Serum-Free B27/Neurobasal Medium Supports Differentiated Growth of Neurons From the Striatum, Substantia Nigra, Septum, Cerebral Cortex, Cerebellum, and Dentate Gyrus

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Two fundamental questions about neuron cell culture were addressed. Can one serum-free medium that was developed for optimum growth of hippocampal neurons support the growth of neurons from other regions of the brain? Is the region specific state of differentiation maintained in culture? To answer these questions, we isolated neurons from six other rat brain regions, placed them in culture in B27/Neurobasal™ defined medium, and analyzed their morphology and growth dependence on cell density after 4 days in culture. Neuronal identity was confirmed by immunostaining with antibodies to neurofilament 200. Neurons from each brain region maintained distinctive morphologies in culture in the virtual absence of glia. Cells isolated from embryonic day 18 cerebral cortex by digestion with papain showed the same high survival as hippocampal neurons, e.g., 70% survival for cells plated at 160/mm². At this age and density, neurons from the septum showed slightly lower survival, 45%. Survival of dentate granule neurons from postnatal day four brains was 30-40%, significantly lower, and relatively independent of plating density. This suggests an absence of dependence on trophic factors or contact for dentate granule neurons. Growth of cerebellar granule neurons isolated from postnatal day 7, 8, or 9 brains in B27/Neurobasal was compared to growth in BME/10% serum. Viability in serum-free medium at 4 days was much better than that in serum, did not require KCl elevated to 25 mM, and occurred without substantial growth of glia. Cerebellar granule neurons plated at 1,280 cells/mm² were maintained in culture for three weeks with 17% of the original cell density surviving. Survival of cells isolated from embryonic day 18 substantia nigra was 50% at 160 cells/mm² after 4 days, similar to that of striatum, but slightly less than hippocampal neuron survival. The dopaminergic phenotype of the substantia nigral neurons was maintained over 2 weeks in culture as judged by immunoreactivity with antibodies to tyrosine hydroxylase. During this time, immunoreactivity was found in the processes as they grew out from the soma. Together, these studies suggest that B27/Neurobasal will be a useful medium for maintaining the differentiated growth of neurons from many brain regions. Potential applications of a common growth medium for different neurons are discussed. © 1995 Wiley-Liss, Inc.

Key words: serum-free medium, culture, development, neuron

INTRODUCTION

Efforts to understand the basis for regional differences in brain neurons would benefit from the ability to grow isolated neurons in culture with common media conditions. Such comparative studies with neurons isolated from particular brain regions would require these neurons to grow by extending new processes and behave according to a characteristic differentiated state. Ultimately, neuron phenotype must depend on input and output connections, local adhesion, and trophic factors, but how much plasticity exists? Would dopaminergic neurons from the substantia nigra maintain their phenotype if grown in a medium optimized for hippocampal neurons, most of which do not produce dopamine transmitter, but rather communicate with glutamate? To answer these questions about phenotypic plasticity and to determine the range of neuron types that can be supported by one growth medium, we have examined the growth and a transmitter phenotype in a common growth medium, the newly developed serum-free Neurobasal™ supplemented with B27 (Brewer et al., 1993).

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attempts at comparative studies of neurons in serum containing media suffer from several disadvantages: lot variations in hormone and growth factor levels, variable proximity to co-cultured glia, ability to support growth only in the presence of a feeder layer of glia (Goslin and Banker, 1989), or only at very high cell densities (Aizenman and de Vellis, 1987). We have developed a serum-free defined medium supplement B27, together with improvements in the base medium, Neurobasal, derived from Dulbecco modified Eagle’s medium (DMEM), which were optimized for 4-day survival of hippocampal neurons at low density (Brewer et al., 1993). In comparison to DMEM, Neurobasal contains less NaCl and less NaHCO₃, resulting in lower osmolarity, and lower cysteine and glutamine, resulting in less proliferation of glia. Neurobasal also contains amino acids and a vitamin missing from DMEM: alanine, asparagine, proline, and vitamin B12. B27 contains optimized concentrations of the ingredients of Bottenstein’s N2 (insulin, transferrin, progesterone, putrescine, and selenium) along with the thyroid hormone T3, fatty acids, insulin, transferrin, progesterone, putrescine, and selenium) along with the thyroid hormone T3, fatty acids, vitamin E, and other anti-oxidants, for a total of 20 ingredients. The B27/Neurobasal combination produces more than 30 day survival of hippocampal neurons at densities down to 80 cells/mm².

In this paper, we compare the density-dependence of survival of neurons from other brain regions to that of hippocampal neurons. Low density cultures are useful for electrophysiology and morphological studies. Also, inferences about growth dependence on cell contact and autocrine factors can be made from comparisons of survival with high density cultures since low density cultures make many fewer contacts with other cells and secreted autocrine factors are diluted in the medium.

**MATERIALS AND METHODS**

**Isolation of Neurons**

Hippocampal, septal, substantia nigral, striatal, and cerebral cortical neurons were isolated from the brains of embryonic day 18 Sprague-Dawley rats (timed pregnant, Harlan Laboratories, Indianapolis, IN) as previously described (Brewer et al., 1993). Substantia nigral cells were obtained from the ventral mesencephalon according to the atlas of Specht et al. (1981b). Instead of mechanical disruption, dissected regions were treated with papain (Sigma, St. Louis, MO P4762) for 20 min, 30°C, 2 mg/ml in Neurobasal (GIBCO/Life Technologies 21003, Grand Island, NY). In order to maintain pH while working outside the incubator, Neurobasal was formulated with 4.4 mM sodium bicarbonate and additional 21.6 mM NaCl to maintain osmolarity (NBL). Tissue was transferred to 2 ml B27/NBL at room temperature and triturated 10–15 times through the fire-polished tip of a nine inch pasteur pipet. After undispersed tissue settled for 3 min, the supernatant was centrifuged 1 min, 200g. The pellet of cells was resuspended in Neurobasal (normal bicarbonate) supplemented with B27 (GIBCO/Life Technologies 17504). Viable cell concentration was determined by mixing an aliquot with trypsin blue and counting in a hemacytometer. Representative cell yields are shown in Table I.

Cerebellar granule neurons were isolated from cerebellum of day 8 neonatal rats (Wilkin et al., 1976). Neonates were anaesthetized on ice for 2 min before isolating the brain. Each cerebellum was placed in 4°C Hanks Balanced Salts (GIBCO/Life Technologies 14060, diluted to 1×) containing 1 mM pyruvate, 10 mM Hepes, pH 7.4 (HBSS), while meninges were removed. Tissue was chopped at 0.4 mm and again at 90°C using a McIlwain chopper and digested for 15 min at 37°C with gentle shaking in 2 ml 0.05% trypsin in T buffer (HBSS, 3 mg/ml bovine serum albumin). After rinsing with 2 ml quench (HBSS, 6.4 μg/ml DNAse, 0.4 mg/ml soy bean trypsin inhibitor; both Sigma), tissue was triturated as above in 2 ml quench. After addition of 2 ml more HBSS and settling of the large amount of nondispersed tissue, the supernatant was layered over 2.5 ml of 4% BSA in HBSS. The pellet of cells was resuspended in HBSS and counted as above. Aliquots were added to wells containing B27/Neurobasal or BME/10% fetal bovine serum (both GIBCO/Life Technologies 21010 and 16140, 2 mM glutamine). KCl (25 mM total) was added as indicated.

The lateral dentate gyrus was dissected as a strip with a single incision 1 mm from the hippocampal fissure after removing meninges from day 4 neonatal rat hippocampus. Dentate granule neurons were isolated with trypsin digestion similar to that described for cerebellar granule neurons.

**Cell Culture and Quantitation of Survival**

Substrates of either 24 well culture polystyrene (for viability studies; Corning, Rochester, NY) or 12 mm diameter glass cover slips (for immunocytology; German
Assistant from Carolina Biological) were coated for 1 hr or overnight with 50 μg/ml poly-D-lysine (Sigma P6407), aspirated, and allowed to dry. Cover slips were placed in wells for growth of neurons in the face up configuration. Wells were filled with 0.4 ml of 2% B27, 98% Neurobasal containing 0.5 mM glutamine, and 25 μM glutamate. Cells were plated at the indicated densities and incubated in a humidified atmosphere of 5% CO₂, 9% O₂, balance nitrogen at 37°C (Forma). After 4 days of growth, survival and viability were determined by staining with fluorescein diacetate and propidium iodide (Brewer et al., 1993). Statistical analyses used Student’s t test at each plating density with rejection of the null hypothesis at P < 0.05.

Semi-Quantitative Immunocytochemistry for Tyrosine Hydroxylase

Cells on slips were rinsed twice with HBSS and fixed for 30 min with 3.7% formaldehyde in PBS. After rinsing, slips were stored in PBS at 4°C until all samples were fixed. Slips were processed concurrently by blocking non-specific binding and permeabiling for 5 min with 1% BSA, 1% normal goat serum, 0.05% Triton-X100. Cells were reacted overnight at 4°C with rabbit anti-tyrosine hydroxylase (1:100 dilution in block; Chemicon, Temecula, CA). After rinsing, cells were reacted for 1 hr at room temperature with goat anti-rabbit IgG (Fab') conjugated to fluorescein (1:200 dilution; Tago 4320). After rinsing, slips were mounted on glass slides with Vectashield (Vector Labs, Burlingame, CA). Six fields were photographed through a 60 X/1.4n.a. Nikon objective, B1A dichroic mirror block, 0.5 neutral density excitation filter on Ektachrome ASA800 film. This permitted digitization of 30 to 90 cells. Cellular immunoreactivity was digitized with Bioquant software (R & M Systems) as previously described (Brewer and Ashford, 1992). Controls without primary antibody, or with an irrelevant primary antibody, were negative. For enhanced contrast, fluorescent cell images on Ektachrome film were projected directly onto photographic paper at identical exposures to produce comparable negative images (see Figs. 1, 5 and 6).

Neurofilament Immunoreactivity

Cells grown for 4 days were processed for immunocytochemistry similar to the above with the following exceptions. Fixative was freshly prepared 4% paraformaldehyde in PBS. Block was 5% normal goat serum, 0.05% Triton. Primary antibody was rabbit anti-neurofilament 200 IgG (dilution 1:50; Sigma N4142), reacted 1 hr at room temperature. Secondary antibody was either the fluorescein conjugate (diluted 1:500) or a peroxidase conjugate (Sigma, diluted 1:1000) and developed with 0.5 mg/ml diaminobenzidine, 0.01% H₂O₂ in 50 mM Tris-Cl, pH 7.2.

RESULTS

Substantia Nigra

The dopaminergic neurons of the substantia nigra are important to coordination of movement and emotions. Neuronal degeneration in this area, associated with Parkinson’s disease, results in loss of dopaminergic function (Cohen, 1983). Therefore, the ability to grow these cells in serum-free culture could provide a culture system for basic pharmacologic and physiologic studies. In preliminary studies, we isolated nigra neurons from rat embryos (18 days gestation), plated them at 400 cells/mm² in B27/Neurobasal, and grew them for 19 days. After loading with 1.2 mM dopamine for 3 hr, cells were examined for catecholamine fluorescence after reaction with glyoxylic acid (protocol VI; Bjorklund, 1983). The bright blue fluorescence indicated the presence of catecholamines and dependence on uptake of dopamine. Unfortunately, the drying required to prevent diffusion of catecholamine glyoxylate resulted in poor preservation of neuron morphology.

For greater specificity and better preservation of morphology, in subsequent studies to identify dopaminergic neurons, we used an antibody to tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. Figure 1 shows TH immunoreactivity in a developmental time series in culture. For cells plated at 640/mm², immunoreactivity was intense 1 day after isolation from embryonic day 18 brains (Fig. 1A). Processes are just beginning to sprout. About 80% of the cells were TH positive. By 4 days, extensive process growth has occurred with TH immunoreactivity present in these processes (Fig. 1B). Process growth continued through 8 days with some evidence of a redistribution of immunoreactivity into puncta (Fig. 1C). This distribution was not noticeably changed at 14 days (not shown). As a control, similar staining of hippocampal neurons after 5 days in culture revealed little TH immunoreactivity (Fig. 1D); these low levels probably represent non-specific staining, but to prove this, we would need to adsorb the antibody with purified TH, which is not available.

As a semi-quantitative measure of relative changes, TH immunofluorescence from 30 to 85 neurons per time point was digitized. Analysis of these measures confirmed the above impressions (Fig. 2). The mean immunofluorescent brightness of individual cells decreases with time in culture. This suggests a 46% decrease in TH concentration inside the cell. However, process growth creates more than a two-fold larger volume into which the enzyme can diffuse (assuming that the imaged volume scales linearly as a cross-section of a volume). Mul-
Fig. 1. Tyrosine hydroxylase immunoreactivity in substantia nigra neurons developing in culture in serum-free B27/Neurobasal. Cells were plated at 640/mm² and processed for immunocytoology at the indicated times: (A) 1 day in culture, (B) 4 days in culture, (C) 8 days in culture, and (D) TH negative hippocampal neurons after 5 days in culture. Arrows in (C) indicate punctate concentrations of immunoreactivity. The scale indicates 20 μm.
Fig. 2. Quantitative analysis of tyrosine hydroxylase immunoreactivity in substantia nigra neurons during growth in culture. Digitization of immunoreactivity in 30 to 85 neurons per time point indicates an initial increase in immunoreactive cell area during sprouting (Student’s t = 11.6, P < 0.001) followed by a plateau, while the concentration of immunoreactivity (brightness per cellular volume) steadily declines (linear regression, P < 0.001). Means ± SE.

tiplying area times mean brightness results in a peak of total fluorescence at 4 days, followed by a plateau from 8 to 14 days in culture. This suggests that TH is maintained for extended periods in culture and reaches a plateau of expression like that in the brain (Coyle and Axelrod, 1972).

The relative ability of substantia nigral neurons to survive in serum-free culture was compared to survival of striatal and hippocampal neurons. Because neurons are sensitive to unidentified trophic factors and possibly to the extent of contact and synapse formation, a comparison of density dependence on survival could reveal differences in the need for these factors. Figure 3A shows neuron survival after 4 days for neurons from the substantia nigra or striatum or hippocampus. Since survival is the live cell count divided by the total cell count (live + dead) and some dead neurons attach from the start, survival does not reach 100%, even at the highest plating densities where autocrine trophic factor concentrations and connectivity are highest. From the slope of density dependence, these neurons appear to be aided by some factors not present in the medium. However, the slope was similar to that of hippocampal neurons by linear regression analysis. Survival of neurons from substantia nigra and striatum was consistently inferior to that from the hippocampus.

Fig. 3. Density dependence of survival of neurons from the indicated brain regions compared to hippocampus after 4 days in culture. A: Substantia nigra and striatum neurons produced less survival than hippocampal neurons (*P < 0.02). B: Septal neurons produced lower survival than hippocampal neurons (*P < 0.01) while cortical neurons were similar to hippocampal neurons. C: Relative lack of density dependence of survival of dentate granule neurons from postnatal day 4 rat brains compared to hippocampal neurons from 18 day embryo brains (*P < 0.001). Means ± SE (n = 6 at each point).

Cerebral Cortex and Septum

Neurons of the septum, striatum, and cerebral cortex are more heterogeneous in their transmitter phenotype than those from the hippocampus. Less extensive
evaluations were made of cultures from these regions. Figure 3B shows that survival of neurons from the cerebral cortex is equivalent to that of hippocampal neurons. The concentration dependence of survival of septal neurons was slightly lower than with hippocampal neurons.

**Dentate Granule Neurons**

Some brain regions develop only after birth; neuroepithelial cells differentiate into neurons during postnatal development. We studied granule neurons from the cerebellum and dentate gyrus. Dentate granule neurons are best isolated from the hippocampal formation of postnatal day 4 rats (Boss et al., 1987). Few of these neurons have been born in the hippocampal formation at embryonic day 18 when pyramidal neurons are usually isolated. Figure 3C shows a relative lack of sensitivity of survival to plating density compared to hippocampal neurons isolated from 18 day embryos. Linear regression analysis indicates a slope of zero. This suggests less dependence on cellular contact and trophic support for survival and differentiation of dentate granule neurons.

**Cerebellar Granule Neurons**

Cerebellar granule neurons are grown most frequently in Basal Medium Eagle (BME) with 10% fetal bovine serum and 25 mM KCl to induce differentiation and promote survival (Balazs et al., 1988). To test the feasibility of serum-free medium, we compared survival of these neurons in serum-containing medium to that in B27/Neurobasal. Figure 4 shows a large improvement in viability of cerebellar granule neurons grown for 4 days in B27/Neurobasal in comparison to BME/serum, both with 25 mM KCl. In normal B27/Neurobasal containing 5 mM KCl, viable cell counts were not significantly different from medium containing 25 mM KCl. A denser neuropil after 4 days in culture suggests that the rate of process extension in B27/Neurobasal was higher than in BME/serum (compare Fig. 5A with 5B). This suggests that specific components in B27 are providing a signal for differentiation that is provided in serum-containing medium by depolarization. There is a sharp decline in viabilities at lower densities of 80 and 160 cells/mm². At these densities few cells are in contact. These results suggest that intercellular contact is needed for survival. They also suggest that autocrine trophic factors that would accumulate at higher densities may aid survival of cerebellar granule neurons.

Qualitative Morphologic Comparisons

The relatively long processes with limited branching of cerebellar granule neurons (Fig. 5C) are in contrast to substantia nigral, septal, hippocampal pyramidal and dentate granule neurons (Fig. 6). The somae of substantia nigra are larger and hippocampal neurons are even larger (Fig. 6A,C). Nigral neurons also produce large tapering dendrites with extensive branching, typical of those in the brain (Fallon and Loughlin, 1985). Septal neurons are characterized by multiple processes emanating from a large ovoid soma (Fig. 6B). There is less branching of these processes, similar to processes in the brain (Shingai et al., 1990). The growth cones of the hippocampal neurons spread over large areas in B27/Neurobasal medium on polylysine substrates (Fig. 6C). Highly branched basi-
Fig. 5. Growth of cerebellar granule neurons in (A) serum-containing or (B) serum-free medium for 8 days after plating at 1,280 cells/mm². Protein is stained with Coomassie blue. Arrows indicate soma of cerebellar granule neurons; arrowheads indicate glia, which are numerous in serum-containing medium and greatly diminished in serum-free B27/Neurobasal. (C) Neurofilament 200 immunoreactivity of 23 day culture revealed with peroxidase and DAB as chromogen. Light colored large flat glia are non-immunoreactive. The scale indicates 50 μm.

lar dendrites, a large tapering and branched apical dendrite, and uniform calibre axons resemble those in vivo (Banker and Cowan, 1979), especially if grown in culture for more than 6 days (Brewer et al., 1993). The small size and spherical shape of the cerebellar granule and dentate granule neurons (Fig. 6D) are maintained. The dentate granule neurons in culture morphologically resemble adult granule neurons in their numerous long dendritic processes emanating from one or more sharply tapering sprouts from a small spherical soma (Seress and Pokorny, 1981). In summary, neurons isolated from different brain regions produced distinctive morphologies in culture, not unlike those produced in vivo.

DISCUSSION

Good growth of septal, cortical, striatal, substantia nigral, cerebellar granule, and dentate granule neurons in one common serum-free medium suggests that these neurons are not entirely dependent on trophic support for survival and neuritogenesis. The high yields suggest that cells that have processes removed during trituration can regenerate processes in B27/Neurobasal. It is surprising that vigorous growth occurs without growth factors other than insulin. Reports of great efficacy of growth factors are obtained with cells grown in defined media with many fewer components than B27 (Ip et al., 1993; Ohsawa et al., 1993). This suggests that growth factors can increase survival of neurons that are stressed or malnourished. Attempts to augment growth in B27 with NGF, β-FGF, NT3, and CNTF have shown no obvious changes in rate of sprouting, process elongation, branching, or survival (Brewer, unpublished).

What does this growth in a common medium imply about the process of differentiation? Does a common medium cause neurons to differentiate into a common phenotype? Our studies with substantia nigral neurons suggest that differentiation is programmed earlier than day 18 and continues in culture. The developmental sequence of tyrosine hydroxylase expression and distribution in neurons from substantia nigra suggests that they had developed this differentiated expression at the time of isolation and that it continued in culture. These cells were already making far more tyrosine hydroxylase than hippocampal neurons and they distributed the enzyme into processes as neuritogenesis proceeded. These in vitro findings agree with the ontogeny in the rat embryo where TH is first seen after 12.5 days gestation (Specht et al., 1981a) and reaches a peak of immunoreactivity after day 18 (Specht et al., 1981b), when TH started to disseminate into dendrites and axons. These processes are removed during isolation by our techniques but regrow rapidly. In culture, total TH immunoreactivity
Fig. 6. Morphologic comparisons after 4 days of culture in B27/Neurobasal of neurons from (A) substantia nigra, (B) septum, (C) hippocampus, and (D) dentate gyrus. Immunoreactivity to neurofilament is seen in A, B, and C. Protein is stained with Coomassie blue in D. Cells were plated at 320/mm² except for hippocampus which was plated at 160/mm². Note the differences in size of the somae, the width, taper, number and branching of the processes, and the sizes of growth cones. For the hippocampus, arrows indicate apparent axons and arrowheads indicate apparent dendrites. The scale indicates 20 μm.
reached a peak at 4 days with rather uniform distribution into processes. The continued detection of TH immunoreactivity through the seventh day, with little change through the 14th day suggests that expression is continuing, although we can not rule out the possibility that previously synthesized TH is being redistributed with no new expression. The latter would require unusually low turnover of protein and message over a 2 week period. Other autocrine or paracrine factors may be needed to maintain expression, as suggested by the density dependence of survival. In support of this possibility, Magal et al. (1993) have demonstrated enhanced expression of TH in substantia nigra neurons cultured in a serum-containing medium after exposure to CNTF and dopamine. Overall, these results support the hypothesis that differentiation of neurons was committed in vivo and is maintained in our serum-free culture medium.

Other evidence supports the hypothesis that appropriate phenotypes are obtained from different brain regions grown in B27/Neurobasal. The density dependence of growth of substantia nigra, striatal, and cerebellar granule neurons is greater than that of hippocampal neurons. This is not surprising since the ingredients in B27/Neurobasal were optimized for growth of hippocampal neurons. The lack of density dependence of dentate granule neurons suggests a differentiated state in which process growth and survival are not dependent on contact or autocrine factors. This does not exclude an influence of other cells on development. The different growth responses suggest that each brain region may require distinct optimal concentrations of components of B27 or Neurobasal or that other factors, possibly recognized growth factors, may be required for better growth. Alternatively, neurons in these regions could have been programmed for apoptotic death before isolation (Sloviter et al., 1993). This would preclude rescue by any growth medium.

Despite the ability of neurons from these regions to differentiate in culture, we have not studied as a function of time in culture possible changes in representation of different cell types. Nor have we studied changes in specific receptor or transmitter types that may be influenced by glia (Qian et al., 1992) or local concentrations of transmitter (Brewer and Cotman, 1989).

The ability to grow neurons from the seven brain regions reported here suggests that other regions may be able to grow in B27/Neurobasal. The long-term growth (>2 weeks) of cerebellar and substantia nigra neurons reported here and of hippocampal neurons reported previously (>8 weeks) suggest that studies of many brain regions will be possible in culture for extended periods of time. The ability to culture different neurons with the same medium should facilitate comparative pharmacological and electrophysiological studies. It will enable rapid screening of pharmacological agents for effects on specific brain regions of interest while minimizing detrimental side-effects on other areas. Culture of substantia nigral neurons may benefit studies of Parkinson's disease. Crafts to repair specific damaged brain regions may be aided by these methods, especially if neurons can be isolated and grown from fully differentiated adult brain surgical tissue (Brewer, in preparation).

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