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Slice cultures of cerebellar, hippocampal and hypothalamic tissue

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Summary. Cerebellar, hippocampal and hypothalamic slices prepared from newborn and 7-day-old rats were cultured by means of the roller-tube technique. Identification of cells was made easier by the fact that at least part of the characteristic cytoarchitecture of the tissue was preserved in vitro. Cerebellar Purkinje cells and neurones of the deep cerebellar nuclei were recognized on the basis of their size, their location within the culture and their dendritic arborization. Pyramidal cells of all hippocampal subfields displayed their characteristic basal and apical dendritic trees with numerous spinous processes. Hippocampal granule cells gave rise to a monopolar dendritic arbor; their axons terminated in the dentate hilus and CA3 region. Golgi-like immuni-peroxidase staining allowed localization of groups of neurophysin-positive neurones in slices prepared from the anterior hypothalamus. These neurones, bilaterally bordering the third ventricle, usually displayed a simple dendritic arborization and fine beaded axons. – Cultivation of brain slices prepared from young rats offers particular advantages in that the cultured cells are organized in an organotypic monolayer and individual living neurones may be directly visualized.

Introduction

Slices and cultures of dissociated cells are clearly the predominant in vitro preparations for pharmacological, electrophysiological and morphological studies on vertebrate brain tissue. Slices offer the advantage of retaining the normal anatomical neuronal relationships, but because of their thickness do not usually allow visualization of single living neurones. Nerve cells in cultures of dissociated cells, on the other hand, can be continuously observed and are highly accessible to exploration with microelectrodes, but often suffer from insufficient cellular differentiation and organotypic tissue organization. I have, therefore, attempted to combine the advantages of the slice and the culture methods by culturing slices from young animals. Such a preparation may be useful for all those studies which require a higher degree of cellular accessibility than that provided by the usual slices. Moreover, it may be the method of choice for investigations where no advantage is gained by first dissociating the cellular elements only to let them reassemble again.

The technique for cultivation of explants by means of the roller-tube method has been previously described[17]. The present paper focuses on the organotypic organization of cultured tissue derived from three brain regions as demonstrated by several histological staining techniques.

Experimental procedures

As a rule, we have used 7-day-old rats for the cultivation of hippocampal and hypothalamic tissue and newborn rats for the preparation of cerebellar cultures. Cultivation of tissue derived from older animals was possible, but complicated by the necessity to oxygenate continuously all media used. Animals were killed by decapitation, the skull opened with fine scissors and the cerebellum and hippocampi aseptically removed (fig. 1). After washing in Geys balanced salt solution (BSS), they were placed on a dry Aclar foil (Allied Chemical) and parasagitally cut into 400-µm-thick slices by means of a McIlwain tissue sectioner. The cerebellum and the hippocampi were then transferred by spatula into a petri dish containing Geys BSS, where the individual slices started to float. For the preparation of hypothalamic cultures, the hypothalamus was first isolated on all 4 sides in situ by cuts with a razor blade (left and right, anterior and posterior border), then it was undercut at a depth of about 1.5 mm and the entire hypothalamus removed en bloc. After washing in Geys BSS, the hypothalamus was placed on a dry Aclar foil, the ventral surface facing upwards, and cut with the tissue sectioner into 9 slices of 400 µm thickness starting from the preoptic area, up to the area including the mamillary bodies. Each slice was placed on a separate coverslip and em-

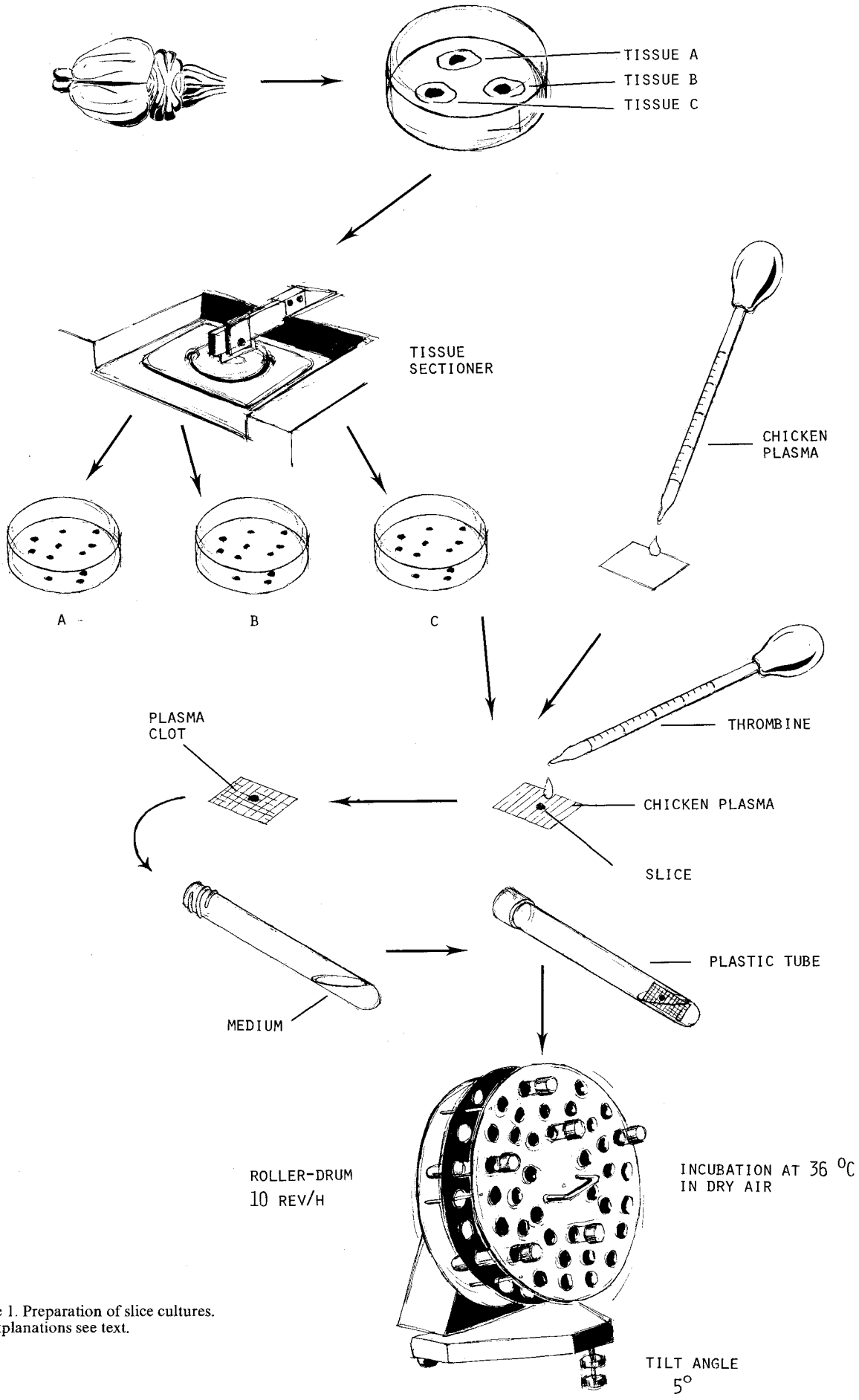


Figure 1. Preparation of slice cultures.
For explanations see text.

bedded in a plasma clot formed by a drop of heparinized chicken plasma (Difco, lyophilized) which had been spread over the entire surface of a glass coverslip and which was coagulated by addition of a drop of thrombin solution (0.2 mg/ml, 20 NIHU/ml, Hoffmann-La Roche). The rectangular coverslips (12 × 24 mm) were previously cleaned as described[17]. Due to the release of proteolytic enzymes, presumably from damaged cells, the plasma was soon lysed in an area immediately surrounding the cultures. With single cultures, this lysis only occasionally led to detachment of parts of the culture, but with co-cultures, where more tissue covered a greater surface area, problems were more severe. We have, therefore, occasionally embedded the co-cultures on coverslips coated with reconstituted rat collagen[11,34].

The slices were cultivated in plastic test tubes (Falcon No. 3033), by means of the roller-tube technique at 36 °C in dry air. The test tubes rotated at approximately 10 revolutions/h. The roller-drum was tilted about 5 ° with respect to the horizontal axis to ensure that cultures were completely immersed in the medium (1 ml) during about half a cycle. The culture medium consisted of horse serum (25%), basal medium (Eagle, 50%) and Hanks' or Earles BSS (25%) supplemented with glucose to a final concentration of 6.5 mg/ml. Addition of phenol red facilitated control of medium pH (about 7.4). The sera were heat inactivated at 56 °C for 30 min. No antibiotics were added to the medium. During the culture period (3–12 weeks), the cultures were fed once per week and inspected without removing the coverslips from the test tubes to prevent contamination of the cultures.

For intracellular injection of horseradish peroxidase (HRP, type IV, Sigma) or lucifer yellow, the cultures were transferred to a microchamber and impaled under visual control. Lucifer yellow was injected by application of a constant negative current of 1–3 nA for about 5 min from an electrode filled with a 4% aqueous solution of the fluorescent compound[16]. HRP was injected by 500-msec depolarizing current pulses of 1–5 nA at 1 pulse/sec[18] for about 10 min from electrodes filled with a 4% HRP solution in 0.05 M tris HCl, pH 8.5. Following injection of HRP, the cultures were incubated for 10 h, then fixed in 2.5% glutaraldehyde and exposed to diaminobenzidine. The reaction product was made darker by heavy metal intensification[2]. Cultures stained with Bodian's protargol impregnation were fixed with 4% formaldehyde. For Timm staining, the cultures were exposed for 10 min to a 1% solution of phosphate buffered sulphide, fixed in 96% alcohol and stained as described[22,41]. Cultures stained for acetylcholinesterase[20] (AChE) were fixed in 4% paraformaldehyde in 0.15 M phosphate buffer.

Results

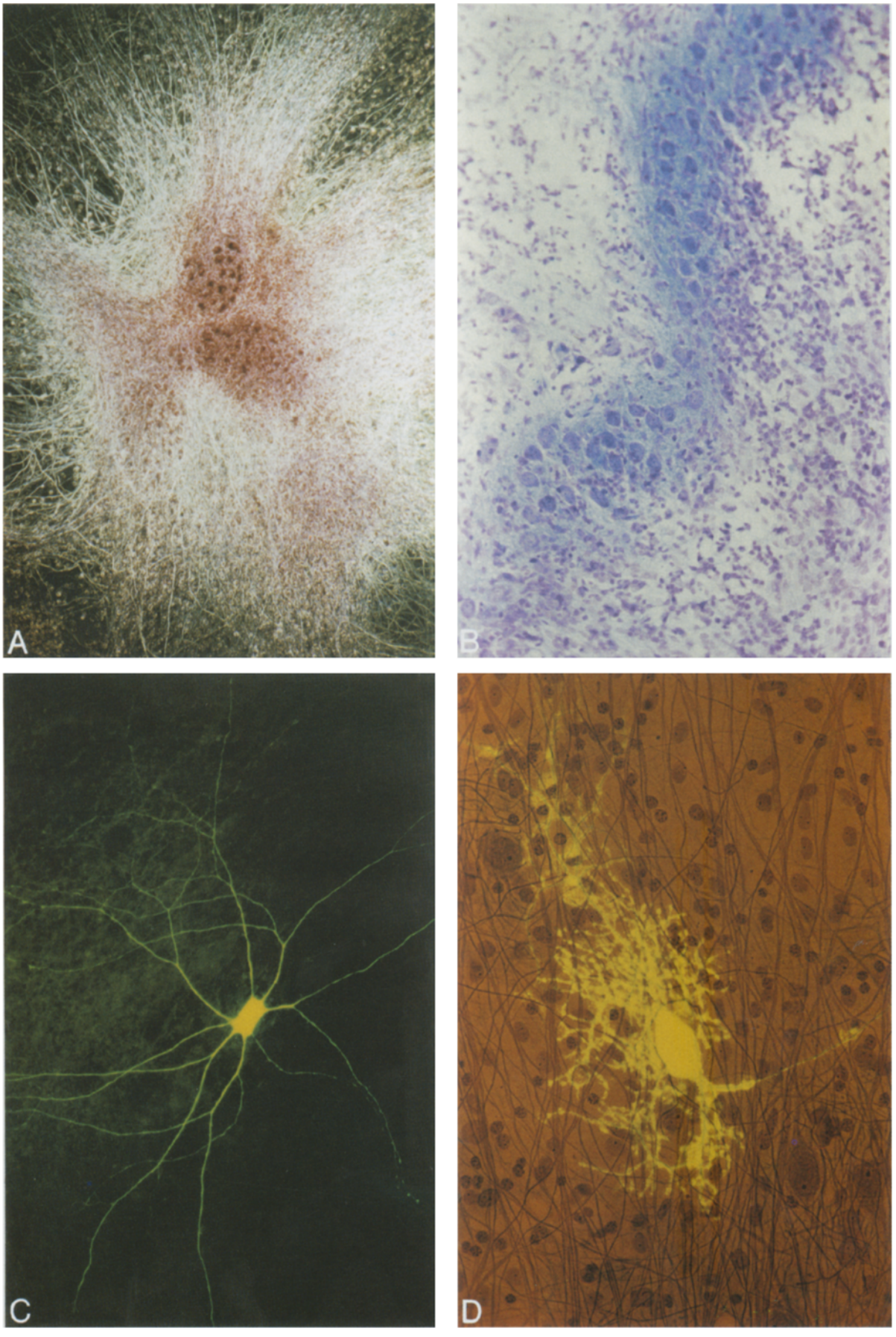
Cerebellum. Purkinje cells were recognized in the living state by their large size and their characteristic dark cytoplasm as visualized by phase contrast microscopy. They were often, but not always localized in laminated structures in which the cells clustered, but usually did not align in a single row as is the case in situ (fig. 2B). Intrasomatic injection of lucifer yellow showed that the cultured Purkinje cells had numerous primary dendrites originating from the cell body. All secondary and in part primary dendrites were covered by spinous processes, but somatic spines were virtually absent (fig. 2D). It was evident that the dendrites of cultured Purkinje cells remained short and that a full arborization reminiscent of adult Purkinje cells was never achieved. The shape of the dendritic arborization of cultured Purkinje cells resembled, however, that observed in experimentally degranulated cerebellum[3,14,24,26,27,40]. Similar observations were made by several authors who cultured explants of cerebellar tissue[12,23,35,36]. The reasons for the failure of Purkinje cells to grow a fully arborized dendritic tree are not fully understood, but a possible relationship to the out-of-phase course of parallel fibers in parasagitally oriented explants has been postulated[10].

The axons of Purkinje cells were myelinated by the end of the 2nd week in vitro. They grew toward the center of the cultures where bundles of fibers could be observed to converge (fig. 2A). This center consisted of tightly packed groups of large neurones which could be distinguished from Purkinje cells by their rarely branching dendrites which covered an area much larger than that of Purkinje cells (fig. 2C). In view of their characteristic location in the center of cerebellar cultures, their dendritic arborization[13], as well as the presence of AChE in their cell bodies (fig. 5A), these neurones were presumably derived from the deep nuclei.

Hippocampus. Cultures prepared from the hippocampus of 7-day-old rats first underwent massive morphological changes since all afferent fibers and many cells lying close to the cut surfaces degenerated. By the end of the 3rd week, most of this cellular debris had disappeared. Due to loss and migration of cells, the large nerve cells eventually became organized as a monolayer structure.

The overall structure of the hippocampal formation with pyramidal (areas CA1 to CA4) and granule cell layers is preserved in these slice cultures (figs 3A, 3B, 5B). Of all the hippocampal subfields, the granule cells of the medial blade of the fascia dentata were the least confined to one cell layer. Interestingly, this coincides with known morphogenetic gradients since in situ the medial blade of the fascia dentata is the latest cell layer to be formed[4,5,25,37,42].

Intrasomatic injection of HRP or lucifer yellow revealed that pyramidal cells in all hippocampal sub-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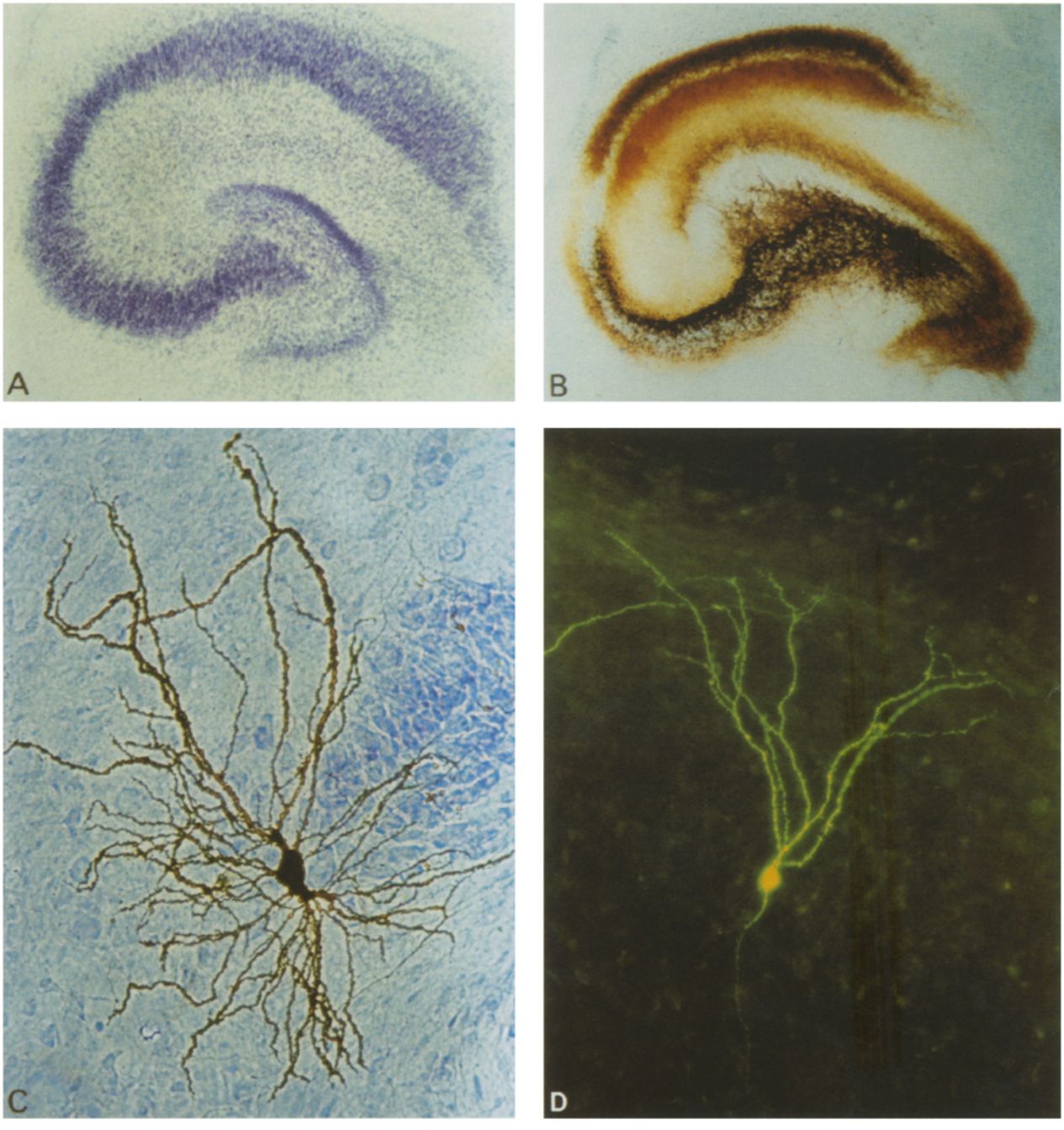

Figure 3. Hippocampal slice cultures. *A* Staining with toluidine blue reveals pyramidal as well as granule cell layers. Age of the culture, 42 DIV. *B* Hippocampal culture after Timms staining shows black mossy fiber boutons mainly in the dentate hilus and in the suprapyramidal layer of CA3. The dentate molecular layer and stratum oriens and radiatum of CA1 showed a more brownish staining. Age of culture, 28 DIV. *C* HRP-injected pyramidal cell of area CA3 in toluidine-blue stained preparation. Age of the culture, 35 DIV. *D* Granule cell of the dentate gyrus injected with lucifer yellow. Age of the culture, 32 DIV. The scale indicates 340 µm (A, B) and 50 µm (C,D).

Figure 2. Cerebellar slice cultures. *A* Center of cerebellar culture. Bodian silver stain, viewed with dark field microscopy, shows groups of neurones derived from deep nuclei and bundles of axons (observed as bright lines). Age of the culture, 28 days in vitro (DIV). *B* Row of Purkinje cells in toluidine-stained preparation. Age of the culture, 27 DIV. *C* Dendritic arborization of living neurone from the deep nuclei injected intrasomatically with lucifer yellow. The axon is difficult to distinguish from the dendrites which lack spinous processes. Age of the culture, 28 DIV. *D* After injection with lucifer yellow and subsequent Bodian silver staining, a Purkinje cell can be seen in its cellular microenvironment. Age of the culture, 25 DIV. The scale indicates 270 µm (A), 100 µm (B), 60 µm (C), 40 µm (D).

fields retained their characteristic shape and bipolar orientation with basal as well as apical dendritic arborizations (fig. 3C). The main dendritic shafts bifurcated frequently and were loaded with spinous processes. After 1 month in vitro, the pyramidal cells appeared fully mature; with respect to size of their dendritic fields, development of spines and diameter of cell bodies, these pyramidal cells closely resembled the ones observed in situ during the late post-natal phase[33]. The dendritic arbor of granule cells was always monopolar, confined to the stratum moleculare and consisted of 2–5 long dendrites which were covered by spinous processes (fig. 3D).

The granule cells grew axons (fig. 3D) which were seen as thin fibers growing toward the hilar region with occasional supragranular mossy fibers terminating in the stratum moleculare.

During prolonged cultivation of nervous tissue, synaptic reorganization might occur. Although the existence of synapses in explants of rat hippocampus had been demonstrated by morphological[29] and electrophysical[19] techniques, the specificity of these connections was usually difficult to evaluate. Using the Timms sulfide silver method which intensely stains the zinc-containing boutons formed by the mossy fibers[22,41], we have been able to show the projection of granule cells to the dentate hilus and the CA3 pyramidal cells where the mossy fibers predominantly terminated within the suprapyramidal layer (J. Zimmer and B.H. Gähwiler, in preparation). Like their in situ counterparts, the mossy fibers respected the CA1/CA3 borderline; thus, no black densely stained mossy fibers were observed in the CA1 area. Instead, the stratum oriens and radiatum contained smaller brownish elements (fig. 3B).

Hypothalamus. For long-term studies of hypothalamo-hypophyseal interactions and investigations of hypothalamic function in vitro, culturing of discrete, identified hypothalamic areas is a necessary prerequisite. The slice culture technique allows reproducible cultivation of the entire hypothalamus of a 7-day-old rat in nine 400-µm-thick slices. Their relative structural interrelationships were retained even after cultivation for periods up to 3 months. Following fixation and Golgi-like immunoperoxidase staining for neurophysin[38], various hypothalamic nuclei could be tentatively identified. In an area derived from the ventral surface of the brain, two neurophysin-positive nuclei could be recognized (fig. 4A). These nuclei, presumed to be the suprachiasmatic nuclei, consisted of small parvocellular cells whose axons formed complicated networks with bundles of fibers passing along the surface of the slice. Other groups of neurophysin-positive cells with large diameter, presumably derived from the paraventricular nuclei, were located bilaterally directly bordering on the third ventricle (figs. 4A, 4B). In fixed preparations, the ventricle was only seen as a cell-free area (fig. 5C), but it was easily recognized in living preparations by the presence of ciliated ependymal cells forming the ventricular walls.

In slices including the supraoptic and paraventricular nuclei, cellular morphology was also studied by injection of HRP into large hypothalamic neurones (figs. 4D–4G). These neurones were found to be morphologically heterogenous. The most frequently encountered cell type displayed a simple dendritic pattern with 2–4 long, rarely branching dendrites and a beaded axon which often emerged from a primary dendrite (figs. 4C, 4E), gave rise to numerous axon collaterals and could be followed for several millimeters (fig. 4E). Other cells showed a more complex morphology with somatic as well as dendritic spines which varied greatly in number and length (figs. 4F, 4G). Many of these morphological features closely resembled those of magnocellular neurones in Golgi preparations[6,7,15,28,30,31] and after Golgi-like immunoperoxidase staining in situ[39].

Conclusions

The slice culture technique yields a preparation of which the main advantages are its organotypic organization and the excellent accessibility of individual cells to experimental manipulation. In cerebellar cultures, Purkinje cells and cells derived from the deep nuclei were recognized on the basis of their size, location and arborization. Identification of hippocampal neurones was eased by the fact that the characteristic hippocampal cytoarchitecture with pyramidal and granule cell layers remained preserved. Golgi-like immunoperoxidase staining of hypothalamic cultures demonstrated that the gross anatomical interrelationships between hypothalamic nuclei were retained and that cultured hypothalamic neurones displayed their characteristic cellular morphology with beaded axons and typical dendritic arbors.

A similar degree of tissue organization could at times be preserved using conventional explant cultures. The introduction of standardized slicing techniques has, however, significantly improved reproducibility and reduced variations between cultures of the same anatomical origin.

Slice cultures appear to provide a tool suitable for a variety of multidisciplinary studies. Firstly, long-lasting intracellular recordings from neurones in slice cultures demonstrated the existence of normal spiking and synaptic activity, and the feasibility of measuring the chemosensitivity of these neurones[18,19]. Secondly, slice cultures can be used for release studies; it was possible to determine levels of arginine vasopressin in tissues as well as in the medium of long-term cultures of hypothalamic slices[8,9]. Thirdly, intracellular dye injection and immunohistochemical staining techniques

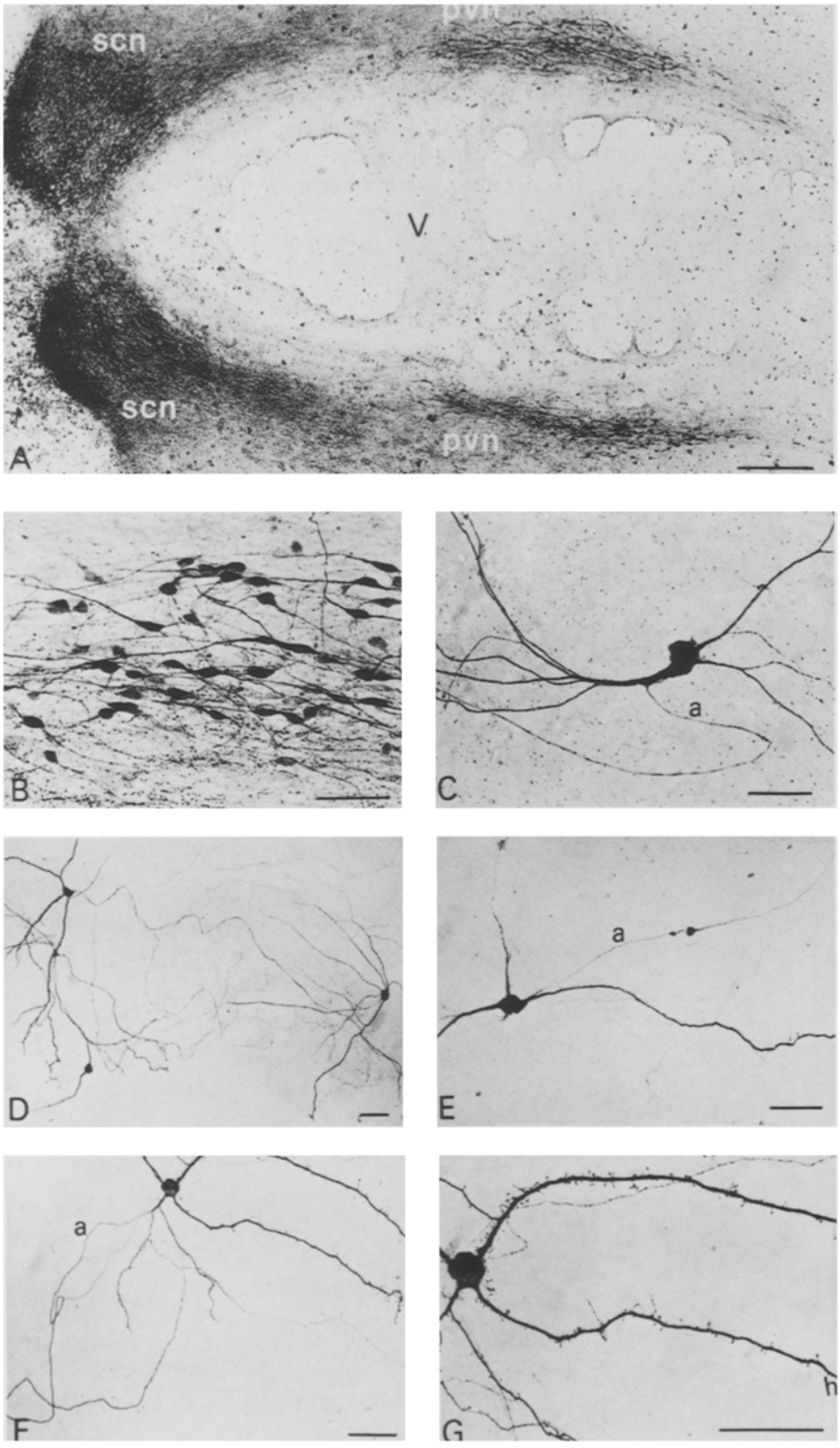

Figure 4. Hypothalamic slice cultures. *A* to *C* are Golgi-like immunoperoxidase stainings for neurophysin. *D* to *G* illustrate cells of the paraventricular and supraoptic region injected with HRP. *A* Survey of slice prepared from the anterior hypothalamus. On the basis of their location within the slice and the small diameter of their cells, the 2 nuclei on the left were tentatively identified as suprachiasmatic nuclei (scn). Directly bordering the 3rd ventricle (V) bilaterally were 2 groups of neurones which were presumably derived from the paraventricular nuclei (pvn). Age of the culture, 32 DIV. *B* Detail of a paraventricular nucleus displayed in A. *C* Large neurophysin-positive neurone in a slice from the posterior hypothalamus. Note the axon (a) emerging from a dendrite. Age of the culture, 33 DIV. *D* 3 cells were labeled by injection of HRP. Age of the culture, 56 DIV. *E* Large nerve cell with beaded axon (a). Age of the culture, 63 DIV. *F* Nerve cell displaying dendrites covered by spinous processes and an elaborate axonal pathway (a). Age of the culture, 35 DIV. *G* Detail of F. The scale indicates 500 μm in A and 100 μm in all other figures.

allowed the study of cellular morphology and axonal pathways.

Further studies with slice cultures may help to clarify the degree to which this model is complementary to those existing techniques such as brain slices, cultures of dissociated cells and brain transplants. I anticipate that slice cultures, and particularly the co-cultivation of slices derived from different brain regions, may be instrumental in elucidating the mechanisms involved in synaptogenesis. Furthermore, using hypothalamo-hypophyseal cultures, it appears to be possible to identify neuronal sites involved in the synthesis and release of hypothalamic factors which influence pituitary function. Finally, the possibility of observing in-

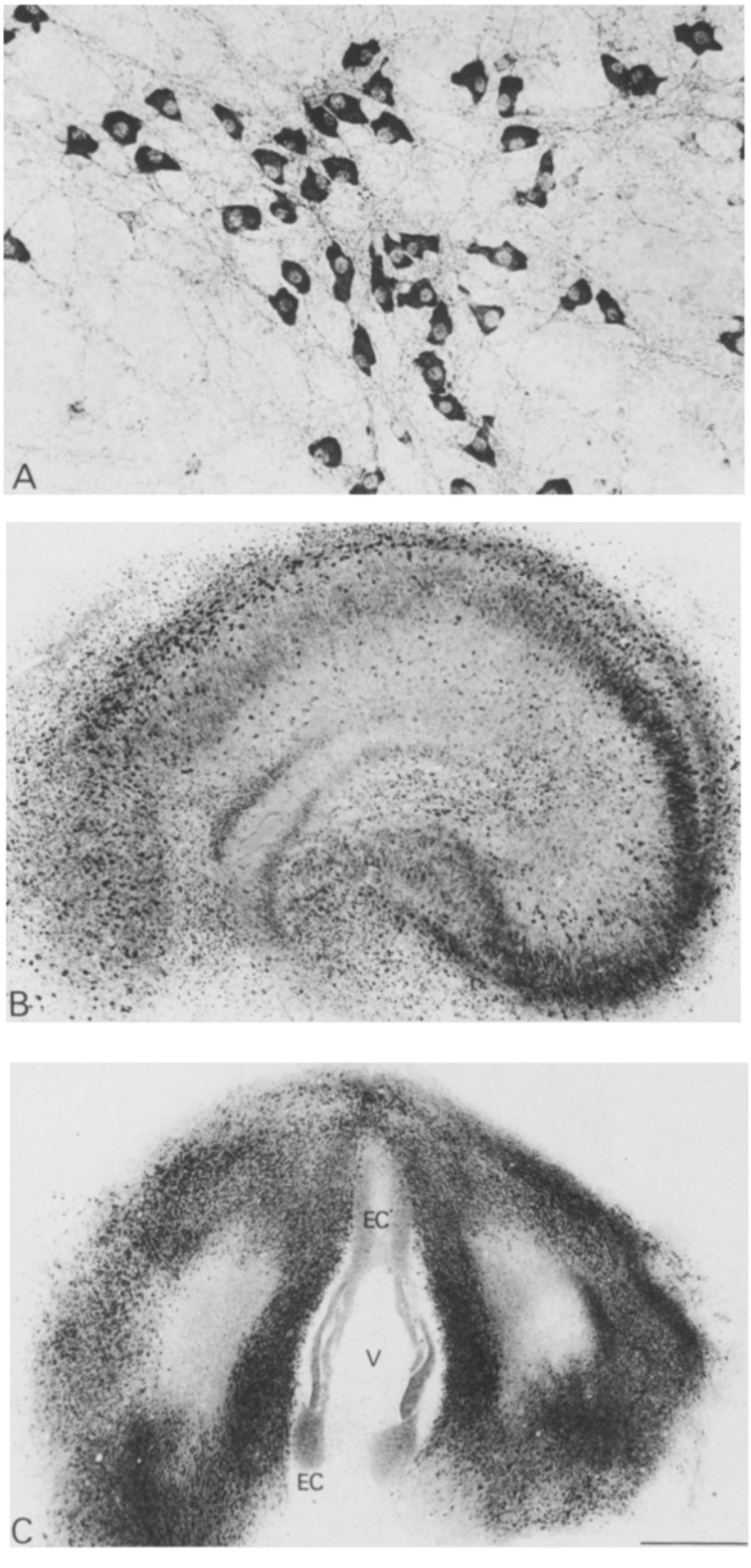

Figure 5. Acetylcholinesterase (AChE) staining of cultures derived from cerebellum (A), hippocampus (B) and hypothalamus (C). *A* AChE-stained cell bodies were only found in the center of cerebellar cultures. Age of the culture, 18 DIV. *B* AChE-positive cells were found in the hilar region and in areas CA1 and CA3 of the hippocampus where they displayed a distribution usually attributed to interneurones. Age of the culture, 25 DIV. *C* AChE-staining demonstrates the cyto-organization of a culture prepared form the medial-basal hypothalamus. Groups of AChE-positive neurones are clustered in an area bordering the 3rd ventricle (V). Note the presence of ependymal cells (EC). Age of the culture, 22 DIV. The scale indicates 100 µm (A), 500 µm (B), 800 µm (C).

dividual neurones within neuronal networks may facilitate the use of advanced electrophysiological techniques such as the patch-clamp[21] for investigations of identified central neurones.

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