A MICROMASS CULTURE METHOD FOR RAT EMBRYONIC NEURAL CELLS

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SUMMARY
A method of culturing early (13-day) rat embryo neural cells is described. Undifferentiated neural epithelium is disaggregated and cultured in small discrete islands. Cells that are destined to differentiate as neurons actively segregate from the other cells in the island and aggregate together into small clumps. Other cells flatten and attach to the substrate and resemble typical fibroblasts throughout the culture period. The clumps of preneuron cells spread out forming large irregular foci. Spreading is mediated by active cell movements. Cells in the foci differentiate as a pure population of neurons identifiable by specific inhibition of $\text{H}^\text{3}$-labelled $\gamma$-amino butyric acid incorporation or by labelling with a monoclonal antibody to GQ-ganglioside. The ganglioside is not found on the cell surface at the start of culture after trypsinization, but emerges during the 5 days of culture. The antigen is similarly not present in the embryonic mesencephalon in vivo at 13 days post coitum, only emerging later in the differentiated midbrain. There is thus an apparent de novo synthesis, which is paralleled in vivo and in vitro.

INTRODUCTION
Cultures of neural tissues for pharmacological or electrophysiological investigations are normally prepared by dissociating differentiated neurons and glial cells from foetal rat brain (Giacobini, 1980; Nelson & Bergey, 1980). Neurons divide slowly and are soon overgrown by the fast-dividing fibroblast-like glial cells. This problem has been solved in a number of ways by growth on poly-L-lysine-coated culture dishes, which favour neuronal adhesion (Pettmann, Louis & Sensenbrenner, 1979); by the use of inhibitors of rapid cell growth such as fluorodeoxuridine (Dimpfel, 1980), or with serum-poor culture medium, which favours more slowly growing cells (Fedoroff & Hall, 1979). Another problem has been dedifferentiation of the differentiated neuron after separation from its tissue and a short period of culture (Giacobini, 1980). A similar problem has been observed with cultured chondrocytes (Holtzer, Abbott, Lash & Holtzer, 1980). This may be a general problem with cultures of differentiated cells. The aim of the present work was to obtain cultures of neurons capable of achieving and maintaining a significant degree of differentiation without the need to modify culture conditions to encourage their growth. We have designed a technique that achieves this aim in which embryonic rat neural epithelium from a stage before the appearance of neurons is cultured so that differentiation of the neurons takes place in culture.

Embryonic cells can be made to differentiate in culture, but often only under conditions of fairly high density (Umansky, 1966). One way of optimizing the number
of high-density cultures obtained from a small quantity of embryonic tissue has been the micromass technique developed by Ahrens, Solursh & Reiter (1977). A modification for use with embryonic neural tissue is described in this paper. One advantage of this technique is that a homogeneous set of cultures is produced in which differentiation may be followed quantitatively by biochemical or morphometric analysis (Hassell, Pennypacker & Lewis, 1978; Flint, 1980).

MATERIALS AND METHODS

Adult virgin albino rats of the Alderley Park strain were mated and vaginal smears were examined to confirm copulation. Pregnant females were killed at 13 days post coitum (p.c.) and embryos removed under sterile conditions. Cultures were prepared essentially as described by Ahrens et al. (1977) and Ede & Flint (1975). Embryos (34–35 somite stage) were submerged in a warm (37°C) mixture of horse serum (HS) and Earle’s Balanced Salt Solution (EBSS) in the proportion EBSS: HS of 1:1 (v/v). Mesencephalon (excluding metencephalon and telencephalon) together with overlying fibroblasts and epithelium were cut away from the rest of the head (Fig. 1).

Tissues were pooled from a number of embryos, washed three times with calcium- and magnesium-free EBSS (CMF-Flow Laboratories, Irving, Scotland) followed by incubation at 37°C in CMF for 20 min. CMF was exchanged for 1% trypsin (DIFCO, 1:250) in CMF and the tissue was then briefly washed in CMF and resuspended in culture medium (Ham’s F12 + 10% foetal calf serum (FCS); L-glutamine, 584.6 mg/l; penicillin, 10 000 000 i.u./l; streptomycin, 100 mg/l, Flow Laboratories, Scotland). Mechanical dissociation was carried out by repeated flushing of the suspension through a 0.7 mm bore glass pipette. A single cell suspension was ensured by passing the cells through a sterile 10 µm nylon mesh (Henry Simon, Stockport). The cells were isolated by centrifugation (1000 rev./min for 3 min) and finally resuspended in sufficient medium to give 1 X 10^5 cells per 20 µl. Five 20 µl samples of this suspension were normally delivered to each 30 mm plastic Petri dish (Falcon, 3001F). After 2 h incubation at 37°C each dish was filled with culture medium to a final volume of 2 ml. At this time, most cells had adhered to the substrate thus resulting in separate 6–7 mm cell islands. During this period, the culture medium was not changed and no overgrowth of one cell island by another was observed. The cultures were incubated for 5 days at 37°C and 100% humidity in an atmosphere of 5% CO₂, 95% air.

Histological preparation

Permanent preparations were made of some cultures by fixation in formol saline followed by washing and brief staining with haematoxylin. Axons and neurofibrils were specifically stained by Bielschowsky’s silver method (Drury & Wallington, 1967). For electron microscopic examination, cultures were grown on 13 mm glass coverslips to which cells had been attached by the method described above. The cultures were fixed with glutaraldehyde (20 min) in 0.1 M-cacodylate buffer made isosmotic with culture medium using a vapour pressure osmometer (Wescor Inc., Utah, U.S.A.). Post-fixation was achieved with Caulfield’s osmium fixative for 20 min (Caulfield, 1957). After dehydration through a graded acetone series, coverslips were dried in a critical-point drier (Polaron Equipment Ltd, Watford), coated with gold in a vacuum coating unit (Edward’s 306, Crawley, Sussex). Cultures were examined with a scanning electron microscope (Jeol JSM 1, Jeol House, London).

Estimation of cell population and total protein content

Cultures were washed carefully twice with EBSS (without phenol red) and then covered with 1 ml 0.5% EDTA in CMF for 20 min at 37°C. Cells still attached to the substrate were detached mechanically and the complete suspension was removed to a test tube where cells were dissociated by aspirating through a 0.7 mm bore pipette. The cell population was estimated from 20 µl samples using a Fuch’s Rosenthal haemocytometer (Gallenkamp). The cells were isolated by centrifugation (3500 rev./min for 5 min) and protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard.
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$^3$H-labelled γ-amino butyric acid uptake

The cultures were exposed to $^3$H-labelled γ-amino butyric acid ($[^3H]$GABA, 50 Ci/mmol) at final concentrations of 1 μCi/ml medium for 1 h. The uptake of radioactivity was assessed by detachment of the cells from the dish, solubilization in Soluene-350 (1 ml) (Packard) and liquid scintillation counting in Dilmulume-30 (10 ml) (Packard). For demonstration of uptake by micro-autoradiography, cells were grown on washed and sterilized glass microscope slides, fixed in glutaraldehyde and coated with Ilford K5 emulsion essentially as described by Marshall, Lamey & Ferguson (1980). The specific inhibitors of neuronal glial and GABA incorporation, β alanine (Aldrich, Gillingham, Kent) and cis-1,3-aminoacyclohexane carboxylic acid (ACHC; Dr N. Bowery, St Thomas’s Hospital), respectively, were included in the culture medium at concentrations of 1 mM.

Inhibition of cell movement

Cultures were treated with cytochalasin B (final concentration 0·01 to 10 μg/ml culture medium added in dimethylsulphoxide).

Quantification of differentiation

Foci of neurons in each cell island were stained with haematoxylin and counted with an Artek 980 colony counter, modified for use with a dissecting microscope. Each focus of neuronal cells in a cell island (see Fig. 2d) scored a number of spots on the television monitor. Spot number per cell island correlated well with differentiated area ($r = 0·98$, $P < 0·01$).

Immunofluorescence determination of neuronal cell-surface ganglioside

Cells were grown as before on glass coverslips, fixed in isosmotic glutaraldehyde and then stained according to the double-layer technique described by Goldman (1968). One drop of 1:40 dilution of mouse monoclonal antibody to GQ-ganglioside (MAS045-Sera Labs., Crawley Down, Sussex) was layered over the cells and incubated for 1 h at room temperature (or overnight at 4 °C in a moisture chamber). The slide was washed three times with fresh phosphate-buffered saline (PBS) and excess fluid was removed. A drop of a 1:40 dilution of anti-mouse immunoglobulin G (IgG)$_i$ + 2-fluorescein isothyocyanate (FITC) conjugate (Meloy-GIBCO Biolcult Ltd, Paisley, Scotland) was layered over the cells. The slides were left for 30–60 min in a moisture chamber at room temperature. They were then washed three times with PBS, mounted in PBS/glycerol (50:50, v/v) and examined with a Leitz Ortholux fluorescence microscope (E. Leitz Instruments, Luton). Control slides were taken through the same procedure except the antibody was omitted.

Cells were also analysed by indirect ELISA (enzyme-linked immunosorbent assay). Cells were grown as already described, but in 200 μl well microtitre culture dishes (Flow), fixed with isosmotic glutaraldehyde and washed with tap water. The cells were overlayed with 100 μl of a 1:1000 dilution of monoclonal antibody and incubated for 2 h at room temperature. The microtitre plates were then rewashed and overlayed with a 1:1000 dilution of rabbit anti-mouse IgG antibody labelled with peroxidase (Nordic Labs., Maidenhead, Berks) for 12 h at room temperature. After a further wash, 200 μl of an orthophenylenediamine/urea peroxide mixture (0·4 μg; 0·23 μg/ml) was added to each well and the enzyme reaction allowed to proceed for approximately 45 min in the dark. The reaction was stopped by the addition of 50 μl of 25 % sulphuric acid. The plates were read on a Multiskan Multispectrophotometer (Flow). Controls in which one or other of the reagents were omitted or were included. A negative control was also employed using another monoclonal antibody to dinitrophenol (MOPC315, originally from Glasgow University Immunology Department).

RESULTS

Description of culture development

A 1 μm section through 13-day rat embryonic mesencaphalon (Fig. 1) had the typical appearance of a pseudo-stratified epithelium with dividing cells adjacent to the
0. P. Flint

Fig. 1. A. Dissected mesencephalon prior to culture. Bar, 400 μm. B. Transverse section of mesencephalon from 34 somite rat embryo (1 μm plastic section). ep, epithelium; pm, primary cranial mesenchyme; nep, neuroepithelium (pseudostratified). Bar, 100 μm.

Lumen of the neural tube. Outside the neuroepithelium is the very sparse cranial mesenchyme and a thin sparsely populated external epithelium. The cells cultured were therefore mainly neuroepithelium (no differentiated neuronal or glial cells) with some fibroblasts and a few epithelial cells. No epithelial cells were seen to attach to the substrate.

Immediately after disaggregation, the cell suspension was mainly composed of single cells (Fig. 2a). One hour later, the cells began to aggregate together into small clumps (Fig. 2b). Between the clumps, a number of rounded single cells remained. After 7 h, the clumps increased in size and the unaggregated cells remaining between clumps began to adhere to the substrate and flatten out (Fig. 2c). At 24 h, the clumps were larger and still distinct but the spaces between were filled with flattened fibroblast-like cells (Fig. 2d). After 5 days, the clumps had spread and flattened, forming small irregular foci within the micromass island (Fig. 3a, b). Focus size was
Fig. 2. Differential contrast micrographs of living mesencephalon cultures at the start of culture: 0 h (a); 1 h (b); 7 h (c) and 24 h (d) later. Bar, 50 μm.
variable within each micromass island, larger foci generally forming towards the centre of each island. When the micromass islands were stained with silver, which is specific for neurons or neuronal filaments (Fig. 3A), the foci stained most intensely. Where foci were at their most dense, the intercolony regions also stained, but weakly.

In living cultures, foci appeared to be slightly raised in differential interference contrast microscopy (Fig. 4A). Networks of attenuated cell projections emerging from each focus lay on top of a background of undefined fibroblast-like cells. The fibres often crossed between closely adjacent foci. Occasionally, the fibres bundled together.
Fig. 4. Morphology of neuronal cells in CNS cultures. A, B. Photographs of living cultures by differential contrast micrography. Bars, 25 μm. C, D. The regions in the squares in A and B are shown by scanning electron micrography after fixation and critical-point drying of the cultures. Bars, 5 μm.
and crossed large tracts of otherwise featureless fibroblast-like cells (Fig. 4B, D). Under reflection contrast microscopy, the fibres and fibre bundles did not appear to make close contacts typical of cell–cell adhesions with underlying cells, except at their tips. Within the colonies there was a very high density of long narrow cell projections as well as many cell bodies (Fig. 4C).

The morphological changes described did not take place when densities at the start of culture were lower than $1 \times 10^5$ cells per $20 \mu l$.

**Growth of the micromass islands**

Total protein and cell number per micromass island, as measures of culture growth, increased linearly between days 1 and 5 (Fig. 5A, B).

**Uptake of $[^3H]GABA$**

*Liquid scintillation counting.* No significant uptake of $[^3H]GABA$ occurred on day 1 of culture. In 5 day cultures there was a marked level of $[^3H]GABA$ uptake (Table 1). $[^3H]GABA$ uptake was unaffected by $\beta$-alanine, but was inhibited (24% control value) by ACHC.

![Graphs showing daily increase in protein and cell number per cell island.](image)
Table 1. $[^3H]$GABA uptake into 5 day central nervous system (CNS) cultures

<table>
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<tr>
<th>Culture type</th>
<th>$[^3H]$GABA uptake (d.p.m./µg protein)</th>
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<tr>
<td>Control</td>
<td>9295 ± 2002-01*</td>
</tr>
<tr>
<td>+ 1 mM β-alanine</td>
<td>8992 ± 1309-62</td>
</tr>
<tr>
<td>+ 1 mM ACHC</td>
<td>2211 ± 124-56†</td>
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* Values are the mean and s.d. of 8-cell islands.
† Significantly different from control ($P < 0.001$, analysis of variance).

Fig. 6. Micro-autoradiograms of cell cultures after $[^3H]$GABA incorporation. A, Within the foci; B, at the periphery of one of the foci. Grain counts above the interfocal cells (a) were not above background levels. Bars, 10 µm.
Fig. 7. Indirect immunofluorescent studies with anti-GQ-ganglioside (MAS045). Concentrations of antibody are as described in Materials and Methods. A phase-contrast micrograph of a 5 day culture fixed with glutaraldehyde is shown in A and the same field, at slightly higher magnification, is shown in B, revealing that the fluorescence is only associated with cells of differentiated neuronal morphology. Frozen, unfixed sections of 13 day embryonic rat mesencephalon did not bind the GQ-ganglioside (D). Sections from older 18 day embryonic mesencephalon did bind the anti-GQ-ganglioside (F). Sections of the mid-brain stained with haematoxylin and eosin (C, E) are shown for comparison with those processed for indirect immunofluorescence (D, F). Bars: A, B, 25 μm; C, D, E, F, 100 μm.
Microautoradiography. After [3H]GABA exposure, a very high density of grains were observed in 5 day cultures over the foci and over most of the long cell extensions crossing the areas between colonies (Fig. 6A, B). Out of 183 such extensions scored, 55% were so densely coated with silver grains that each grain touched the next. Grains were more sparingly scattered along the cell extension in 32% and no grains were found in 13%. Grain counts over the cells lying between colonies did not rise above background levels.

Formation of GQ-ganglioside on neuronal cell surface

The mouse IgG antibody against neuron cell antigen (MAS045) binds exclusively to a GQ-ganglioside found on the plasma membrane of neurons (Eisenbarth, Walsh & Nirenberg, 1979) and transformed neurons–human neuroblastoma cell lines (Kennet & Gilbert, 1979). On day 5 of culture using indirect immunofluorescence, antibody bound to the surface of the neuron-like cells and not to the fibroblast-like cells (Fig. 7A, B).

Quantification of antibody binding by the ELISA technique indicated no binding at 2 h after the start of culture but an essentially linear increase over the following 5 days. Anti-dinitrophenol (MOPC315) did not bind to the 5 day cultures (Fig. 8). Using indirect immunofluorescence, the antibody did not bind to 13 day embryo mesencephalon (which is essentially pseudo-stratified neuroepithelium (Fig. 7c, d)), but did bind to 18 day mesencephalon (Fig. 7e, f).

Effect of cytochalasin B on culture development

Cytochalasin B was added to cultures either with the initial medium (day 0), or on days 1, 2, or 3.
An exponential concentration-dependent inhibition of differentiation was found whether cytochalasin B was added at the start or any day of culture thereafter (Fig. 9). Estimates of the concentration of cytochalasin B inhibiting differentiation by 50% (ID$_{50}$) showed that cultures are far more sensitive to the action of this compound on days 0 and 2 than on days 1 and 3 of culture (Fig. 9).

**DISCUSSION**

The growth of each cell island in terms of protein content and cell number was essentially linear between days 1 and 5. Cell growth within the micromass cell islands, during the 5 days of culture, is therefore similar to the log phase of growth observed in more conventional cultures prior to contact inhibition at confluence (Levine, Becker, Boone & Eagle, 1965; Ceccarini & Eagle, 1971; Sefton & Rubin, 1970). In addition to cell multiplication and growth, three other events were noted within the cell islands: (1) active aggregation of cells into small discrete masses; (2) active spreading of these aggregates into larger but still discrete foci; and (3) differentiation of cells within the foci to form neurons.

The small aggregates of cells formed on day 1 of culture were later replaced by flattened foci containing neurons (see below). Cytochalasin B at low concentrations (approx. 0.5 μg/ml) has been shown to disrupt reversibly the cytoskeletal microfilaments involved in active cell movement (Yamada, Spooner & Wessels, 1970; Spooner, Yamada & Wessells, 1971; Carter, 1972). The action of cytochalasin B at different stages of neural culture development showed that active cell movement was involved in the formation of both the aggregates and in spreading.

The aggregation phase up to the end of the first 24 h in culture coincided with a high sensitivity to the action of cytochalasin B, with an ID$_{50}$ of 0.83 μg/ml (Fig. 9). This
value is within the narrow range of those concentrations (approx. 0.5–1.5 µg/ml) normally associated with the reversible inhibition of movement in cultured cells (Carter, 1972). On day 1 cultures were less sensitive, entering a quiescent phase where differentiation was only inhibited by concentrations within the normally cytotoxic range of cytochalasin B concentrations (Carter, 1972). During day 2 of culture and the phase of aggregate spreading, cultures were again sensitive to those low concentrations of cytochalasin B that normally reversibly inhibit cell movement (Carter, 1972). After spreading of the aggregates the cultures were again very refractory to the action of cytochalasin B except at cytotoxic concentrations. Aggregation of cultured embryonic mouse cerebellar and midbrain cells has previously been reported (Hatten & Sidman, 1978), but was not followed beyond the aggregation phase.

The darkly staining foci (Fig. 3) seen on day 5 of culture were the end result of spreading of the aggregated cell clusters. The evidence clearly suggests that the foci consist of differentiated neurons and that outside the foci only fibroblast-like cells can be found attached to the culture dish. First, silver staining (Fig. 3A) suggested that the foci contained differentiated neural cells. Second, cell morphology suggested typical neurons (Fig. 4). Some of the cell extensions, leaving the foci and lying loosely on the intervening fibroblasts, were combined into structures similar to embryonic nerves (Fig. 4D). Staining with axonal- and neurofibril-specific stain was largely restricted to the foci, but where these were closest there was some intervening staining probably connected with the network of cell extensions crossing between foci (Fig. 3A). Third, the focal cells bound antibody against neuronal GQ-ganglioside and, fourth, specifically incorporated [3H]GABA, illustrated qualitatively by microautoradiography and quantitatively by liquid scintillation counting. None of the interfocal cells incorporated [3H]GABA, whereas 88% of those cells with neuronal morphology did. None of the interfocal cells bound anti-GQ-ganglioside.

Hosli & Hosli (1980) and Hosli, Andres & Hosli (1980) have shown that neuronal cells, but not glial cells, bind GABA in culture. Hauser & Bernasconi (1980) and Hauser, Balcar & Bernasconi (1980) have also shown that GABA incorporation and GABA-transaminase activity is largely restricted to neurons in mixed cultures of neurons and glial cells. Inhibition of [3H]GABA uptake by cis-1,3-aminocyclohexane carboxylic acid, a specific inhibitor of neuronal GABA uptake (Bowery, Jones & Neal, 1976), and not by β-alanine, an inhibitor of glial GABA incorporation (Schon & Kelly, 1974), confirmed that GABA incorporation in these cultures is restricted to neuronal cells and that glial cells have not, as yet, differentiated. As 12% of the neuronal cells did not incorporate GABA or choline, it is possible that other neurotransmitters are incorporated into this population of cells.

GABA is an inhibitory substance in the invertebrate and vertebrate nervous system (Iversen & Kelly, 1975). GABA incorporation has been shown to be associated with the differentiation of neurons, particularly with respect to synaptogenesis (Wolff, Rickman & Chronwall, 1979; Crain & Bornstein, 1979). This latter aspect may be a reason for its incorporation into the differentiating focal neurons.

Although the aggregated cells later differentiated as neurons, three factors marking these cells as neurons were not present at the start of culture: (1) morphologically, the
cell population introduced into culture consisted of the undifferentiated pseudo-stratified neuroepithelium plus some fibroblasts on the outside of the neural tube (Fig. 1). These fibroblasts were derived from the primary embryonic mesenchyme (Johnston, 1966) unlike much of the cranial mesenchyme, which is of neural crest origin; (2) $[^3]H$GABA uptake, a marker of the differentiated neuron, was very low on day 1 of culture; (3) anti-GQ-ganglioside did not bind to any cells, including the aggregates that were forming as early as 1–2 h after the start of culture (Fig. 8). It should be noted that the increase in anti-GQ-ganglioside binding over 5 days of culture simply parallels the increase in cell number. It is only the cells that start the cultures that do not bind antibody at all. The GQ-ganglioside was also not detected in intact 13 day embryos (Fig. 7). The hypothesis that trypsinization removed this antigenic determinant from cells already differentiated (in this respect) as neurons, can be excluded.

The active aggregation of cells into clusters that later differentiated as neurons, while the surrounding cells remained apparently undifferentiated, can only be interpreted as a sorting out of one cell type from another. Hatten & Sidman (1978) did not observe sorting out of non-neuron from pre-neuronal cells, probably because they used a lower cell density and a different culture technique, which cannot reveal sorting out. Such a phenomenon was classically observed by Steinberg (1970) in rotation cultured aggregates of mixed cell populations and later in monolayer cultures of liver cells (Garrod & Steinberg, 1973). Active sorting out by mutual recognition suggests an embryonic antigen or antigens, distinct from the GQ-ganglioside, which label future neurons at the earliest stages of neural tube formation.

The formation of neurons, the binding of neural cell projections (axons) into nerve-like structures (Fig. 2), the synthesis of neuronal cell antigen and $[^3]H$GABA uptake studies confirm that the micromass culture system may be a very good model of in vivo neuronal differentiation. Another advantage is that, in terms of the amount of neuronal differentiation, there are only small differences between one cell island and the next within one batch of cultures (Flint, unpublished observation). The micromass technique optimizes the number of homogeneous cell cultures that could be generated from a limited pool of embryonic cells. The effects of various agents, such as cytochalasin B, on differentiation within these cell islands can be quantitated.

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