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Effects of glia-conditioned medium on primary cultures of central neurons

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Summary. The effects of glia conditioned media on the survival and differentiation of embryonic neuron cultures of the Central Nervous System is described. We established glial cultures of peritumoral areas and the culture medium was changed serially and collected as glia conditioned medium (GCM). Neuron enriched cultures plated on poly-L-lysine coated Petri dishes and after 4 days in vitro, the neuronal cultures were treated for the inhibition of glial cells. We established two groups of neuronal cultures that were respectively exposed to the GCM and to conventional culture medium. We evaluated the survival and differentiation of neuronal population in each group of cultures by contrast-phase microscopy and cellular uptake of Horse Radish Peroxidase. The cell cultures exposed to GCM showed a survival during the in vitro stages and an organization and differentiation pattern of mature neurons larger than the control cultures. This fact suggests that the glial cultures produce a diffusible neurotrophic factor that influences the neuronal response in vitro.

Key words: Neurotrophic effect, Glia conditioned medium, Tissue culture

Introduction

There has been considerable acceptance of proliferation of glial constituents of the CNS through the life span of individual mammals (Biesold et al., 1976; Mares and Brueckner, 1978; Schultze and Korr, 1981; Korr et al., 1983; Sinués, 1985). The neurons may respond to the stimuli capable of inducing neurotrophic effects and these could be mediated through either direct cell contact or the microenvironment (Sakellaridis et al., 1986). Tissue culture techniques eliminate some of the in vivo disadvantages and allow the study of individual factors that influence cell development. Primary cultures enriched in astroglial cells are an experimental model to study growth regulation in the CNS. Also, primary nerve cell cultures offer the possibility of choosing the brain region and the age of the animal at the time of cell seeding (Hansson, 1984).

In this paper, the effects of glia conditioned medium (GCM) on primary nerve cell culture are described, after characterization of the glial cultures using cell specific marker. We evaluated the survival and differentiation of neurons by morphological patterns and the cellular uptake of Horse Radish Peroxidase as a neuronal marker.

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Materials and methods

Material of glial cultures: Characterization

Tissue adjacent in the white matter to glioblastoma multiforme was obtained from the operating room within a few minutes. Sections of 10 μm of this tissue were stained with H-E according to conventional method.

This tissue was tested for the glial fibrillary acidic protein (GFAP), an astrocyte and immature oligodendrocyte marker (Choi and Kim, 1984). Fragments were frozen and stored before staining. Frozen sections 10 μm thick were fixed with 70° C acetone for 30 secs and then washed twice with PBS. The sections were then incubated with an antiserum to GFAP (a gift from Prof. A. Bignami, Harvard) at a dilution of 1:20. FITC-conjugated secondary antibody (Miles-Yeda, Lab.) was added at a dilution of 1:15 and the sections were viewed under a Leitz microscope with FITC interference (500 nm) and filters.

Glial cultures from human peritumoral tissue

The peritumoral tissue was washed with Hank's solution, minced into small pieces and digested with

0.125% trypsin for 15 min at room temperature, the cell suspension obtained was washed with F-12 medium and passed through a Pasteur pipette. The pelleted cells, without removing cellular microaggregates, were resuspended and placed in tissue culture flasks. Initially, F-12 medium, supplemented with 10% fetal bovine serum (FBS), without antibiotics, was used. Cell cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2 in air

Characterization of human glial cultures

Morphology: The human peritumoral glia was grown on coverslips. After several phases of proliferation (3-8 days), it was washed with Hank's solution and fixed for 15 min in methanol, washed with distilled water and stained with H-E. The cells were dehydrated successively until finally reaching xylol. Coverslips were mounted on slides and examined with a Leitz light microscope.

Identification of GFAP: The GFAP was assayed by indirect immunofluorescence in cell cultures with successive stages of confluence. Cells were fixed with cold ethanol/acetic a. (95:5). The next steps were similar to step previously described for the peritumoral tissue.

Reactive glia-conditioined medium (GCM)

The human astroglial cultures were changed to fresh serum-free F-12 when the cells reached confluence after 5 days in vitro. Subsequently, the cultures were serially changed twice a week and the GCM was collected and filtered through a 0.22 µm filter. The GCM was frozen and stored at -20 ° C prior to use.

Neuronal primary cultures

Neuron-enriched cultures were prepared according to the method of Dutton et al. (1981) by dissociation of cerebral hemispheres of 18-day fetal rats (Wistar strain). Following decapitation, the cerebral hemispheres were collected, pooled and cleaned of meninges and blood vessels. All dissociation media were based on Ca^{2+}- free Earle's basic salt solution containing bovine serum albumine (fraction V, Sigma), glucose and $MgSO_4$. The cell suspension was plated on 1.5 µgr/ml of poly-lysine (Sigma) coated Petri dishes (35 mm. Nunc, Denmark), at a density of 10^5 cells/ml. The culture medium, Eagle's MEM, was supplied with 10% FBS, 30 mM glucose, 5 µgr insulin/ml, 200mM L-glutamine, 25 mM K^+ and 10^{-5} M cytosine-arabinoside (Sigma). The cell cultures were incubated at 37° C in a humidified atmosphere with 95% air and 5% CO_2

Experimental culture conditions

After the methodology above described, the neuronal cultures were divided into two groups. Each group of cultures was respectively exposed to the following media: GCM and Eagle's MEM. In both groups of cultures the medium was changed every 5 days.

To determine the percentage of glial contamination, both groups of cultures were characterized on the 7th day using indirect immunofluorescence with GFAP antibody.

We evaluated the survival and differentiation of neuronal population in each group of cultures by examination with a contrast-phase microscope every 24 h and cell uptake of HRP (type VI, Sigma). The HRP was dilluted in culture medium at 3%. After incubation the cultures were washed 3 times with PBS, fixed in methanol, rehydrated and stained with DAB (Sigma) according to the method of Eng and Rubinstein (1978).

Results

Peritumoral tissue

Peritumoral areas in the white matter, stained with H-E, were characterized by numerous reactive astrocytes. They were oriented along myelin fibers. By indirect immunofluorescence, reactive astrocytes were GFAP positive. They showed many processes with strong GFAP-expression (Fig. 1A).

Characterization of glial cultures

Morphology: The cell cultures showed morphological differentiation from the early stages in vitro. The logarithmic growth displayed stellate cells with long processes linked together and polygonal cells with bipolar processes (Fig. 1B). The cells were frequently of different sizes and with one central nucleus with two or more nucleoli. In the next days of development the cultured astroglial cells formed a confluent monolayer (Fig. 1C). The majority were flat epithelial-like cells with large nucleus and scanty cytoplasm and slight processes.

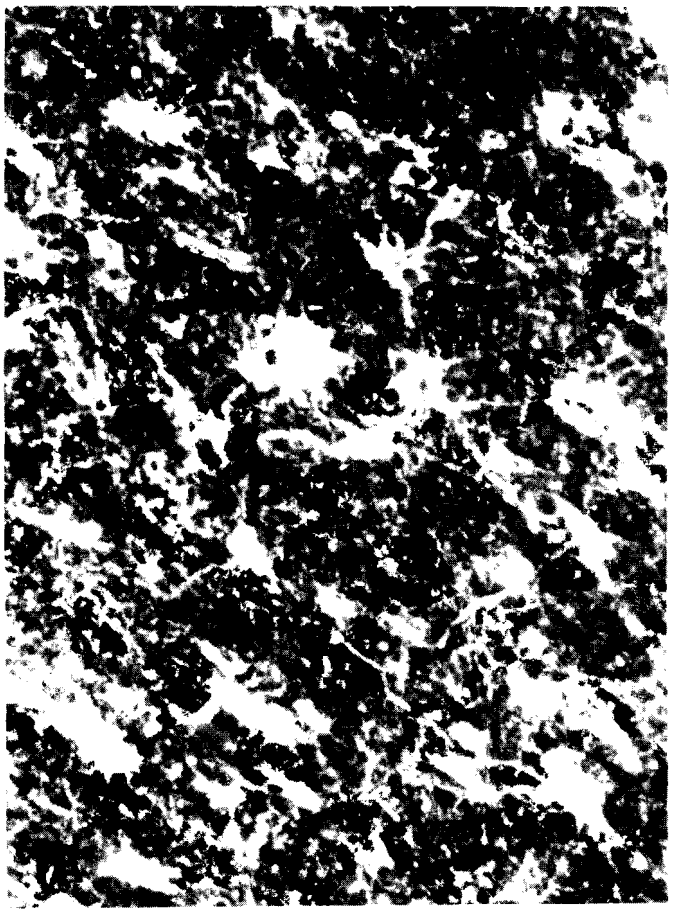
Primary monolayer cultures expressed GFAP in 90% of the cells, with a characteristic fibrillary pattern (Fig. 1D). Once the almost confluent cultures were established, 7 days in vitro, the GFAP-expression appeared to be within cells usually showing a weaker homogeneous fluorescence than in the first days. The GFAP-positive cells reached 100% in culture.

Neuronal cultures. Identification of neurotrophic factors

In the neuronal cultures we observed that considerably less than 2% of the cells stained positively with GFAP-antibody, a marker of astrocytes and immature oligodendrocytes.

The morphological evolution of the primary neuronal cultures was evaluated by phase-contrast microscopy. In the first stages, 5 days in vitro we visualized cellular clusters made up of refracting cells attached to the culture dishes; later short filiform processes closely apposed to clusters had emerged (Fig. 2A).

In order to determine the glia-dependent neurotrophic activity, after this initial period in culture, we evaluated the differences of neuronal survival and differentiation between the two groups of neuronal cultures: After longer periods in culture, 40 days in vitro, the cultures

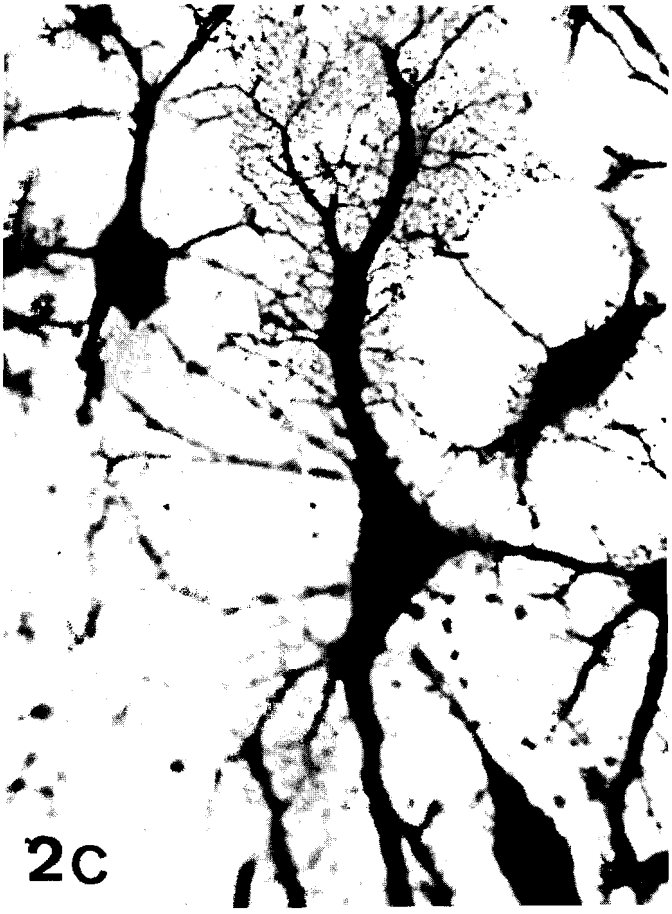



1C

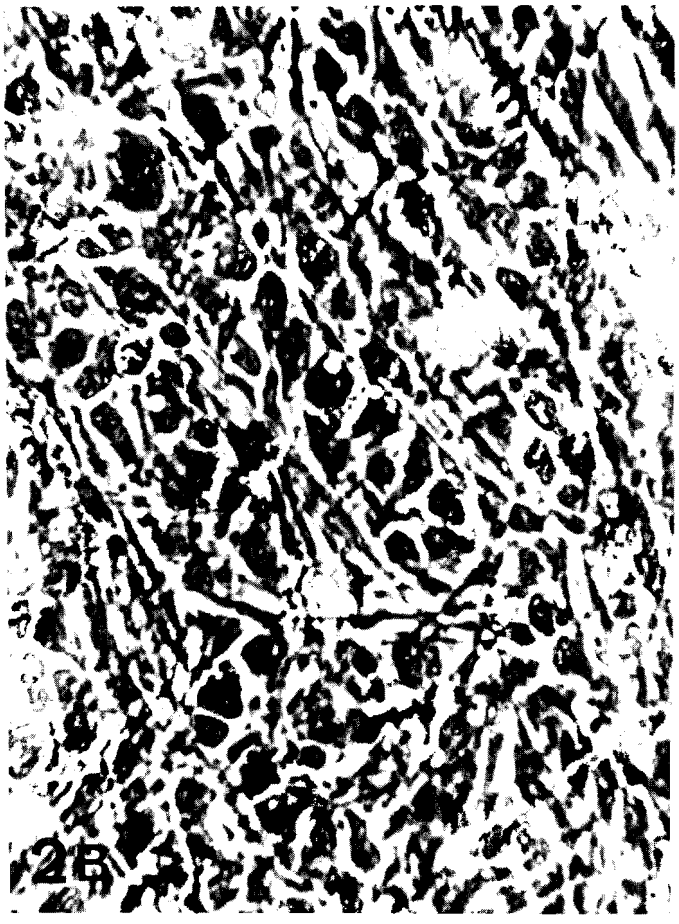

1B


1D

2A



2C



2B

2D

Fig. 1A. Peritumoral tissue. GFAP determination by indirect immunofluorescence. 10μm section. × 200

Fig. 1B. Astroglial culture, 3 days in vitro. H-E. × 100

Fig. 1C. Astroglial culture, 9 days in vitro. Monolayer with mature astrocytes. Phase contrast microscopy. × 200

Fig. 1D. Astroglial culture, 6 days in vitro. GFAP-positives astrocytes. Indirect immunofluorescence. × 400

Fig. 2A. Neuronal culture, 7 days in vitro. Neuronal cells with filiform processes. Phase contrast microscopy. × 200

Fig. 2B. Neuronal culture, 18 days in vitro. Cellular monolayer with bundle of axons. Phase contrast microscopy. × 100

Fig. 2C. Neuronal culture, 10 days in vitro. HRP uptake. Neuron with a profuse neuritic network. × 400

Fig. 2D. Neuronal culture, 18 days in vitro. HRP uptake. Morphological pattern of mature neurons. × 200

exposed to Eagle's MEM showed a lower population of cells possessing neurites; cells identified as neurons.

Both groups of cultures showed a similar cellular differentiation pattern during the first days in culture. Beginning on the 5th day, a number of characteristics had been observed in the cultures exposed to GCM: cultures became composed predominantly of cells with neuritic branchings that established a network together with the neuronal somas. In later stages of maturation, 18 days in vitro, the neuronal population showed dense neuron clusters with bundles of axons so that their microscopic image was similar to histological organization pattern (Fig. 2B). After a month in culture the microscopic control determined a greater number of adult neurons to the cultures exposed to GCM. This finding was ratified by the cellular uptake and neuronal transport of HRP: neurons with apparent nuclei with a subtle and very dense profuse neuritic network that in some areas was similar to fibers. (Figs. 2C, D).

We observed through our study a stable morphological pattern in the cultures exposed to Eagle's MEM: cells with interconnected branching processes. These neuritic processes became increasingly complex; however, these cultures showed levels of development and differentiation considerably inferior to that of the cultures exposed to GCM.

Discussion

The astroglial cells play an important role for the function of the brain. Astroglia have been shown to act as K^+ buffer (Waltz and Hertz, 1983), to participate in uptake and metabolism of amino acid transmitters (Hansson, 1984; Hansson et al., 1984) and to bear receptors for various neurotransmitters and neuromodulators (Van Calker and Hamprecht, 1980). Epigenetic factors involved in the regulation of neuronal plasticity may also be closely associated with the expression of glial cell function (Hansson, 1986).

The microenvironment factors influence the growth and neuronal differentiation. So, in the cellular culture systems, a glial monolayer affects neuronal survival (Denis-Donini et al., 1984). However, the studies with references to CNS are limited (Schonfeld et al., 1981; Benfey and Aguayo, 1982; Kligman, 1982; Muller and Seifert, 1982). These studies suggest an in vivo role for peripherally derived factors in stimulating central neuron regeneration. Nerve growth factor (NGF) is a neurotrophic protein for sympathetic and some sensory neurons (Levi-Montalcini and Angeletti, 1986). In the CNS, mRNA of NGF (Shelton and Reichart, 1984) and a low level of NGF (Korschcing et al., 1985) have been detected, suggesting that NGF acts as a neurotrophic factor in the CNS as in the PNS.

In this report, we suggest that reactive glia from peritumoral tissues, containing predominantly GFAP-positive protoplasmic astrocytes, produces diffusable neurotrophic factor(s) which promote survival and neurite out-growth from embryonic cerebral neurons in vitro. We are assuming that the input of fibrillary neuroglia, the most common reaction of the brain in the peripheral areas of gliomas (Schiffer et al., 1986), to the neuronal microenvironment is mediated in culture through the glia conditioned medium.

In our study we based the establishment of pure neuronal cultures on: use of fetus chosen at a critical phase of early development, while there are many still immature neurons that possess ability to survive under tissue culture conditions and to differentiate; use of cytosine-arabinoside, a specific inhibitor of the DNA synthesis, to kill all still dividing cells at a critical time in the culture differentiation (Sotelo et al., 1980); and the plating surface and cell density. So, poly-lysine was essential for cell growth and neurite extension (Aizenman and de Vellis, 1987).

The functional activity of the cultures exposed to GCM is shown by uptake and cellular transport of HRP; this inespecific neuronal marker shows a different intracellular pattern of distribution depending on glial or neuronal uptake.

Furakawa et al. (1986) have found that astroglial cells cultured from the whole mouse brain synthesize and secrete a material with NGF-like immunoreactivity (NGF-LI) into their culture medium, this material showed neurite outgrowth stimulatory activity. We propose that the GCM contain a diffusable neurotrophic factor(s) produced by GFAP-positive cells.

It has been reported that the distribution of NGF and its mRNA is region-specific in the brain (Korshcing et al., 1985) and neutrophic factors have been described in the brain of developing or adult rats after injury to the nervous system (Nieto-Sampedro et al., 1982). So, the astroglial cells in different regions of the brain or in different levels of maturation produce neurotrophic factors as proper supply to neurons for their growth, survival and maintenance of function.

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References

Aizenman Y. and de Velis J. (1987). Brain neurons develop in a serum and glial free environment: effects of transferrin, insulin, insulin-like growth factor-1 and thryoid hormone on neuronal survival, growth and differentiation. Brain Res. 406, 32-42.

Barde Y.A., Edger D. and Thoenen H. (1983). New neurotrophic factors. Ann. Rev. Physiol. 45, 601-602.

Benfey R. and Aguayo M. (1982). Extensive elongation of axons into peripheral nerve grafts. Nature 11, 150-152.

Biesold D., Bruckner G. and Mares V. (1976). An autoradiographic study of gliogenesis in the rat lateral geniculate nucleus. Brain Res. 104, 95-301.

Choi B.H. and Kim R.C. (1984). Expression of glial fibrillary acid protein in immature oligodendroglia. Science 223, 467-468.

Denis-Donini S., Glowinski J. and Prochiantz A. (1984). Glial heterogenety may define the tree-dimensional shape of mouse mesencephalic dopaminergic neurons. Nature (London) 307, 641-643.

Dutton G.R., Currie D. and Tear K. (1981). An improved method for the bulk isolation of viable perikarya from postnatal cerebellum. J. Neurosci. Methods 3, 421-427.

Eng L. and Rubinstein L.J. (1978). Contribution of immunochemistry to diagnosis problems of human cerebral tumors. J. Histochem. Cytochem. 26, 513-522.

Furakawa Y., Furakawa S., Satoyoshi E. and Hayashi K. (1984). Nerve growth factor secreted by mouse heart cells in culture. J. Biol. Chem. 259, 259-1264.

Furakawa S., Furakawa Y., Satoyoshi E. and Hayashi K. (1986). Synthesis and secretion of nerve growth factor by mouse astroglial cells in culture. Biochem. Biophys. Res. Commun. 136, 57-63.

Hansson E. (1984). Enzyme activities of monoamine oxidase, cathechol-0-methyl transferase and γ -aminobutiric acid transaminase in primary astroglial cultures and adult rat brain from different brain regions. Neurochem. Res. 9, 45-47.

Hansson E. (1985). Primary cultures from defined brain areas; effects of seeding time on cell growth, astroglial contents and protein synthesis. Develop. Brain Res. 21, 187-192.

Hansson E. (1986). Co-cultivation of astroglial and neuronal primary cultures from rat brain. Brain Res. 366, 159-168.

Hansson E., Isacsson H. and Sellostrom A. (1984). Some characteristics of dopamine and GABA transport in primary cultures of astroglial cells. Acta Physiol. Scand. 121, 333-341.

Hansson E., Ronnback L., Lowental A. and Noppe M. (1985). Primary cultures from defined brain areas, effects of seeding time on the development of beta-adrenergic and dopamine-stimulated cAMP-activity during cultivation. Develop. Brain Res. 21, 175-185.

Kligman D. (1982). Neurite outgrowth from cerebral cortical neurons is promoted by medium conditioned over heart cells. J. Neurosci. Res. 8, 281-287.

Korr H., Schilling W., Schultze B. and Maurer W. (1983). Autoradiographic studies of glial proliferation in different areas of the brain of the 14-days-old rat. Cell Tissue Kinet. 16, 393-413.

Korsching S., Heumann R., Thoenen H. and Hefti F. (1986). Cholinergic denervation of the rat hippocampus by fimbrial transection leads to a transient accumulation of NGF without change in mRNA NGF content. Neurosci. Lett. 15, 66, 175-180.

Levi-Montalcini R. and Angeletti P. (1986). Nerve growth factor. Physiol. Rev. 48, 534-569.

Mares V. and Brueckner G. (1978). Postnatal formation of nonneuronal cells in the rat occipital cerebrum: an autoradiographic study of the time and space pattern of cell division. J. Comp. Neurol. 177, 519-528.

Muller H.W. and Seifert W. (1982). A neurotrophic factor (NGF) released from primary glial cultures supports survival and fiber outgrowth of cultured hippocampal neurons. J. Neurosci. Res. 8, 195-204.

Muller H.W., Beckh S. and Seifert W. (1984). Neurotrophic factor for central neurons. Proc. Natl. Acad. Sci. U.S.A. 81, 1248-1252.

Nieto-Sampedro M., Lewis E.R., Cotman C.W., Manthorpe M., Skaper S.D., Barbin C., Longo F. and Varon S. (1982). Brain injury causes a time-dependent increase in neurotrophic activity at the lesion site. Science 217, 860-861.

Pettman B., Louis J.C. and Sensebrener M. (1979). Morphological and biochemical maturation of neurons cultured in the absence of glial cells. Nature (London) 281, 378-380.

Sakellaridis N., Nangoura D. and Vernadakis A. (1986). Effects of neuronconditioned medium and fetal calf serum content on glial growth in dissociated cutures. Develop. Brain Res. 27, 31-34.

Sensebrener M. and Mandel P. (1984). Behaviour of neuroblasts in the presence of glial cells, fibroblasts and meningeal cells in culture. Exp. Cell Res. 87, 159-167.

Schiffer D., Giordana M.T., Germano I. and Mauro A. (1986). Anaplasia and heterogeneity of GFAP expresion in gliomas. Tumori 72, 163-170.

Schonfel A.R., Thal L., Morowitz S. and Katzman R. (1981). Heart conditioned medium promotes central cholinergic regeneration in vivo. Brain Res. 229, 541-546.

Schultze B. and Korr H. (1981). Cell kinetic studies of different cell types in the developing and adult brain of the rat and the mouse; a revier. Cell Tissue Kinet. 14, 309-325.

Shelton D.L. and Reichart L.F. (1984). Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. Proc. Natl. Acad. Sci. U.S.A. 81, 7951-7955.

Sinués E. (1985). Proliferación postnatal de células gliales y endoteliales en el colículo superior. Universidad de Zaragoza, Facultad de Medicina.

Sotelo J., Gibbs C.J., Gajdusek D.C., Toh B.H. and Wurth M. (1980). Method for preparing cultures of central neurons: cytochemical and immunochemical studies. Proc. Natl. Acad. Sci. USA 77, 653-657.

Van Calker D. and Hamprechet B. (1980). Effects of neurohormones on glial cells. In: Advances in Cellular Neurobiology. vol. 1. Federoff S. and Hert L (eds). Academic Press. New York. pp 31-67.

Vernadakis A., Nidess R., Culver B. and Arnold E.B. (1975). Glial cells: modulators of neuronal envoronment. Merch. Aging Develop. 9, 553-566.

Vernadakis A., Parker G. and Norenberg M. (1980). Changes in biochemical properties of glial cells in culture; in vitro differentiation. In: Tissue Culture in Neurobiology. Giacobini E. Vernadakis A. and Shahar A. (eds) Raven Press. New York. pp 411-426.

Waltz W. and Hertz L. (1983). Comparison between fluxes of potassium and of chloride in astrocytes in primary cultures. Brain Res. 277, 321-328.

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