentiation while still in the intermediate zone; the latter not until they lie in the cortical plate. The specification of the entire hippocampus would be complete by birth, causing late-appearing markers of field identity to develop in a complete field without a poles-inward progression.

The present study concerns the signals that specify CA field identity in the hippocampus: when are such signals likely to

act, and what are their possible sources? An alternative set of questions would concern instead the target of the specifying signals, the embryonic hippocampal cells themselves. Over what embryonic period are hippocampal cells responsive to specifying signals? When do cells cease to be responsive and become committed to a particular fate? To test commitment, cells must not simply be removed from potential specifying signals as in the present study, but moved to different environments in which the cells have the opportunity to respond to alternate signals (Eagleson et al., 1997; Levitt et al., 1993; McConnell, 1995).

CONCLUSION

Our findings suggest the hypothesis that patterning of the hippocampus is induced by signals that occur at least as early as E14.5 in the mouse, and may emanate from neighboring structures. Such a picture accords with the manner in which other parts of the embryo and neural tube are patterned in development (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). A hypothesis of this type would be difficult to test with respect to much of the cerebral cortex, however, because of the scarcity of area-specific molecular markers. Patterning mechanisms of the spinal cord and brainstem have been established by manipulating candidate sources of patterning cues and determining the effects on the expression of region- and cell type-specific morphological and molecular markers (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). The robust, field-specific hippocampal markers identified in the present study should, therefore, prove a major advantage in determining the molecular and cellular mechanisms of hippocampal patterning.

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