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Authors

Tsai, ES
Haraldson, SJ
Baratta, J
[et al.](#)

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BASAL FOREBRAIN CHOLINERGIC CELL ATTACHMENT AND NEURITE OUTGROWTH ON ORGANOTYPIC SLICE CULTURES OF HIPPOCAMPAL FORMATION

E. S. TSAI,^a S. J. HARALDSON,^a J. BARATTA,^a A. D. LANDER,^b J. YU^c and R. T. ROBERTSON^{a*}

^aDepartment of Anatomy and Neurobiology, College of Medicine, University of California, Irvine, CA 92697-1280, USA

^bDepartment of Developmental and Cell Biology, University of California, Irvine, CA 92697, USA

^cDepartment of Physical Medicine and Rehabilitation, University of California, Irvine, CA 92697, USA

Abstract—Distributions of somata and neurites of cholinergic neurons were studied after seeding dissociated cells onto organotypic slice cultures. Slice cultures were made from hippocampal formation and adjacent cortical regions from rats or mice. Dissociated cell suspensions of basal forebrain tissue from rat or mouse fetuses were seeded onto the slice cultures. Combined cultures were maintained for 1–21 days *in vitro*. Cultures processed for acetylcholinesterase (AChE) histochemistry demonstrated non-random patterns of cholinergic cells and their neurites. Labeled cells appeared most frequently in the molecular layer of the dentate gyrus, and in the deeper layers of cortical regions adjacent to the hippocampus. Neurites extending from these labeled cells appeared to target the dentate molecular layer and the cortical subplate layer. By 4 days *in vitro*, AChE-positive basal forebrain cells display several short and thick neurites that appear to be dendrites, and one long process that appears to be an axon. By 5 days *in vitro*, dendrites are well developed; by 7 days the presumed axon has extended widely over the cortical target zone. These neurites are maintained through 3 weeks in culture. Distributions of cells varied with the age of the slice. AChE-labeled cells were not seen overlying hippocampal tissue when dissociated cells were seeded on slice cultures made from day 0 rats, but a few labeled cells were seen when seeded on slices from day 2 rats. Clear non-random patterns of labeled cells and neurite outgrowth were seen on slice cultures from day 5 or older pups. The non-random distribution seen with AChE-positive neurons was not seen using other techniques that labeled all cells (non-selective fluorescent labels) or all neurons; these techniques resulted in labeled cells scattered apparently homogeneously across the slice culture.

These studies demonstrate a non-random pattern of attachment or differentiation of basal forebrain cholinergic neurons when these cells are seeded onto cultured cortical slices; this pattern mimics the normal patterns of basal forebrain cholinergic projections to these cortical regions. These data suggest that the factors that normally guide basal forebrain-derived cholinergic axons to their target cells *in vivo* are present and detectable in this model system.

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Key words: axon, cell adhesion, cell cultures, dentate gyrus, differentiation, neocortex.

It is well recognized that neuronal circuitry is established through the formation of appropriate synaptic connections in the developing brain. Recent research has demonstrated that formation of these connections is likely to be influenced by a variety of factors, including attractive, repulsive, and guidance cues (Goodman and Shatz, 1993; Johansen et al., 1994; Lander, 1990; Landmesser, 1994). These factors serve to bring the axons and dendrites of two different cells into close proximity so that cell–cell interactions can allow the growing axons to recognize their target neurons (Goodman and Shatz, 1993; Johansen et al., 1994; Lander, 1990; Landmesser, 1994).

Studying these putative cell–cell recognition processes in the whole developing animal is a challenging problem that has prompted the use of more reduced *in vitro* approaches (Alderson et al., 1990; Amaral and Witter, 1995; Baratta et al., 1996a,b; Distler and Robertson, 1992, 1993; Dreyfus, 1989; Ha et al., 1996; Hartikka and Hefti, 1988). Recently, we have introduced a method in which dissociated cells are seeded onto organotypic slice cultures of known target tissue (Robertson et al., 1997). Some of the seeded neurons attach and differentiate on the slice culture. The locations of the seeded cells, and the patterns of their axonal growth, can be used as indicators of the distributions of factors within the slice tissue that promote, guide, or repulse cell attachment, differentiation, or outgrowth (Robertson et al., 1997).

Development of cholinergic projections from the septum to the hippocampal dentate gyrus (Amaral and Kurz, 1985; Armstrong et al., 1987; Deller, 1998; Förster et al., 1997; Lewis and Shute, 1967; Luiten et al., 1987; McKinney et al., 1983; Milner et al., 1983; Shute and Lewis, 1967; Storm-Mathisen and Blackstadt,

*Corresponding author. Tel.: +1-949-824-6553; fax: +1-949-824-1105.

E-mail address: rrobert@uci.edu (R. T. Robertson).

Abbreviations: AChE, acetylcholinesterase; BDNF, brain-derived neurotrophic factor; BFCN, basal forebrain cholinergic neuron; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; FLM, fluorescent latex microspheres; GFP, green fluorescent protein; NT-3, neurotrophic factor 3; P, postnatal day.

1964; Wenk et al., 1980; Woolf, 1991; Zimmer et al., 1986) provides an excellent model system for studies of axon targeting. The reasons are as follows. First, the axons of septal cholinergic neurons form a distinct pattern of termination in the inner molecular layer of the dentate gyrus (Amaral and Witter, 1995; Baratta et al., 1996a,b; Deller, 1998; Förster et al., 1997; Lewis and Shute, 1967; Storm-Mathisen and Blackstadt, 1964; Zimmer et al., 1986), suggesting that communication between the septal cholinergic axons and the dentate gyrus granule cells is important for establishing this pattern of connectivity. Second, septal and other basal forebrain cholinergic neurons (BFCNs) express high levels of acetylcholinesterase (AChE), which is present throughout the neurons (Levey et al., 1983; Woolf, 1991), including their axon terminals. The presence of AChE provides a convenient and sensitive endogenous marker for cholinergic axon growth (Baratta et al., 1996a,b; Levey et al., 1983). Third, the septal cholinergic projection to dentate gyrus develops largely postnatally in the rodent (Armstrong et al., 1987; Brady et al., 1989; Milner et al., 1983; Semba and Fibiger, 1988; Super and Soriano, 1994), making it easily accessible for experimental study.

The goal of the present investigation was to determine whether dissociated BFCN somata and their neurites are distributed in non-random patterns that mimic the normal patterns of cholinergic ingrowth seen *in vivo*. Such patterns would be interpreted as indicating the presence of local factors that attract, repel, or nurture cholinergic elements. Portions of these data have been presented previously in abstract form (Haraldson et al., 1997).

EXPERIMENTAL PROCEDURES

Animals

Fetal and infant rats and mice were used in these experiments. Rats were of the Sprague–Dawley strain from Simonsen Labs, and included fetuses of gestational age 15–17 days (G15–17) and newborn pups of postnatal age 4–7 days (P4–7). Both wild-type (C57BL/6) and transgenic (see below) newborn (P5–7) and fetal (G15–17) mice were used. All experiments were carried out to minimize animal suffering and to reduce the number of animals used. All use of animals was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and was approved by the University of California, Irvine, CA, USA, Institutional Animal Care and Use Committee.

Materials

All reagents were purchased from Sigma Chemical (St. Louis, MO, USA) unless specified otherwise.

Organotypic slice culture preparations

Organotypic slice cultures using the ‘membrane technique’ (Gähwiler, 1981; Gähwiler et al., 1998; Stoppini et al., 1991; Yamamoto et al., 1989, 1992) were prepared from brains of P0–7 rat or mouse pups, using sterile techniques under a laminar flow hood. Animals were cryoanesthetized and killed by decapitation, and the brains were quickly removed. Transverse or horizontal 400- μ m slices of brain tissue were cut on a Vibratome and placed into cold Dulbecco’s modified Eagle’s medium

(DMEM). Under a dissecting microscope, a fine knife was used to isolate the hippocampus in transverse sections, or the hippocampus and adjacent entorhinal cortex and perirhinal cortex in horizontal sections. Each slice was placed alone on a 30-mm Millicell culture insert (Millipore, MA, USA). Four culture inserts with tissue slices were placed into a 100-mm \times 20-mm Petri dish containing 7 ml of culture medium. The medium was made with 100 ml basal Eagle medium (with Earle’s salts), 50 ml Earle’s balanced salt solution, 50 ml heat-inactivated horse serum, 1 ml 200 mM L-glutamine, and 2 ml 50% glucose.

Slice cultures were maintained in a 36°C incubator with 5% CO₂ for 1–4 days before the addition of suspensions of dissociated cells.

Dissociated cell culture preparations

Pregnant rats or mice of gestational days 15–17 were deeply anesthetized with sodium pentobarbital (50 mg/kg). Anesthetized fetuses were surgically removed from the uterus and decapitated. The brains were then removed and placed in cold DMEM. Under a dissection microscope the basal forebrain region, including septum and diagonal band, was exposed and removed surgically, using sharp forceps (Ha et al., 1996; Hartikka and Hefti, 1988). Basal forebrain tissue pieces were incubated in 0.09% trypsin in DMEM at 36°C for 30 min. The tissue was centrifuged at 3000 r.p.m. for 5 min, and then the supernatant was removed and replaced with serum-based medium. The tissue was dissociated by trituration with fire-polished fine barrel pipettes and suspended in the culture medium. The cell suspension had a concentration of approximately 2×10^2 cells/ μ l. A volume of 5–20 μ l of the cell suspension was carefully pipetted onto the previously prepared slice cultures. These combined cultures were allowed to survive for periods of 1 day to 3 weeks.

Prelabeling of dissociated cells

In some cases cell suspensions were pre-labeled so that cells could be detected after seeding on slice cultures. After incubation with trypsin, cell suspensions were mixed for 20 min at 37°C with either rhodamine-labeled fluorescent microspheres (Fluorospheres; Molecular Probes, Eugene, OR, USA) at 1 μ l/ml, or Vibrant DiI cell tracker (Molecular Probes, Eugene, OR, USA) at 5 μ l/ml. After labeling, cells were centrifuged and washed five times at 5 min each before being suspended again in tissue culture medium and seeding onto organotypic slices.

In other cases, basal forebrain tissue from transgenic mice engineered to carry their own endogenous marker was dissociated; these included strains that strongly express either green fluorescent protein (GFP; Okabe et al., 1997) or β -galactosidase (β -gal). As described by Davies et al. (1999) the GFP expressing transgenic mice were maintained on a C57BL/6N background, with the gene for the GFP protein under control of a chicken β -actin promoter (Okabe et al., 1997). In these animals, virtually all neurons, along with other cells, express GFP. Other transgenic mice were the genetically engineered *tattler IV* (an acronym for T- α 1-tubulin-tau-LacZ-expresser) strain (Murray et al., 2002), in which neurons express a tau- β -gal fusion protein under direction of the neuron-specific T- α 1 alpha tubulin promoter (Gloster et al., 1994). These mice were maintained on an outbred CD-1 background.

After fixation with 4% paraformaldehyde, the DiI and fluorescent latex microsphere (FLM)-labeled cells were detected directly using the fluorescence microscope, using a rhodamine filter. Similarly, GFP-positive cells were detected directly, using a fluorescein filter. The β -gal-positive cells from *tattler* mice were detected immunocytochemically. After fixing with 4% paraformaldehyde in 100 mM sodium phosphate-buffered saline (PBS) for 1 h, cultures were rinsed in PBS and processed for immunocytochemistry using a primary polyclonal antibody to β -gal (5 prime 3 prime) at 1:1000 followed by either a biotinylated or a rhodamine-labeled secondary antibody at 1:200. The biotinylated secondary antibody was followed by the ABC Elite

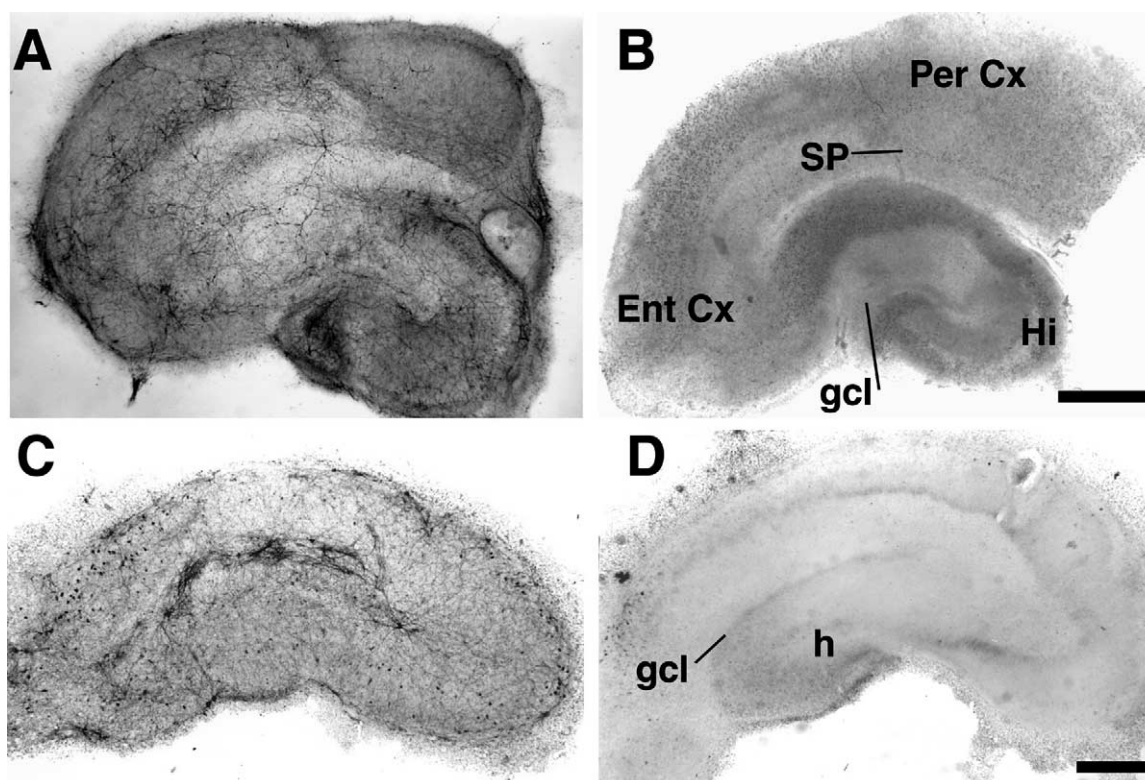


Fig. 1. AChE-stained organotypic slice cultures. (A) Slice culture of horizontal section of hippocampus and adjacent cortices with seeded AChE-positive BFCNs grown in culture for 1 week. (B) Cultured slice of comparable hippocampus and adjacent tissue without seeding of BFCNs; no AChE-positive neurons are present. (C) Slice culture of a transverse section of hippocampus, with seeded AChE-positive BFCNs, after 10 days in culture. (D) Cultured transverse slice, comparable to 'C', without seeded BFCNs. Calibration bar in 'B' = 1 mm for 'A' and 'B'; bar in 'D' = 1 mm for 'C' and 'D'.

kit (Vector) for 1 h each and then the reaction was visualized by 0.02% 3,3'-diaminobenzidine with 0.001% H_2O_2 in Tris-saline for 6–10 min.

AChE histochemistry

Cultures were fixed by immersion in 4% paraformaldehyde and 0.5% glutaraldehyde in 100 mM sodium phosphate buffer overnight at 4°C and processed for AChE histochemistry as described by Tago et al. (1986). Cultures were rinsed in three changes of 100 mM maleate buffer (pH 6.0) for 10 min each, and then incubated in 50 ml maleate buffer, with 30 μ l 86.5 mM acetyl-thio-choline iodide, 100 μ l 30 mM tetra-isopropyl pyrophosphoramidate and 500 μ l Karnovsky and Roots solution (made up from 2.5 ml 100 mM sodium citrate, 5 ml 30 mM copper sulfate, 8 mg potassium ferricyanide and 42.5 ml 100 mM maleate buffer). After 5 h incubation in the dark, the cultures were rinsed in three changes of 50 mM Tris buffer (pH 7.5) and then reacted in 0.05% 3,3'-diaminobenzidine, 0.003% hydrogen peroxide and 0.2% nickel ammonium sulfate in 50 mM Tris buffer for 5–10 min when stained fibers appeared. Cultures were rinsed in several changes of 50 mM Tris buffer, mounted on gelatin-coated slides, dehydrated in ethanol, and cleared in xylene before coverslipping.

Analysis

Histochemically stained cultures were examined under bright-field microscopy and documented photographically using Kodak technical pan film. Images from negatives were scanned and imported into Adobe Photoshop. Individual images were adjusted for consistent brightness and contrast for each photographic plate; no other modification of the images was done.

In addition to the photographic documentation, drawings were made of the location of labeled AChE-positive neurons and axons on composite drawings of the cultures. Drawings were made of individual AChE-stained neurons, using a camera lucida and either a 40 \times or 63 \times objective lens.

RESULTS

Appearance of AChE-stained cultures

AChE histochemistry was used to identify BFCNs seeded onto the cultured slices. Examples are shown in Fig. 1. A case with AChE-positive BFCNs on a cultured

Abbreviations used in the figures

Ent Cx	entorhinal cortex	ml	molecular layer (of dentate gyrus)
gcl	granule cell layer (of dentate gyrus)	Per Cx	perirhinal cortex
h	hilus (of dentate gyrus)	SP	subplate layer of cortex
Hi	hippocampus		

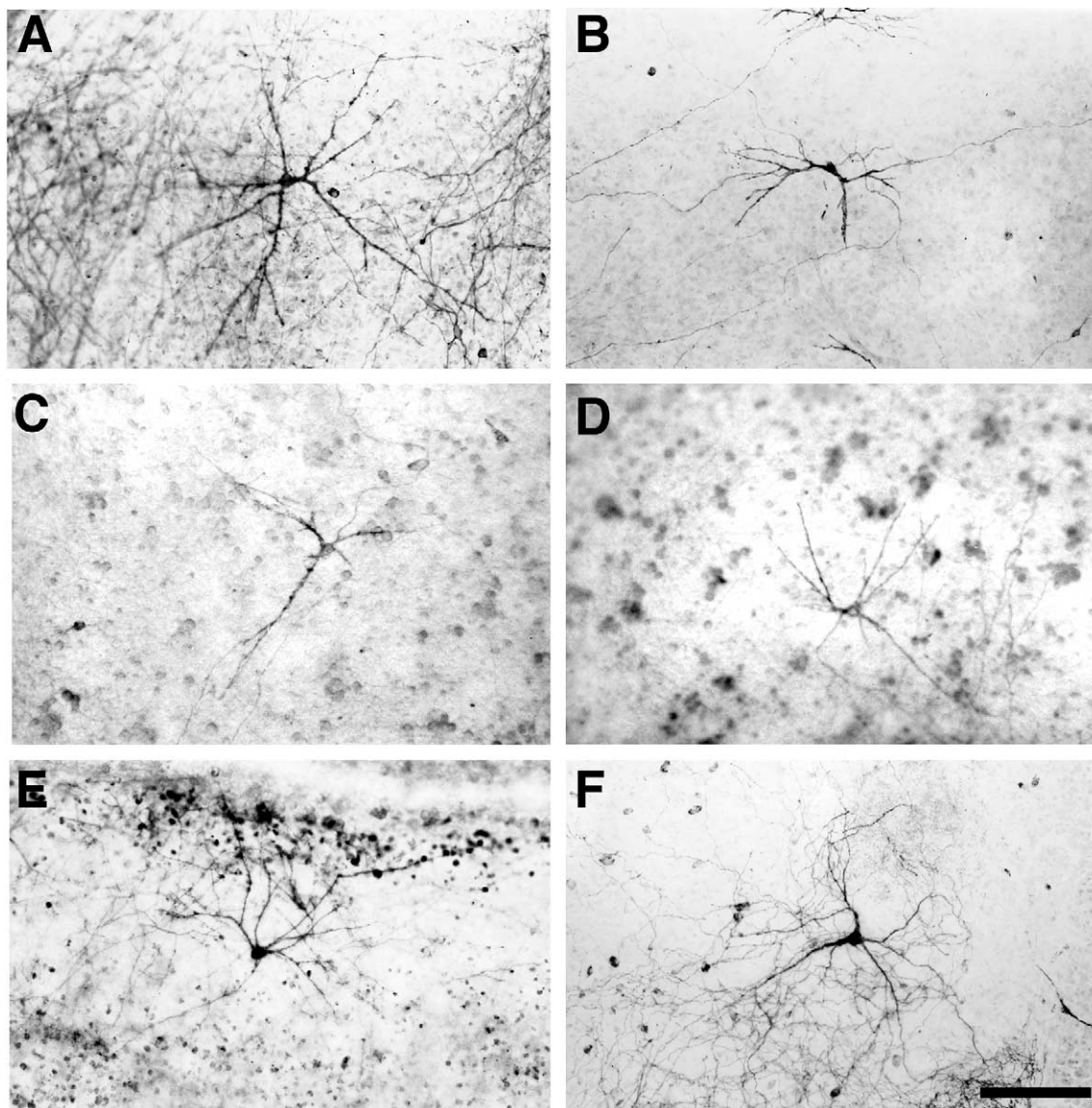


Fig. 2. Photomicrographs of AChE-positive BFCNs seeded on cultured slices of hippocampus. (A) 11 DIV; (B) 11 days; (C) 3 days; (D) 4 days; (E) 7 days; (F) 3 weeks *in vitro*. Calibration bar in 'F' = 100 μ m for 'A', 'B', 'F' and 50 μ m for 'C', 'D', 'E'.

horizontal slice through the temporal pole of cerebral cortex is shown in the photomicrograph Fig. 1A, and a similar case, but with BFCNs seeded onto a transverse slice of hippocampus, is shown in Fig. 1C. These cultures were maintained for 7 days (Fig. 1A) or 10 days (Fig. 1C) following the seeding of basal forebrain cells onto the slice. Close inspection reveals the presence of several darkly stained AChE-positive neurons and neurites on the slices. Comparable photomicrographs of cultured slices without the added BFCNs are shown in Fig. 1B, D. While some AChE staining is present, the slice cultures shown in Fig. 1B, D lack the large distinct AChE-positive neurons that are seen following seeding with dissociated BFCN cells. Thus, the histochemical techniques employed here do not result in strong staining of the intrinsic AChE-positive neurons that might confound the analysis of the seeded BFCNs.

Quantitative analyses revealed a range of 4–21 AChE-

positive BFCNs on each horizontal slice culture; these BFCNs were distributed over the hippocampal formation and adjacent cortices. The number of detected neurons on each slice varied with the volume of cell suspension seeded onto the cultured slice. Counts made from 15–20 cultures for each condition revealed that a 5- μ l suspension of cells resulted in 4.8 ± 1.2 (mean and S.D.) AChE-positive neurons per slice; 10 μ l resulted in 7.8 ± 2.0 cells; and 20 μ l resulted in 16.7 ± 4.3 cells per slice culture.

Most (70–80%) of the AChE-positive cells were large multipolar neurons, with an isodendritic pattern of dendrites (e.g. Fig. 2A, C, F) with three to six primary dendrites. More rarely, stained cells appeared with tufted bipolar morphological features (e.g. Fig. 2B). These features are similar to those described for BFCNs *in situ* (Amaral and Witter, 1995; Houser et al., 1983; Shingai et al., 1990; Wenk et al., 1980; Woolf, 1991), and similar

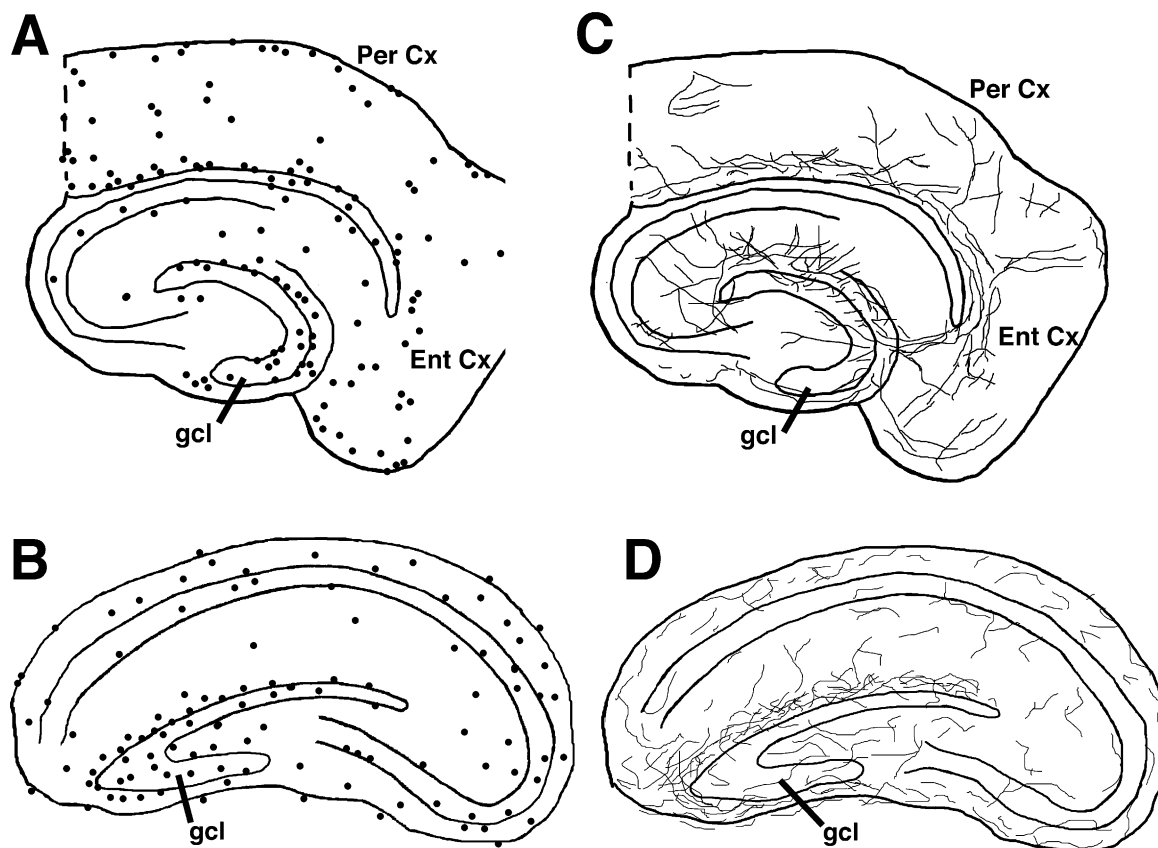


Fig. 3. Composite camera lucida drawings of distributions of AChE-positive somata seeded on slice cultures of hippocampus from horizontal sections (A) and transverse sections (B). Also shown are composite distributions of AChE-positive neurites, as seen on cultures from horizontal (C) and transverse (D) sections.

to the features described for these cells in both dissociated cell cultures (Dreyfus, 1989; Ha et al., 1996; Shingai et al., 1990) and in slice cultures (Baratta et al., 1996a,b; Distler and Robertson, 1992, 1993; Gähwiler et al., 1987).

Distribution of AChE-positive neuron somata on slice cultures

The AChE-positive neurons were distributed in a non-random pattern across the cultured slices of hippocampus and adjacent regions. Figure 3A presents a composite camera lucida drawing showing the distribution of all these neurons from a total of 20 cultures made from horizontal sections through the temporal pole, while Fig. 3B presents a similar composite from a total of 15 cultures made from transverse sections of the hippocampus. In the horizontal slices (Fig. 3A) labeled cells were distributed mainly on the dentate gyrus of the hippocampus and on regions along the inner layers of perirhinal and entorhinal cortices. Counts of labeled cells on cultures made from horizontal sections and from transverse sections reveal that AChE-positive cells were found on the dentate gyrus at approximately two to three times the density (labeled cells per unit area) as cells found over the hippocampal CA fields. Within the dentate gyrus, the AChE-positive cells tend to appear over the inner molecular layer. Furthermore, counts revealed that labeled

cells were found over the deep layers of perirhinal cortex at approximately twice the density as was seen over superficial layers of neocortex.

Distribution of neurites

Neurite outgrowth also appeared in non-random patterns. The photomicrographs in Fig. 4 illustrate the finding that AChE-positive neurites appear concentrated in the dentate gyrus in general (Fig. 4A), and in particular in the dentate molecular layer (Fig. 4B–D). Staining of neurites in the hilus varied considerably between cultures, but AChE-positive neurites were a consistent feature in the molecular layer.

Figure 3 also presents composite drawings of AChE-positive BFCN neurite outgrowth for cultures made from horizontal (Fig. 3C) and transverse (Fig. 3D) slices through the hippocampal formation and adjacent regions. The pattern of AChE-positive neurites is similar to the pattern of AChE-positive somata. Examples of distributions of AChE-positive neurite outgrowth also are illustrated in the camera lucida drawings presented in Fig. 5.

Development and maintenance of neurites

The development and differentiation of BFCNs and the growth of their neurites were studied in cultures

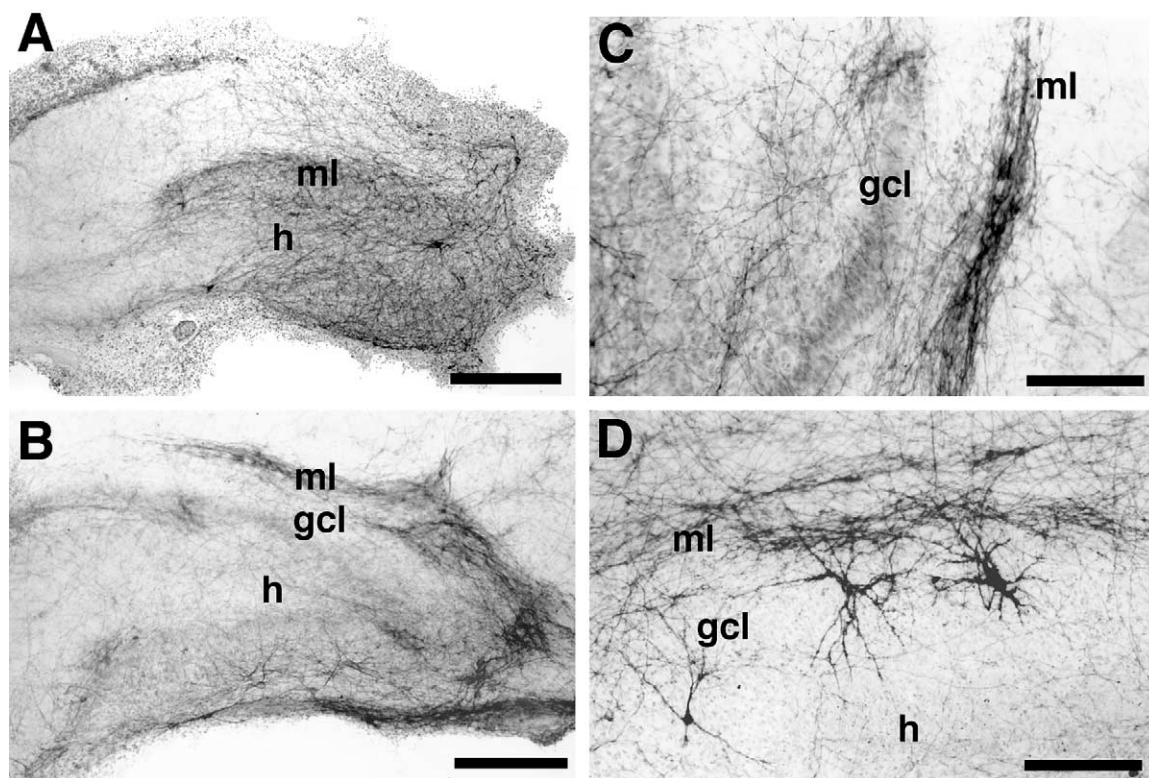


Fig. 4. Photomicrographs of AChE-stained cultures made from transverse slices of hippocampus, showing the pattern of BFCN neurites at 7–10 days in culture. (A) After 1 week in culture, note AChE-positive fibers in dentate hilus and the molecular layer. (B) 10 days in culture, note patterns of fibers in dentate molecular layer. (C) Higher magnification of culture shown in 'B'. (D) 10 days in culture, note the two AChE-positive cells, and neurite plexus in the molecular layer. Calibration bars in A: 500 μm ; B: 250 μm ; C: 100 μm ; D: 100 μm .

maintained for 1 day to 3 weeks. Examination of AChE-stained cultures 1 or 2 days following the seeding of dissociated basal forebrain cells revealed no detectable BFCNs on the tissue (data not illustrated). Three days following the seeding of dissociated cells on the cultured slices, a few small and lightly AChE-positive cells with poorly developed neurites could be seen (Fig. 2C). By 4 days *in vitro* (DIV) however, AChE-positive neurons were more prominent (Fig. 2D), with clearly visible somata and AChE-positive neurites. Most of the neurons at this stage had relatively short neurites, which rarely extended farther than 50 μm from the soma. In cultures of more than 4 DIV, labeled cells had several neurites, including a single thin and long (greater than 100 μm) axon-like process and several shorter and thicker dendrite-like processes.

By 7 DIV, AChE-positive neurons were commonly observed; these cells displayed larger somata and more extensive neurite outgrowth (Fig. 1E) than those from shorter culture periods. Continued growth of AChE-positive neurites was apparent in cultures maintained for longer than 1 week in culture (Fig. 2A, B) with an accompanying increase in the intensity of the AChE histochemical reaction product. Cultures maintained for 3 weeks displayed strongly AChE-positive neurons (Fig. 1F), with neurites that were much longer and more complex (Fig. 1F). Although the morphological features of individual neurons appeared more complex in older cultures, the distributions of AChE-positive

cells across the tissue slice were similar to those observed with shorter culture periods. No evidence of degeneration of AChE-positive neurons was detected with the longer (3 weeks) culture periods.

With culture periods of 4 days up to 3 weeks *in vitro*, patterns of neurite outgrowth appeared similar to those seen with shorter culture periods, although the AChE-positive neurites appeared more profuse at 3 weeks than at 7 days. Axon projections were maintained after 3 weeks in culture, indicating that axonal innervation into dentate gyrus, entorhinal cortex, and inner cortex was not transient.

Development of adhering or growth promoting aspects of the hippocampus

The non-random distribution of BFCNs on the cultured slice suggests that the seeded BFCNs are attaching, surviving, or differentiating by some factor(s) produced by the slice tissue. A set of studies examined the role of maturation of the hippocampal tissue on the patterns of BFCNs. Figure 6 presents examples of slices of hippocampus taken from animals at P0 (Fig. 6A), P2 (Fig. 6B) and P7 (Fig. 6C). BFCNs from the same age fetal tissue (gestational day 17) were seeded onto each of the slices after the slice had been maintained 1 day in culture; the combined cultures then were maintained for an additional 7 days. Note that no AChE-positive BFCNs were seen on the slice taken at P0 (although a few labeled

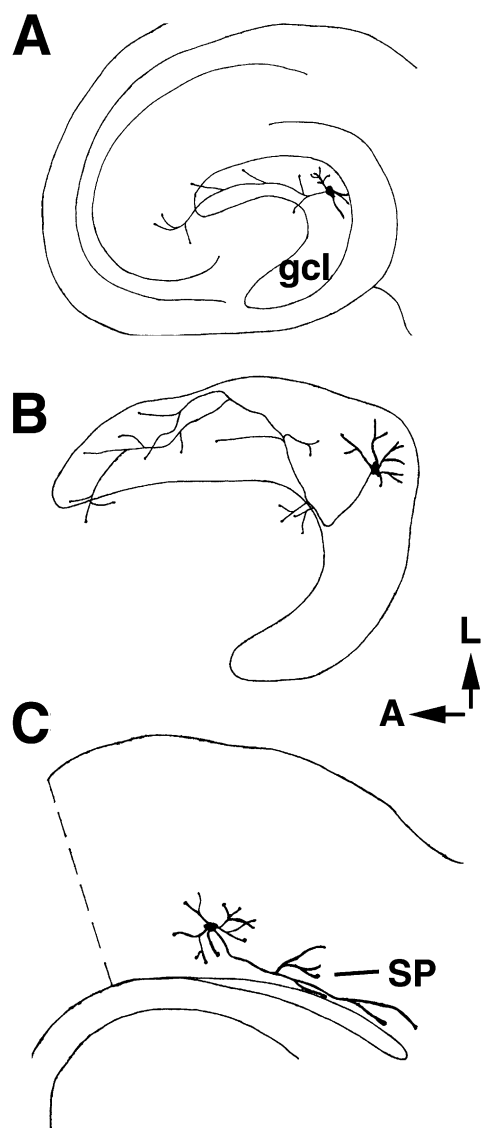


Fig. 5. Camera lucida drawings of AChE-positive individual neurons. In each case, tissue was fixed 1 week after seeding BFCNs. Presumed dendrites are indicated as the thicker processes; presumed axons are indicated by the thinner processes. (A) AChE-positive cell on the granule cell layer, with axon branching mainly on the gcl. (B) Another example of an AChE-positive cell, on gcl. (C) AChE-positive cell on the subplate layer of perirhinal cortex; the axon branches mainly on the subplate.

cells could be detected around the edge of the cultured slice). A few AChE-positive cells were seen on the slice taken at P2, and the stereotypical pattern of BFCNs was seen on the slice taken at P7. These data suggest that some aspect of the hippocampal formation must mature prior to the addition of the dissociated basal forebrain cells, in order for the BFCNs to attach, survive, or differentiate on the cultured slice.

Does the non-random distribution of AChE-positive neurons result from initial non-random attachment of neurons?

The data presented in the previous section demon-

strate that postnatal maturation of hippocampal tissue is important for the non-random distribution of AChE neurons. Selective adhesive factors demonstrated in other models (Emerling and Lander, 1994; Förster et al., 1998; Förster, 2001) may be important here. Dissociated cells prelabeled with the DiI cell tracker or with FLM were seeded onto slices from P5 animals, left undisturbed for 2 h, and then gently rinsed with culture medium. These cases showed no evidence of the presence of labeled cells 2 h after washing, and no evidence of AChE-labeled cells several days after washing (data not illustrated). Thus, these data suggest that initial cell attachment does not play an essential role in the later distribution of AChE-positive cells.

Is the non-random distribution displayed by AChE-positive neurons simply one example of the non-random attachment of all cells?

The data indicate that AChE-positive cholinergic neurons are distributed non-randomly on slices of hippocampal formation and neocortex. Experiments with prelabeled dissociated basal forebrain cells explored whether this pattern was characteristic of cholinergic neurons specifically, or whether all cells would display this pattern. The photomicrographs in Fig. 7 present examples of labeling of cells with a variety of techniques, including cells from transgenic animals that carried endogenous labels (β -gal: Fig. 7B or GFP: Fig. 7E) and cells that were labeled by exposure to direct cell labeling techniques while in dissociated cell suspension (DiI: Fig. 7D or FLM: Fig. 7F). Cells labeled by each of these methods did not display the pattern typical of the AChE-positive neurons, but rather were distributed apparently randomly across the culture. Related studies using double labeling techniques demonstrated that the majority of FLM-positive cells were likely to be microglia, as they displayed a positive reaction to the tomato lectin (Eliason et al., 2002). Vibrant DiI-labeled cells appeared to be both neuronal and glial, with glial cells (co-labeled either by tomato lectin or GFAP immunocytochemistry) being the majority.

Both neuronal (Fig. 7E) and non-neuronal cells from the GFP expressing transgenic mice displayed GFP fluorescence. GFP-positive cells with neuronal morphological features appeared distributed homogeneously across the slice culture, although these labeled cells appeared slightly more frequently over the region of the hilus of the dentate gyrus, and slightly less frequently over the dentate gyrus molecular layer. The β -gal immunoreactive cells (Fig. 7B) virtually all had morphological features of neurons, and displayed a homogenous distribution pattern similar to that of the GFP-labeled cells.

DISCUSSION

The present results demonstrate that AChE-positive BFCNs and their neurites are found in non-random distributions after dissociated basal forebrain cells are seeded on slice cultures of hippocampus and adjacent

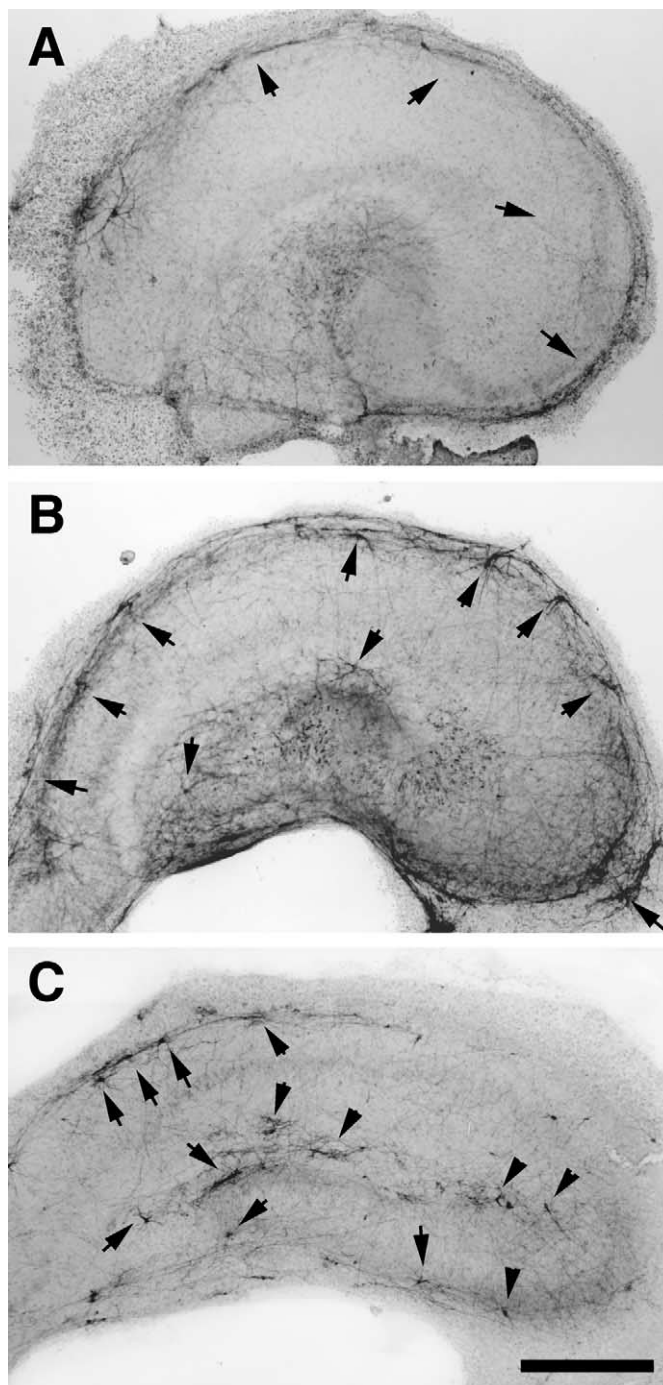


Fig. 6. AChE-stained slice cultures made from transverse slices of brains taken at P0 (A), P2 (B), and at P7 (C). Note that while AChE-positive BFCNs (arrow heads) are found at the edge of the P0 culture, they appear absent from the P0 hippocampal tissue. A few AChE-positive cells are seen over the hippocampus and dentate at P2 (B), and are more prominent on the culture taken at P7. Calibration bar = 500 μ m.

tissue. This finding is important because the distributions of BFCN somata and the neurites in this experimental test situation may provide information regarding mechanisms by which cholinergic axons normally find their targets in the cerebral cortex.

Technical considerations

Histochemical techniques for detecting AChE have

long been used to identify BFCNs (Butcher, 1983; Distler and Robertson, 1992; Lewis and Shute, 1967; Robertson et al., 1997; Wenk et al., 1980; Woolf, 1991). While a number of instances have been documented in which AChE is expressed by non-cholinergic cells (Greenfield, 1984; Layer and Willbold, 1995; Lehman and Fibiger, 1979; Levey et al., 1984; Robertson et al., 1988; Robertson and Yu, 1993; Rye et al., 1984; Silver, 1974; Small, 1990), co-localization

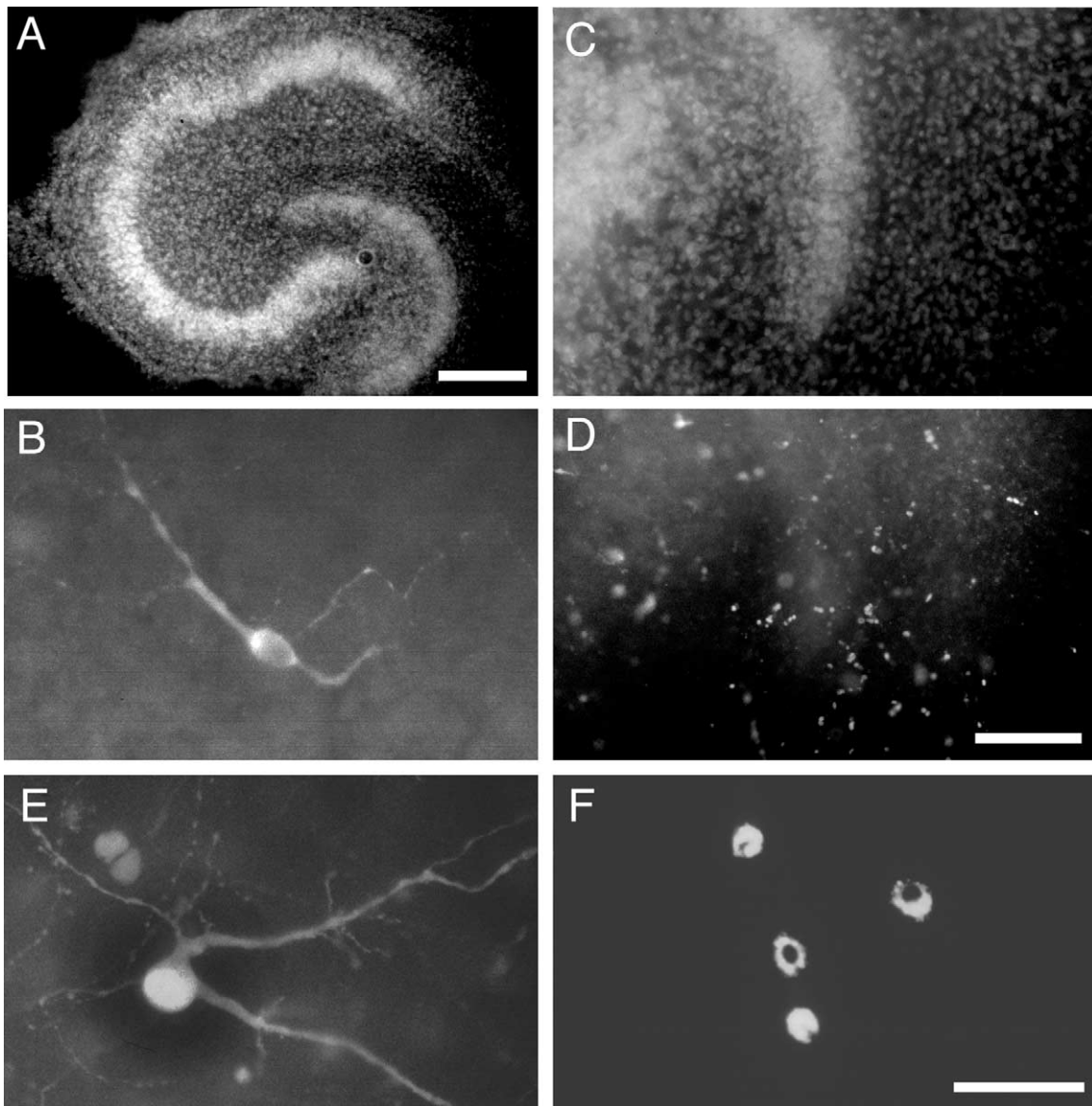


Fig. 7. Fluorescence photomicrographs of labeled neurons on horizontal slice cultures maintained *in vitro*. (A) BoPro-labeled hippocampal slice culture after 7 DIV. Note the organotypic organization. Calibration bar = 200 μ m. (B) Neuron showing β -gal immunoreactivity, 7 DIV. (C) Fluorescein filter image of a BoPro-stained portion of the dentate gyrus, with hilus on the left, 7 DIV. (D) Rhodamine filter image of the same section as in 'C', showing DiI-labeled cells distributed without a clear pattern. (E) GFP-labeled neuron, 7 DIV. (F) Cells labeled by rhodamine-labeled latex microspheres, 4 DIV. Calibration bar in 'A' = 250 μ m; bar in 'D' = 200 μ m for 'C' and 'D'; bar in 'F' = 50 μ m for 'B', 'E', and 'F'.

studies have demonstrated that virtually all BFCNs express AChE (Levey et al., 1983; Woolf, 1991). AChE is expressed by the neuronal somata and dendrites, and also by the axons of cholinergic neurons, making it a reliable and valid marker for revealing the presence and morphological features of BFCNs. AChE-positive neurons also are a normal component of cortical regions, including the hippocampus, entorhinal cortex and neocortex (Levey et al., 1984; Lysakowski et al., 1989; Robertson et al., 1988, 1990; Seress et al., 1987). Interestingly, these AChE-positive neurons appear not to be cholinergic (Levey et al., 1984). However, morphological criteria can be used to distinguish these AChE-positive intrinsic cortical neurons from the seeded basal forebrain-derived AChE-positive neurons; the cortical neu-

rons display smaller somata, more limited dendritic fields, and much lower levels of AChE histochemical activity than do the seeded BFCNs. With the techniques employed in the current study, few of the intrinsic AChE-positive cells were stained in most of the cultures. The greater level of AChE histochemical staining of the BFCNs may be due to their larger size and higher levels of endogenous AChE, and because the BFCNs were seeded on top of the slice and consequently had more direct access to the histochemical reagents.

The seeding of dissociated cells onto slices of potential target tissue is a powerful method of investigating cell-cell interactions in developing brain (Emerling and Lander, 1994; Förster et al., 1998; Förster, 2001; Halloran and Kalil, 1996; Laywell et al., 1996;

Robertson et al., 1997). In normal brain development, of course the somata and dendrites of basal forebrain neurons do not come in contact with cortical structures. It is of particular interest, then, that BFCNs not only display neurite (presumed axonal) outgrowth that forms patterns similar to the patterns formed by basal forebrain-derived cholinergic afferents, but also that the BFCN somata themselves show patterns similar to those of the normal cholinergic axonal projections. Thus, the patterns displayed by BFCNs after seeding onto cultured slices are remarkably similar to the pattern displayed by BFCN axonal ingrowth *in vivo*, suggesting that the mechanisms that serve to attract, retain, or nurture the cholinergic axons to their target sites can be influential on the whole neuron.

Normal pattern of basal forebrain cholinergic afferents to cerebral cortex

Cholinergic neurons in the basal forebrain, including the medial septum, diagonal band complex, substantia innominata, and medial globus pallidus, send axonal projections to the cerebral cortex in a loosely organized topographic pattern (McKinney et al., 1983; Mesulam et al., 1983; Wenk et al., 1980; Woolf, 1991). The cells of the medial septum project to the hippocampal formation, the diagonal band to the medial cortex and olfactory bulb, and the substantial innominata and medial globus pallidus project to the lateral neocortex (Amaral and Kurz, 1985; Calarco and Robertson, 1995; Luiten et al., 1987; McKinney et al., 1983; Mesulam et al., 1983; Rye et al., 1984; Wenk et al., 1980; Woolf, 1991). Although the basal forebrain cholinergic projections to cortex have often been viewed as poorly organized or 'non-specific', regional differences in laminar patterns of innervation between cortical regions have been demonstrated (Lysakowski et al., 1989; Mechawar et al., 2000; Mechawar and Descarries, 2001).

The projection from medial septum to the dentate gyrus displays one of the more clearly layered patterns of termination by cholinergic afferents (Amaral and Witter, 1995; Baratta et al., 1996a,b; Deller, 1998; Deller et al., 1999; Förster et al., 1997; Makuch et al., 2001; Storm-Mathisen and Blackstadt, 1964; Super and Soriano, 1994; Zimmer et al., 1986), and it is this layered quality that makes this region an ideal model system for this study. Septal cholinergic projections to the dentate gyrus terminate principally in the inner molecular layer, although termination also occurs in the outer molecular layer and, to some extent, within the hilus (Amaral and Witter, 1995; Deller et al., 1999; Makuch et al., 2001; Milner et al., 1983; Super and Soriano, 1994). Patterns of BFCN distribution, as well as the pattern of AChE-positive neurite outgrowth, mimic the normal pattern of cholinergic afferents. It should be noted that septal projections to the dentate gyrus are not exclusively cholinergic (Amaral and Kurz, 1985; Manns et al., 2001; Rye et al., 1984). GABA-ergic basal forebrain neurons also project to the hippocampal formation (Köhler et al., 1984; Peterson et al., 1987) and appear to target specifically the GABA-ergic local circuit neurons in the hilus

(Gulyás et al., 1999). At present, we have no information regarding the distributions of basal forebrain GABA-ergic or glutamatergic neurons following the seeding procedure.

Mechanisms leading to the non-random distribution

The non-random distribution of BFCNs after several days in culture may stem from differential attachment of the BFCNs, differential survival, or region-specific differentiation of the AChE-positive phenotype. This issue of the basis of the differential distribution of AChE-positive BFCNs is important for understanding mechanisms that guide the formation of connections in the developing or regenerating nervous system, but at the present time it is difficult to distinguish between these possible mechanisms; indeed, more than one of these may be operating.

As a first possibility, cell attachment or adhesion is likely to be important. Previous work by Emerling and Lander (1994) on distributions of carbocyanine dye-labeled thalamic neurons seeded onto slices of neocortex, demonstrated that the thalamic neurons were found in non-random patterns in short term (a few hours) cultures, suggesting that selective adhesion factors may lead to differential attachment. However, the present experiments reveal little evidence for the initial role of attachment factors for basal forebrain neurons, as all neurons could be effectively removed from the slice with a very low turbulent wash.

Possibly countering the action of putative attraction factors, we have shown that repulsive factors may also be important in some cases. Repulsion factors were suggested by evidence showing populations of neurons that normally do not project to the cultured slice tissue do not adhere or survive when seeded in this protocol (Robertson et al., 1997). For example, basal forebrain-derived cholinergic neurons are not detected when seeded onto slices of striatum, although tyrosine hydroxylase expressing neurons from ventral midbrain are detected readily. Thus, a combination of factors that attract some cells and repel other cells may play important roles in the non-random distribution of the seeded dissociated cells. Recent work has demonstrated that the semaphorins (Chédotal et al., 1998; Steup et al., 1999) act as repulsive agents for some hippocampal afferents, and it may be that the absence or reduced numbers of BFCNs on some regions of cortex is related to the expression of a repulsion factor.

Adhesion factors have been clearly demonstrated for other systems (Emerling and Lander, 1994; Förster et al., 1998; Förster, 2001). Although the present data indicate that adhesion factors of the type demonstrated by Förster and colleagues (Förster et al., 1998; Förster, 2001) may not play a role in initial attachment, this does not mean that adhesion factors do not develop over longer periods of time. Cell adhesion molecules have received considerable attention in recent years (Lander, 1990; Landmesser, 1994; Pinkstaff et al., 1999; Wilson and Snow, 2000). Although candidate molecules, including the integrins, semaphorins, and chondroitin sulfate proteoglycans, are present in the

hippocampus, little information is currently available regarding whether these molecules may be functional in this case. It may be of interest that the AChE that is expressed by the BFCN axons has itself been implicated as serving a cell adhesive function (Layer and Willbold, 1995; Robertson et al., 1998; Robertson and Yu, 1993; Sharma et al., 2001; Small, 1990) although recent work by Mesulam and colleagues (Mesulam et al., 2002) demonstrates that septal projections to dentate gyrus develop normally in AChE knock-out mice.

The AChE-positive phenotype does not become detectable until after 2–3 DIV in these cultures. Because the dissociated cells were taken from brains of animals at gestational day 17, this 2–3-day delay may simply reflect the normal sequence of development of these neurons (Amaral and Witter, 1995; Brady et al., 1989; Ha et al., 1996; Milner et al., 1983; Shingai et al., 1990; Thal et al., 1991). However, it remains possible that the development of the cholinergic phenotype occurs preferentially in cells that have settled down in a supportive environment or have established appropriate adhesive contacts on the slice. Other studies have reported expression of the cholinergic phenotype in dissociated cultures (Dreyfus, 1989; Hartikka and Hefti, 1988; Li et al., 1995; Nonomura et al., 1996; Shingai et al., 1990), however, the presence of exogenously applied neurotrophic factors or the presence of normal target cells is important for expression of the cholinergic phenotype and for survival (Dreyfus, 1989; Ha et al., 1996; Hartikka and Hefti, 1988; Li et al., 1995; Morse et al., 1993; Nonomura et al., 1996; Shingai et al., 1990). Grown in isolation, without target contacts or supplemental neurotrophins, the BFCNs do not thrive.

Differential survival or differential expression of phenotype may also be important here. Two of the neurotrophins, neurotrophic factor 3 (NT-3) and brain-derived neurotrophic factor (BDNF), have been shown to be

expressed by dentate gyrus granule cells (Lauterborn et al., 1994; Makuch et al., 2001). Indeed, both the spatial and the temporal patterns of expression of NT-3 and BDNF mRNAs correspond closely to the spatial and temporal patterns of septally derived cholinergic afferents to the dentate gyrus, and to the patterns of BFCN somata and neurites after seeding. If BFCN axons are targeting the cells that express, and presumably release, NT-3 or BDNF, then those cells that contact the dentate granule cells may be stimulated by one or both of these neurotrophins to differentiate to the AChE-positive phenotype. If this is the case, then BFCNs seeded onto slices of hippocampus taken from animals genetically engineered to eliminate the gene for NT-3 or BDNF would be expected not to form the pattern characteristic of the wild-type animal.

Conclusion

The distributions of BFCNs, and their neurites, following seeding onto cultured slices of hippocampus and adjacent tissues are remarkably similar to the distributions of basal forebrain-derived cholinergic axons in the normal brain. It may be that this characteristic pattern results from one or more factors, including selective attachment and selective differentiation stemming from response to neurotrophin production by cells in the cultured slice. Seeding of dissociated cells onto cultured slice tissue may provide a useful means of investigating mechanisms that normally determine the patterns of termination of afferent projection systems.

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