Isolation and culture of adult rat hippocampal neurons

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Abstract

Inability to culture adult central neurons and the failure of injured neurons to regenerate in the brain could be due to genetic controls or environmental inhibitors. We tested the environmental inhibitor hypothesis by attempting to regenerate adult rat neurons in B27/Neurobasal™ culture medium, a medium optimized for survival of embryonic neurons. To isolate neurons from their numerous connections, papain was the best of six different proteases screened on slices of hippocampus for survival of isolated cells after 4 days of culture. Use of a density gradient enabled separation of oligodendroglia and some enrichment of neurons and microglia from considerable debris which was inhibitory to sprouting and viability. With these techniques, about 900 000 viable neurons were isolated from each hippocampus of any age rat from birth to 24–36 months, near the median mortality. FGF2 was found to enhance viability at least 3-fold to 40–80%, independent of age, without affecting the length of the processes. Neurons were cultured for more than 3 weeks. These methods demonstrate that hippocampal neurons can regenerate axons and dendrites if provided with adequate nutrition and if inhibitors are removed. They also will enable aging studies. Therefore, the concept of environmental growth restriction may be more appropriate for neurons in the brain than the concept of a genetic block that precludes regeneration of processes. © 1997 Elsevier Science B.V.

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1. Introduction

The inability to isolate and culture neurons from the adult mammalian brain and the general failure of neurons to regenerate in the lesioned brain have contributed to the concept that adult neurons do not regenerate. Differentiated cells are posited to have set genetic switches that preclude redifferentiation. Yet adult neurons maintain enormous capacity for changing their connections, for synaptic plasticity (Pons et al., 1991; Cotman et al., 1994; Stroemer et al., 1995). To demonstrate that isolated adult neurons are capable of regeneration, four major technical problems must be overcome: (1) Isolation of cells from an intertwined network of thousands of adhesive contacts without causing irreversible damage of sheared axons and dendrites. This may be the primary benefit of starting with embryonic tissue with fewer connections (Banker and Cowan, 1979). (2) Separating away possible growth inhibitory factors (Faissner and Steindler, 1995), needed in the adult brain to maintain homeostasis. (3) Separating the different cell types, especially neurons from glia, to be able to assign experimental effects to specific cell types. (4) Providing an adequate substrate for attachment and adequate nutrients for continued viability in culture.

We have attempted to solve the isolation problem by comparing several proteases for yield of isolated cells following trituration in an isolation medium optimized for osmolarity. This medium also contains anti-oxidants to reduce reactive oxygen damage to membranes, proteins and DNA. We have also used density gradient fractionation to enrich for neuronal cell types and to

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separate cells from inhibitory factors. These studies were spurred by our successful optimization of a serum-free medium for embryonic hippocampal neurons (Brewer et al., 1993). This B27 medium supplement contains essential fatty acids, antioxidants, vitamins and hormones that enable culture of embryonic neurons from numerous regions of the rat brain for weeks at high viability (Brewer, 1995). Use of B27 and adequate isolation methods have permitted us to isolate spherical remnants of hippocampal neurons from any age rat and promote their regeneration of axon and dendrite-like processes. A neuron-like morphology is maintained over several weeks in culture.

Isolated neurons offer several advantages for studies outside the complexity of the brain. First, enriched populations of neurons decrease the ambiguity of interpreting the cellular target of various test substances. Second, isolated neurons in culture allow direct access for application of pharmacological agents and electrophysiological recording. Third, cell culture allows precise control of the environment affecting the cell. These uniform conditions promote greater reproducibility in physiological measurements than whole animal studies. Fourth, cell culture allows continuous visual access for studies of morphological characteristics such as neuritogenesis, connectivity and toxicity. In contrast to adult neurons, embryonic neurons have been used for these studies because they can be easily isolated and maintained for periods of weeks (Mattson et al., 1988; Goslin and Banker, 1989; Brewer et al., 1993). However, many diseases occur only in mature neurons, the same target for most pharmacological interventions. Therefore, we have developed techniques for culture of adult neurons.

2. Materials and methods

2.1. Cell release from hippocampal tissue

For each experiment, a single male Sprague–Dawley, or Fischer 344 rat (Harlan, Indianapolis) of the indicated age was anaesthetized with ether and decapitated by guillotine. Later experiments also used an F1 hybrid of Fischer 344 × Brown Norway (Lipman et al., 1996). In early experiments, all solutions were gassed with 100% oxygen. Later experiments proved oxygenation to be unnecessary, so most experiments were conducted in ambient oxygen. The hippocampi were rapidly dissected from the brain in 2 ml HibernateA/B27 at 4°C in a 35 mm diameter dish. HibernateA (Life Technologies, Gaithersburg, MD) (Brewer and Price, 1996) is a form of Neurobasal (Brewer et al., 1993) with common salts, glucose, pyruvate, 17 amino acids, nine vitamins and buffered for use with adult neurons at ambient CO2. It was further supplemented with 0.5 mM glutamine from a frozen stock solution. B27 is a serum-free medium supplement containing 20 ingredients (Life Technologies # 17504; Brewer et al., 1993). Meninges and excess white matter were removed in the same medium in a second dish at 4°C. Hippocampi were transferred onto sterile filter paper prewet with a few drops of HibernateA/B27 on the cooled stage of a MacIlwain tissue chopper. Slices of 0.5 mm thickness were cut perpendicular to the long axes of the hippocampi and transferred to a tube at 4°C containing 5 ml HibernateA/B27. Average weight of transferred slices from two hippocampi was 133 ± 25 mg (mean ± S.D., n = 14). For comparisons between methods, equal numbers of slices were apportioned for each variable. After shaking 8 min at 30°C, slices were transferred with a wide bore pipet to another tube at 30°C containing papain. Papain (Worthington 3119, 15–23 U/mg protein) was not activated with cysteine, which was found to be cytotoxic, but was prepared by dissolving 12 mg in 6 ml HibernateA, warmed for 5 min at 37°C, filter sterilized and stored at 4°C in a 50 ml sterile plastic centrifuge tube for no more than 3 h. Slices were incubated for 30 min in a 30°C water bath with a platform rotating at 170 rpm, or enough to keep the slices suspended. Slices were transferred to a 15 ml tube containing 2 ml HibernateA/B27 at 30°C. After 5 min at room temperature, slices were triturated 10 times (in about 30 s) with a siliconized 9 inch pasteur pipet with the tip barely fire polished to an opening of 0.7–0.9 mm D (superior) or with a 1 ml blue polypropylene pipet tip with an opening of 0.9 mm D (inferior). After 2 min to let pieces settle, the supernatant was transferred to another tube. Twice more, the sediment was suspended in 2 ml HibernateA/B27, triturated 10 times, allowed to settle 2 min and the supernatants combined. This resulted in a cell suspension in 6 ml HibernateA/B27.

2.2. Gradient isolation of cells

The cells released by trituration were applied to the top of a gradient of Nycoprep 1.15 (Life Technologies #100-2420) or in earlier experiments, 1 ml 4% bovine serum albumin in HibernateA/B27 in a 15 ml centrifuge tube. The Nycoprep gradient was made in four 1 ml steps of 35, 25, 20 and 15% Nycoprep in HibernateA/B27 (v/v). The cell suspension was centrifuged 15 min at room temperature at 1900 rpm (800 × g). Debris above 4 ml was discarded. Fractions containing cells were collected with the trituration pipet (Fig. 3) and diluted into 5 ml HibernateA to remove the Nycoprep. Fraction 1 contained the top 2 ml. Fraction 2 contained the most dense band of cells and fragments. Fraction 3, below this band, was enriched in neurons. The pellet, fraction 4, was resuspended in 2 ml B27/NeurobasalA. Fractions were centrifuged for 1 min, 1100 rpm (200 × g). The cell pellets were resuspended in 2 ml B27/Neu-
robosatalA. NeurobasalA is Neurobasal (Life Technologies #21103; Brewer et al., 1993) made for adult neurons by increasing osmolarity from 235 to 260 (± 10) mosM with an additional 12.5 mM NaCl. It was completed by addition of 0.5 mM glutamine. No glutamate was added. After mixing 20 μl of cell suspension with 20 μl 0.4% Trypan blue, live (dye-excluding) cells were counted in a hemacytometer.

2.3. Cell plating

Cells were plated in 60 to 120 μl B27/NeurobasalA at the indicated densities on glass coverslips previously coated with 50 μg/ml poly-D-lysine in water (135 kDa, Sigma). The coverslips (12 mm diameter Assistent brand from Carolina Biological, Burlington, NC) had been autoclaved without further cleaning. Tests showed that cleaning in concentrated nitric acid produced no improvement in cell survival. The polylysine was applied overnight, aspirated, rinsed once with water and allowed to dry about 1 h. One h after plating and incubation (5% CO2/95% O2; Forma Scientific, Marietta, OH), unattached cells and debris were drained as the coverslip was quickly transferred to 0.4 ml B27/NeurobasalA in a 24-well plate at 37°C. The medium was aspirated to remove additional debris, rinsed once with 0.4 ml warm HibernateA and replaced with 0.4 ml growth medium, B27/NeurobasalA with 0.5 mM glutamine, no glutamate, 50 units penicillin, 0.05 μg/ml streptomycin, 5 ng/ml FGF2 (Life Technologies), unless stated otherwise. Volumes for 96 well cultures were 50 μl/well. For culture longer than 4 days, one-half of the medium was removed every 3 or 4 days and replaced with fresh medium to equal the original volume. For medium changes, the FGF2 concentration in the fresh medium was double the original concentration so that the final concentration was the same, assuming that the old FGF was totally consumed.

2.4. Viability assay

Viable cells were determined by uptake and hydrolysis of fluorescein diacetate (Brewer et al., 1993), usually after 4 days of growth. Briefly, cultures were rinsed twice with Hank’s Balanced Salts (Life Technologies) and treated with fluorescein diacetate (15 μg/ml, Sigma) and propidium iodide (4.6 μg/ml, Sigma). With blue excitation, live cells fluoresce green and dead cells fluoresce red. For statistical analysis, at least six consecutive 0.313 mm² (20 ×) fields were counted in each of two cultures, followed by Student’s t-test or ANOVA with rejection of the null hypothesis at P < 0.05. All experiments were repeated for qualitative confirmation, but data were not combined for statistical analysis because of variations in plating efficiency.

2.5. Other proteases

The method of Kay and Wong (1986) was modified as follows (White et al., 1993). In comparison to the above treatment with papain in HibernateA, slices were first treated for 5 min at 30°C with 5 ml proteinase K (Sigma P0390) in Kay’s medium followed by a brief rinse and 30 min in 5 ml trypsin (Sigma). Kay’s medium consists of 115 mM NaCl, 5 mM KCl, 20 mM Pipes buffer, pH 7.0, 1 mM CaCl2, 4 mM MgCl2, 25 mM glucose, 0.5 mg/ml bovine serum albumin. Proteinase K was filter sterilized from a solution of 6 mg 0.2 ml 5 mM CaCl2 and further diluted into the slices to 0.2 mg/ml. Trypsin was filter sterilized from a solution of 10 mg/0.33 ml PBS and further diluted into the slices to 1 mg/ml.

The method of Swartz and Bean (1992) uses protease XXIII from Aspergillus oryzae (Sigma). We used this protease at 3 mg/ml in place of papain as described above. Early experiments were conducted with the low chloride buffer of Swartz and Bean: 82 mM Na2SO4, 30 mM K2SO4, 5 mM MgCl2, 10 mM Hepes buffer, pH 7.4, 10 mM glucose, 0.001% phenol red. Two other proteases were tested after dissolution in HibernateA and filtration: dispase (0.5 mg/ml, 6 units/mg; Boeringer-Mannheim) and collagenase (1 mg/ml, 153 U/mg; Life Technologies).

2.6. Immunocytochemistry

Cells for staining with anti-neurofilament 200 and anti-GFAP antibodies were rinsed free of medium with PBS and fixed for 20 min at room temperature with 10% glacial acetic acid, 90% ethanol (for neurofilament or GFAP) or 4% paraformaldehyde (all others) in PBS. After rinsing twice with PBS, cells were permeabilized for 5 min with 0.5% Triton X-100 in PBS. After rinsing with PBS, non-specific sites were blocked and cells permeabilized with 5% normal goat serum, 0.5% Triton X-100 in PBS. Cells were incubated overnight at 4°C with mouse anti-neurofilament 200 (Sigma N3589, diluted 1:40) and/or rabbit anti-cow GFAP (Dako Z334, diluted 1:2000) in the blocking solution. Other primary antibodies were rabbit anti-tau (Sigma, 1:20), mouse anti-MAP2 (Sigma, 1:50), rabbit anti-galactosyl cerebroside for oligodendroglia (Sigma, 1:50), mouse anti-rat complement receptor 3 (OX-42; Sera-labs, 1:20) for microglia, or rabbit anti-rat neuron-specific enolase (Boeringer-Mannheim, 1:3000). After rinsing four times with PBS, appropriate secondary antibodies were incubated for 60 min: affinity purified rhodamine-conjugated F(ab')2 goat anti-rabbit IgG (Biosource #4420, Camarillo, CA, diluted 1:500) and fluorescein-conjugated F(ab')2 anti-mouse IgG (Biosource #4340) in blocking solution. After rinsing four times in PBS, slips were mounted in Aquamount (Scientific Products) and
photographed with Ektachrome P800 with Nikon 60X/1.4 n.a. oil immersion objective and Nikon B1A and G1B filters. Controls without primary antibody were negative.

2.7. Process length

Cells grown for 4 days were fixed and stained for tau, as above. Immunofluorescence was photographed through a 40 × objective with a total magnification of 100. As a measure of process length, the linear distance from the center of the soma to the tip of the most distant process was measured with a graticule.

3. Results

3.1. Proteolytic release of neurons

The loose adhesion of cells in the embryonic hippocampus, with minimal synapses and axons, permits mechanical dissociation of cells without enzymes (Brewer et al., 1993). Adult brain tissue is obviously connected by multitudinous synapses, bundled axons and glial-neuronal adhesion. The neurons of neonatal brain are intermediate in their adhesion. To extricate live cells from the three dimensional adult network is a formidable task. Attempts to acutely isolate neurons from neonatal brain for short term studies have used several kinds of proteases. We tested some of these for their ability to release cells from juvenile and adult brains. From a 6-week old animal, viable cells were obtained from slices with each protease protocol. The yield of cells from four slices was similar for each treatment: trypsin/proteinase K (0.15 million), protease XXIII (0.16), papain (0.17), collagenase (0.12) and dispase (0.19). However, after 4 days of growth, viability was superior with cells isolated with papain (Fig. 1).

3.2. Glutamate and optimum osmolarity

The optimum glutamate concentration for starting cultures of embryonic hippocampal neurons is 25 μM (Brewer et al., 1993). This relatively high glutamate may be a useful mimic of the leaky blood brain barrier of embryos. Expression of glutamate receptors, especially of the NMDA type, requires about a week of development in culture for E18 embryonic hippocampal neurons (Peterson et al., 1989). After glutamate receptors are expressed, neurons are susceptible to excitotoxicity from excess glutamate. Therefore, we compared survival of neurons isolated from older rats with and without glutamate in the culture medium. For neurons isolated from a 2- or a 6-week old rat, survival without glutamate was 34 or 25% better than with 25 μM glutamate, respectively (P < 0.05). After confirmation, all subsequent experiments used medium without glutamate.

Initial cell isolations were performed in Neurobasal medium with osmolarity 235 (± 10) mosM. We observed sticky strands of material following protease digestion or trituration of slices. This suggested that osmotic shock caused cells to release DNA. Therefore, the osmolality of Neurobasal which was optimized for embryonic neurons may be too low for adult neurons. Also, since some of the procedures for isolating neurons are conducted in ambient CO₂, buffering pH at 7.4 requires lowering the bicarbonate to 0.037% from 0.22% (Umbreit, 1964). This further requires raising NaCl to compensate. Experiments were conducted to optimize osmolarity by varying the amounts of NaCl. For this purpose, we compared sister media made for use in ambient CO₂ and 5% CO₂ with the same osmolality. Fig. 2 shows the effect of five different concentrations of [NaCl + NaHCO₃]. Levels at or lower than 80 mM, similar to the embryonic optimum of 77.6 mM found in Neurobasal, produced significantly lower viable cells after 3 days of growth. Therefore, all subsequent procedures used NeurobasalA with 90 mM total NaCl + NaHCO₃.

3.3. Activity and time of digestion with papain

The full proteolytic activity of papain is produced after activation in reducing agents and 5 mM cysteine. Preparation of the enzyme in this way resulted in the same yield of viable cells after trituration (90% of control), but viability after 4 days in culture was only

Fig. 1. Comparison of different proteases for isolating neurons from hippocampal slices. Papain digestion produced significantly higher viability after 4 days of growth. Hippocampi were obtained from a 6-week old Sprague–Dawley rat. Digestion conditions are described in Materials and methods (Section 2). After each treatment, cells were separated from debris into a step of 4% BSA. Cells were plated at a density of 120 cells/mm². After 4 days of growth, live cells were counted and divided by the number of cells plated to give the viability. Values here and subsequently are means ± S.E. (n = 12 microscopic 0.3 mm² fields, about 200 live cells, total/point). Similar results were obtained in a second experiment.
3.5. Inhibition of sprouting

As expected, trituration of adult brain tissue results in a large amount of cellular debris along with some viable spherical somata. Two approaches were used to purify the cells: rinsing non-adherent material by media changes and density gradient centrifugation. After plating, the majority of cells adhere to the polylysine substrate within 2 h. At this time there is still considerable debris floating just above the cells. Removing this debris had dramatic effects on cell sprouting. Coverslips or plastic substrates with attached cells were rinsed twice with warm HibernateA and returned to B27/NeurobasalA. After 5 days in culture, comparisons to un-rinsed cells indicate an increase from 4% sprouting to 27% sprouting ($P < 0.001$). The seven-fold increase in sprouting suggests that cellular debris contains inhibitory factors (Faissner and Steindler, 1995). These results were confirmed in a second experiment.

3.6. Gradient isolation to remove debris and enrich cell types

Density gradient fractionation has the potential advantage of enriching for different types of cells such as neurons and glia as well as separation from debris. Initial experiments were performed with embryonic neurons over a wide range of densities available with Nycoprep (neat = 1.15 g/ml). Nycoprep is a non-ionic tri-iodinated benzoic acid (5-((N-2,3-dihydroxypropylacetamido)-2,4,6-tri-iodo-N,N%-bis(2,3-dihydroxypropyl)isophthalamide). A 3-step gradient from 83.5% to 16.5% Nycoprep in HibernateA/B27 yielded neurons near the middle of the gradient. Subsequent trials of shallower gradients with triturated adult cell suspensions produced distinct bands over a range of 35 to 15% (densities 1.052 to 1.022). A typical separation is shown in Fig. 3. A large amount of acellular debris resides above the gradient (not shown). Fraction 1, at the top, contains considerable debris and some cells. Fraction 2 is a dense band with a relatively high density of cells and debris. Fraction 3 appears flocculent but contains live cells with some vascular remnants and much less debris. The pellet, fraction 4, is nearly free of debris and contains smaller, more dense cells and nuclei. For comparisons at equal plating densities, fractions were assayed for phase bright cells that exclude Trypan blue. We compared sprouting in unfractionated vs. gradient fractionated cells. After 4 days in culture, 21% of the viable unfractionated cells from a 16 week old rat sprayed while Fraction 3 produced 78% sprouting ($P < 0.001$). Similar comparisons with cells from a 318-g adult (estimated age of 22 weeks) produced a similar improvement by the gradient from 16 to 55% cells with a clear neuron-like morphology (multiple branched pro-
cesses in 5 ng/ml FGF2). This further suggests inhibition of sprouting by certain cell types or by higher concentrations of debris. Immunocytochemical characterization of these fractions for cell types is described below.

3.7. Regrowth of isolated neurons

Cells isolated with papain, fractionated on a density gradient and placed in B27/Neurobasal medium predominantly start as spherical cells that begin to grow processes within hours (Fig. 4A). This reexpression of neuritogenesis continues in culture for a number of days. Fig. 4B shows the extent of neurite outgrowth after 4 days in B27/Neurobasal + 10 ng/ml FGF2. Fig. 4C shows healthy neuron-like cells after 3 weeks in culture. Many have a pyramidal shape with active growth cones seeking to expand the neuronal network.

3.8. Analysis of gradient fractions for different cell types

Fig. 5 shows results of immunofluorescent labeling of cells from each fraction and quantitative analysis. In the presence of FGF2, most viable cells after 4 days of culture were found in fractions 2 and 3 (Fig. 5A). The total viable cell recovery from three preparations after 4 days in culture averaged 66%. As markers for neurons, we used neurofilament 200, MAP2, tau and neuron-specific enolase. Immunoreactivity to neurofilament was quantified across the entire gradient. For astroglia, we used glial fibrillary acidic protein (Bignami et al., 1972); for oligodendroglia, we used galactosyl cerebroside (Raff et al., 1968); and for microglia, we used Ox-42, the rat complement receptor (Robinson et al., 1986). Oligodendroglia were greatly enriched in fraction 1 (Fig. 5D). Neuron-like cells were found in all fractions with some enrichment in fractions 2 and 3 (Fig. 5B). With FGF, they represented 60–70% of the cells in these fractions. Astroglia were present at low levels in all fractions, an average of 9% (Fig. 5C). Microglia were more abundant than astroglia and present in all fractions (Fig. 5E). Microglia were enriched in fraction 4. Scoring for these four antigens accounted for an average of 91% of the cells present after 4 days in culture.

Typical immunostain morphologies are shown on the right in Fig. 5. Neurofilament positive cells had several asymmetric tapered processes with multiple branch points. Astroglial processes were smoother, with few branches, if any. Oligodendrocytes displayed a characteristic circular symmetry with multiple rapidly tapering branching processes, similar to those reported for embryonic cells (Benjamins and Nedelkoska, 1994). Morphologies of microglia were more diverse, mostly flat and round without multiple processes (Giulian and Baker, 1986).

3.9. Effect of FGF2 on survival and morphology

Hippocampal neurons are reported to be responsive to basic fibroblast growth factor (FGF2) (Wallicke et al., 1986). We find a differential effect of FGF2 on the survival of different cell populations (Fig. 5). The number of neurofilament positive cells greatly increases in the presence of FGF2 (Fig. 5B). Since no other cell type decreases by 20 to 40% with FGF, the increase in cells reactive for neurofilament must be due to a trophic effect of FGF on neurons. Astroglia and microglia appear largely unaffected by FGF2, with the possible exception of cells in fraction 1. FGF2 is clearly inhibitory to survival of oligodendrocytes (Fig. 5D).

Although there were fewer neurons without FGF2, those that did grow neurites over 4 days did so at least as well as those with FGF2. For cells immunostained with tau, a measure of the chord distance to the tip of the longest neurite was used to quantify neurite length. The number of processes emanating from the soma was used to quantify sprouting. For cells grown with or without FGF2, the mean number of primary processes was similar: 3.8 ± 0.2 (S.E., n = 34 neurons), and 3.7 ± 0.3 (n = 23), respectively. For cells grown in FGF2, the mean distance to the tip of each process was 60 ± 3 μm (S.E., n = 34). For cells grown without FGF2, the mean distance was 74 ± 6 μm (n = 23). By two-tailed t-test,
Fig. 4. Hippocampal neurons from a 19-month old Fisher rat plated at 90 cells/mm² (A) after 3 h in culture showing early sprouting, and (B) after 4 days in culture in B27/NeurobasalA + 10 ng/ml FGF2 showing extensive regeneration of branching processes. (C) Hippocampal neurons from a 36-month old Fisher-Brown Norway F1 rat after 3 weeks in culture. Slices were digested with papain, triturated in HibernateA/B27 and fractionated on a Nycoprep density gradient. Cells were photographed with phase contrast optics. Bar = 50 μm. Magnification of (A) and (B) are the same.

the 23% increase in process length in cells without FGF2 is significant (P < 0.05). This suggests that FGF2 promotes survival, but not the rate or extent of process outgrowth or the number of processes.

3.10. Oxygen independence

Experiments with hippocampal slices are routinely performed in 95% to 100% oxygen (Aitken et al., 1995). Therefore, methods to acutely isolate neurons from adult slices have also used this high level of oxygen (Kay and Wong, 1986; Huettner and Baughman, 1986). In contrast to cells in a slice, isolated cells have unlimited access to the medium. For this reason and simplicity, we tested the complete isolation procedure with all solutions gassed with 100% oxygen or left unaltered in the 21% ambient oxygen. After 4 days in culture, survival of cells with neuron-like processes from a 10-month old rat was 56 ± 2% without extra oxygenation and 54 ± 2% with oxygenation (mean ± S.E., n = 12 fields). Since these values are not significantly different, all subsequent experiments were conducted without additional oxygenation. We have previously demonstrated that embryonic hippocampal neurons grow better at 9% oxygen, a physiological concentration (Brewer and Cotman, 1989). All cultures described in this report were performed at 9% oxygen.

3.11. Isolation of neurons is independent of the age of the rat

Two hippocampi were dissected from rats ranging in age from embryonic day 18 (3 days from birth) through age 24 months, near the median 28 month mortality for Fisher rats (Solleveld et al., 1984) or out to 35 months, near the median mortality of Fisher × Brown Norway
Fig. 5. Mean distribution over the density gradient of immunostained cells after 4 days of growth (DIV) from 3 adult male Sprague–Dawley rats weighing 283, 318 and 368 g, estimated 4, 5, and 8 months old. Cultures were started at 320 cells/mm² with or without 5 ng/ml FGF2. Cultures were fixed for immunostaining after 4 days in culture. Total cells in (A) is the sum of results from the four stains. For each rat, three coverslips were immunostained as indicated (double-staining with neurofilament and GFAP were analyzed on one slip). For each condition, 12 fields with cells were analyzed with a 60× objective. The majority of neuron-like cells are in fractions 2 and 3. FGF causes an increase in the number of neuron-like cells. Bars without standard errors were less than 2%. Bars at zero do not display. Scale = 20 μm.

F1 hybrid rats (Lipman et al., 1996). Each time, the number of viable cells in fraction 3 was determined by exclusion of trypan blue. Fig. 6 shows that the number of isolated cells from fraction 3 is independent of age for 38 separate animals. The mean yield from fraction 3 was about 500,000 cells from two hippocampi. If counts from fraction 2 are included, the yield is also independent of age, with a mean of 1.8 million cells from 2 hippocampi. Based on the culture results in Fig. 5, about 60% or 1 million of these can survive and regenerate as neurons. The yield of viable cells from old animals is as good as that from young animals.
3.12. Effect of FGF concentration and age on survival

The concentration and age-related effect of FGF2 on neuron survival was measured in cells isolated from an 11- and a 36-month old Fisher × Brown Norway rat. Fig. 7 shows a 5- to 7-fold effect of FGF2 to increase the yield of viable neuron-like cells for both ages, measured as a percent of total live cells (A) and as a percent of total cells (B). The lower values in (B) are due to adding dead cells in the denominator. The majority of viable non-neuron-like cells were flat and large, like microglia. FGF2 is clearly neurotrophic for adult neurons with an optimum efficacy between 2 and 10 ng/ml and an ED$_{50}$ of 0.3 ng/ml. Even though the same number of cells were plated from the 11 and the 36 month old rats, the 36 month rat produced 15% fewer live neurons in response to FGF (ANOVA for the five highest concentrations, $F = 17.7$, $P < 0.0001$). Similar results were obtained in a second experiment.

3.13. Scale-up for isolation of cortical neurons

Neurons were isolated from the cerebral cortex of a 35-month old rat with a density gradient in a 50-ml centrifuge tube. The Nycoprep volumes were doubled for each step. The wet weight of the sliced tissue was 732 mg, about 6 times that of the hippocampi. The yield of cells in fractions 2 and 3 was 11 million, again 6-fold higher. Fig. 8 shows a good yield of neuron-like cells after 5 days in culture. Also evident are large, flat microglia-like cells.

Fig. 7. Age and FGF concentration dependence of neuron-like viability after 4 days in culture. Both ages are highly dependent on FGF. Cells from the older age animal are slightly less responsive to FGF. Hippocampal cells isolated from an 11-month and a 36-month old Fisher × Brown Norway rat were plated at 320 cells/mm$^2$ in a 96-well plate. The indicated FGF2 concentrations were added at the start. After 4 days in culture, neuron-like cells were assayed for viability. Means ± S.E. ($n = 2$ fields/well, 6 wells/treatment, total $n = 12$). Similar results were obtained in a second experiment.

Fig. 6. Yield of viable cells from three strains of rats of various ages. Cells isolated from gradient fraction 3 were counted viable if they were phase bright and excluded trypan blue. Each circle represents the yield from two hippocampi from one rat. Linear regression (dashed line) indicated that isolated cells = 492 + 1.44 × age ($R^2 = 0.007$). The slope is not significant.

4. Discussion

By comparison of different proteases for isolation of neurons from brain tissue and use of an isosmotic medium which contains anti-oxidants, we have improved on the pioneering work of Kay and Wong...
(1986), Huettner and Baughman (1986) and Xie and Barrett (1991) to isolate individual cells from hippocampal tissue. In contrast to neonatal tissue, from which cells can be isolated with dendritic rudiments, our procedure produces largely spherical cells. Nevertheless, in the serum-free medium, B27/NeurobasalA, we can elicit regrowth of processes and maintain viability for weeks. For isolation of cells and tissue viability, oxygenation with 90–100% oxygen or 5% CO₂ are not required. With supplementation of FGF2, a larger fraction of neurons survive, but with no greater extent of process growth than without FGF. These effects on adult neurons are consistent with the neurotrophic effects of FGF2 on embryonic neurons (Morrison et al., 1986; Walicke et al., 1986). Although hippocampal progenitor neurons are known to proliferate in response to FGF2 (Ray et al., 1993), the adult neurons whose survival we measured after 4–5 days in culture did not appear to be dividing. At later times, some cells with neuronal morphologies begin to divide (Brewer, manuscript in preparation). We are attempting to determine whether these derive from neuron progenitors or whether they arise from neurons that re-enter the mitotic cycle.

An important part of this procedure is the use of a density gradient to separate cells from debris and enrich for certain cell types. Early work on separation of neurons and glia from adult tissue used filtration through nylon cloth and stainless steel screens followed by a sucrose gradient (Iqbal and Nagel, 1972). Some intact apical dendrites were reported from post-mortem human brain tissue, but not from rat brain. Later reviews of a variety of sucrose, Ficoll and Percoll gradients indicate general difficulty in maintaining viability of isolated CNS neurons in culture, especially neurons from animals older than a few weeks (Althaus and Neuhoff, 1982; Schaffner and Schnaar, 1983).

Neurons from embryonic or postnatal brain are routinely isolated for cell culture. A critical question remains whether these embryonic neurons behave in culture like their fully developed adult counterparts. Although many basic characteristics of neurons derived from embryonic tissue appear similar to the mature adult tissue (Bartlett and Banker, 1984; Rothman and Samaie, 1985), the temporal and environmentally controlled genetic switches in these neurons are likely to be different in culture than they are in the brain. Therefore, some investigators have approached an adult-like anatomy with culture of whole slices, pioneered by Gahwiler (Zimmer and Gahwiler, 1984). The main advantage of slice culture is that the histotypic relationship of dentate, CA3 and CA1 regions are maintained in a single cell layer with easy access for pharmacological and electrophysiological studies. The main disadvantage of slice cultures is the inability to readily observe the connections of one cell to another, and the inability to view morphology of individual cells without injection of a dye. Also, the presence of mixed neuron and glial populations often precludes identification of specific cellular targets.

Another approach to the adult condition is to use proteolytic enzymes to acutely isolate neurons from postnatal animals (Kay and Wong, 1986). Huettner and Baughman (1986) were able to culture neonatal rat cortical neurons isolated from animals up to 2 weeks old. These neurons survived for several weeks in culture, but neurons from older animals did not survive. Unlike our culture medium, their cultures were dependent on co-culture with a layer of glial cells. Kaneda et al. (1988) found about the same age restriction but were unable to maintain viability more than 10 h. A caveat for acutely isolated cell preparations is the assumption that cells isolated by proteolysis from postnatal slices will have the same receptors and physiology as they had before proteolysis.

Other brain regions may be amenable to our procedure. The basic growth medium of B27/Neurobasal

Fig. 8. Large-scale isolation of cortical neurons from gradient fractions 2 and 3. Phase contrast image after 5 days in culture. Note the numerous cells with phase bright borders and multiple processes, most with numerous branches, some rapidly tapering like dendrites and others longer with uniform caliber like axons. Note also larger flat microglia-like cells without processes. Scale = 50 μm.
supports growth of embryonic and postnatal neurons from the cerebral cortex, dentate gyrus, striatum, substantia nigra, septum, and cerebellar granule cells (Brewer, 1995). We have also successfully isolated and cultured adult human cortical neurons from five surgical specimens, one as old as 70 years (Brewer and Pencek, manuscript in preparation). This extends the precedent established by Silani et al. (1988) who succeeded in culturing neurons from biopsy of human caudate nucleus in the presence of human fetal neuron extracts.

A surprising aspect of this procedure is the independence of cell yield with age of the animal. This achievement should facilitate aging studies to distinguish intrinsic genetic capabilities from influences of the aging physiologic environment. Our initial comparison of the trophic effects of FGF2 on neurons from middle age and elderly rats indicated similar responsiveness. Although neuron survival was about 15% less from the older brain with FGF2, the absence of a larger difference should encourage in vivo attempts to provide damaged tissue with FGF trophic support. In another test of age-dependent differences, we have found that hippocampal neurons isolated from aged rats are more susceptible than those from young adults to glutamate toxicity and lactic acid acidosis (Price and Brewer, 1995).

Until these cultured neurons are analyzed by physiologic and molecular techniques, we do not know how well these cells represent their in vivo condition before isolation. Studies of electrophysiology are underway to address this issue. Preliminary results from electron microscopy indicate characteristic clusters of synaptic vesicles at regions of contact. Many more questions remain. Do these cells maintain adult types of transcription and translation? Or do they re-express embryonic and postnatal patterns of transcription that are needed for the original sprouting of dendrites and axonal differentiation (Goslin and Banker, 1989). We have evidence with antibodies to nestin that regrowth of adult neurons appears to occur through a process of redifferentiation involving reexpression of nestin, the intermediate filament protein thought to be characteristic of neuroepithelial stem cells (G.J. Brewer and R.D.G. McKay, unpublished observations).

Our results of regeneration of a substantial proportion of adult hippocampal and cortical neurons extend the concept of the regenerative capacity of adult mammalian brain beyond that of differentiation of a few resident stem cells (Cattaneo and McKay, 1990; Reynolds and Weiss, 1992; Kilpatrick and Bartlett, 1993; Gage et al., 1995). Although B27 increases survival of these stem cells (Davis and Temple, 1994; Svendsen et al., 1995), we find that B27 promotes survival of a larger population of cells, some of which were neurons before isolation. The large numbers of isolated cells also argues against neurogenesis, a process recognized by the above laboratories to occur at low levels in the adult brain. Continued birth of new neurons is especially well documented in the dentate gyrus of adult rats (Gould et al., 1992). In these studies, adrenalectomy, which lowers corticosteroids, caused increases in neuron-like cells that incorporated [3H]thymidine. In culture, we find that corticosterone, together with the other ingredients in B27, promotes neuron survival. Since we did not measure incorporation of thymidine and in vitro conditions are vastly different from those in vivo, it is difficult to compare these results. Our results of a large number of cells isolated from rat brains, independent of age, is in contrast to the observed 10-fold decline in neurogenesis with age in the dentate gyrus of the rat brain (Kuhn et al., 1996). This suggests that the cells that we isolate and culture are mostly unrelated to the resident stem cell population. Alternatively, deficits in hormonal stimulation/response of neurons in the aging brain may be compensated in culture by uniform and sufficient media components.

Our work with B27/Neurobasal A culture medium suggests that this medium is capable of not only providing nutritive maintenance of viability, but also of stimulating regeneration of differentiated polarity. Further work is needed to determine the molecular extent of genetic reprogramming. Adult neurons may not be terminally differentiated as much as they are growth restricted by their environment. This concept is supported by some expression of neuronal proteins in cells isolated and cultured in serum-containing medium from adult rat (Black et al., 1994) and human brains (DeGiorgio et al., 1994). Other evidence against an immutable ontogenetic neuronal program of differentiation has been reported for neuroblasts from rat sympathetic ganglia (DeCicco-Bloom et al., 1990). The hypothesis of environmental growth restriction seems more appropriate than terminal differentiation or programmed loss of ability to elongate, as recently interpreted from the failure of retinal axons to reinnervate tectal tissue in culture (Chen et al., 1996).

These methods for isolation and culture of adult neurons should facilitate new studies on the development and extent of plasticity of the adult brain. The yield of hippocampal cells, about 1.8 million/brain or 900 000 hippocampus is about 85% of the neurons present in a rat hippocampus (Boss et al., 1987). This should be sufficient for many cytochemical and electrophysiological studies. Use of larger volumes of cortical tissue should permit some biochemical and non-amplified molecular studies. Cultured adult neurons may be useful for neurotoxicology, pharmacology, and brain grafts.
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