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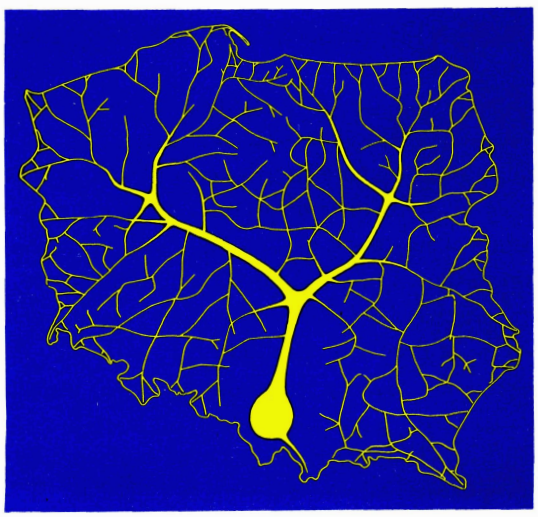

THE ROLE OF GLIA IN CNS PATHOLOGY AND REPAIR : BASIC AND CLINICAL ASPECTS

INTERNATIONAL SYMPOSIUM

Organizers :
Jan ALBRECHT (Warsaw) and Barbara ODERFELD-NOWAK (Warsaw)

Warsaw, June 14-15, 1993

A B S T R A C T S

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Preface

The last few decades have brought about an enormous progress in our understanding of the functions of the different classes of glial cells:astrocytes, oligodendrocytes and more recently microglial cells, in the CNS. Long considered to play only perfunctory, supportive roles, glial cells have turned out to be active partners in neural transmission, neuroregulation and, by most recent accounts, information storage. Of course, recognition of the multiple glial functions has begun to broaden our views on the different - both beneficial and detrimental - roles of glia in various pathological conditions of the brain. However, the literature concerning the pathology of glia is dispersed to the degree that makes it very difficult to follow the progress in an effective, selective and systematic way. We therefore felt it is just the right moment to organize a meeting on glia, covering and bridging all its aspects.

An International Symposium "The role of glia in CNS pathology and repair" coorganized by the Polish Neuroscience Society, Medical Research Centre of the Polish Academy of Sciences, and the Nencki Institute of Experimental Biology of the Polish Academy of Sciences, was held in the Nencki Institute in Warsaw, in June (14-15) 1993. The Symposium hosted about 80 participants from Poland and abroad. As we feel can be concluded from the attached abstracts, we were most fortunate to have with us guest speakers, many of the most learned and respected specialists in the biology and pathology of glia: some of them came really a long way to provide us with the most updated, often labbench views on the state of the art. We witnessed vivid exchange of opinions, both during the sessions and at the "summing up" round table held under the slogan "Astrocytes in brain damage : The good, the bad or (just) the ugly"? Notably, the discussions at the round table went far beyond the scope circumscribed by the slogan. We have reasons to assume that the sessions opened new cooperative ventures. We also hope that the symposium multiplied the ranks of believers in the crucial role of glia in the biology and pathology of the CNS.

We wish to express our special thanks to Professor Małgorzata Kossut, the President of the Polish Neuroscience Society, Professor Witold Karczewski, the President of the Committee of Scientific Research, Professor Mirosław Mossakowski, the Director of the Medical Research Centre, and Professor Maciej Nałęcz, the Director of the Nencki Institute, for their genuine interest, any kind of assistance and for providing generous financial support to the Meeting. We gratefully acknowledge the following companies for their contribution and support: Alab, Beckman, Candela, Comed, Inlab, Linegal Chemicals, Panalytica, and Pharmitalia.

At last but certainly not least, we owe the warmest words to our colleagues and associates, who devoted all their abilities and even more time they have ever had, to make the "small things" work and keep in shape: Hanna Borkowska, Małgorzata Pawłowska, Wojciech Hilgier, Lidia Faff-Michalak, Wojciech Jegliński and Andrzej Bacia. All the organizational efforts were skilfully supervised by the Administrative Director of the Nencki Institute, Dr. Zbigniew Przygoda.

Jan Albrecht
Barbara Oderfeld-Nowak

PLENARY LECTURE

PATHOMORPHOLOGY OF ASTROCYTES: A NATURAL HISTORY

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The presentation, predominantly based on the author's own clinical and experimental material, reviewed the morphological, ultrastructural and immunocytochemical manifestations of astroglial reaction in an array of primary and secondary brain pathologies. The conditions under consideration included: hereditary and acquired hepatic encephalopathy, Creutzfeld-Jacob disease, polyglycosan disease, Alexander's disease, PML, Alzheimer's disease, aging and cerebral ischemia. The author's reflections have focused on those astroglial changes that possibly contribute to the neurological manifestations of the particular conditions, or alternatively participate in repair processes.

SESSION I : PROLIFERATIVE AND TROPHIC RESPONSES

CELL PROLIFERATION INDUCED BY BRAIN INJURY: IDENTIFICATION AND QUANTITATION

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Some proliferating glioblasts are known to exist in the adult CNS, and we have shown that GD_3 ganglioside+ glial precursors cells can be isolated from mature brain (1). In culture these cells differentiate to become astrocytes and oligodendrocytes. Do such cells contribute to glial proliferation following injury? Adult (250g) rats were subjected to unilateral stab wounds. At various days post-injury (dpi) sections were stained for PCNA (a marker for S-phase) and for various markers: GFAP (astrocytes), *Griffonia Simplicifolia* (GSA) lectin (microglia), and GD_3. By 2 dpi the ipsilateral side showed not only a typical reactive astrocyte response, but also a dramatic increase in number of GD_3+/GFAP- small cells with few processes. These cells were assumed to be glioblasts, but we now find they are mostly reactive microglia. Our initial studies showed that they did not react with the microglial markers ED-1, OX-42 or biotinylated GSA, but new studies show that near the wound GD_3 is present on a subpopulation of microglia that stains with GSA conjugated to HRP. At 2 dpi PCNA+ proliferating cells were mostly microglia and GD_3+ cells; by 3-4 dpi both GFAP+/GD_3- and GFAP+/GD_3+ astrocytes were also dividing; by 7 dpi PCNA+ cells were greatly reduced. Kinetic studies were then done in the same model. Four groups were injected with ^{3}H-thymidine (Thy) at 1, 2, 3 and 4 dpi and sacrificed at 2 h, 4 dpi and 7 dpi. If GD_3+ /GFAP- cells acted as precursors for astrocytes then such cells labeled at 1-2 dpi with Thy would decrease with time and GFAP+/Thy+ cells would increase. At 2,3 and 4 dpi about 1/3rd Thy+ cells were GD_3+/GFAP-, 50% were lectin+ microglia and the remainder astrocytes. By 7 dpi the GD_3+/GFAP-/Thy+ cells decreased by 50%, but GFAP+/Thy+ cells did not increase, nor did Thy+ microglia. Thus there is little generation of astrocytes from glioblasts. No dividing oligodendrocytes were detected. We conclude that injury induces transitory GD_3 expression on reactive microglia, that pre-existing astrocytes re-enter the cell cycle, and that some then acquire GD_3 expression. Cultures of Thy-labeled normal and injured brain confirm that GD_3+ precursor cells replicate in normal adult brain. Their numbers increase following injury and *in vitro* they differentiate to become both astrocytes and oligodendrocytes. In normal brain GD_3 is found on precursors in the sub-

ventricular zone, but in injured brain it is induced on both reactive microglia and some reactive astrocytes. These observations complicate the analysis of the fate of glioblasts *in situ* following injury.

Supported by USPHS grants NS02476 and NS23705.

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INJURY-INDUCED PROLIFERATIVE ACTIVITY OF ASTROCYTES IN THE DEVELOPING AND MATURE BRAIN

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I. Changes in the expression of glial fibrillary acidic protein (GFAP) and vimentin (VIM) were examined in astrocytes proliferating in the injured cerebral hemisphere of adult mice. The injury was followed by ^{3}H-thymidine injections at different time intervals. The brain sections were doubly immunostained for GFAP and VIM, and autoradiographed. Using the method, three types of proliferating cells were distinguished: GFAP+VIM+, and GFAP+VIM- astrocytes, and GFAP-VIM+ astrocyte-like cells. One day after the injury proliferation of GFAP+VIM+ and GFAP+VIM- astrocytes could only be seen as statistically insignificant phenomena. On day 2 the reactive proliferation of each cell type was maximal, then gradually decreased and its last signs were recorded on day 8. On day 2, among all the proliferating GFAP+ astrocytes, 67.2% were also VIM+. Later, the proportion declined to 50.7% and 38.5% on days 4 and 8 respectively. In astrocytes starting their proliferative response, VIM expression did not precede the expression of GFAP. The proliferating GFAP-VIM+ astrocyte-like cells were located closest to the lesion margins. In comparison, the GFAP+VIM+ and GFAP+VIM- astrocytes proliferated in regions progressively farther from the lesion site. The results were considered as arguments against the hypothesis that reactive astrocyte division induces a two-stage increase in the cytoskeletal protein level with the elevated VIM synthesis proceding that of GFAP. VIM or GFAP expression appears, therefore, to depend on the astrocyte location in relation to the injury site but not on the cell cycle.

II. The intensity of astrocyte proliferation in the injured cerebral hemisphere was examined in newborn, 6 and 30-day-old rats. ^{3}H-thymidine was injected at different time intervals following the injury. Brain sections were immunostained for S-100 protein and subjected to autoradiography. Thereafter, locations of autoradiographically labeled and S-100 protein-immunoreactive astrocytes were recorded. In newborn and 6-day-old rats, the reactive astrocyte proliferation began on the first posttraumatic day, whereas, in 30-day old rats it was observed on day 2. The maximal increase in the astrocyte proliferative activity recorded in 6 -day-old rats was about three times higher than that recorded in newborns and nearly twice as high as that in 30 day-old rats. The results suggest, therefore, that the intensity of astrocyte proliferative response to injury cannot be regarded as directly proportional to the developmental advancement of the injured brain.

This work was supported by the State Committee for Scientific Research, Grant No. 4 1780 91 01.

REMOTE ASTROCYTIC RESPONSES TO BRAIN LESION : INDUCTION OF NEUROTROPHIC ACTIVITY

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Most studies describe astrogliosis in the vicinity of brain wound. Remote responses of astroglia to brain lesions are less known. In our studies we have performed a partial lesion (mechanical or electrolytic) of the septohippocampal pathways in the rat, and we have investigated the responses of astroglia in structures

distant to the site of the lesion : in the septum (the source of innervation) and in the hippocampus (the target tissue). We have used the double immunostaining technique allowing a simultaneous localization of glial fibrillary acidic protein (GFAP) and nerve growth factor (NGF) immunoreactivity. A strong astrogliosis was noted one week after the lesion in both investigated structures. Moreover, many reactive astrocytes became NGF-immunoreactive. We have observed similar effects also one week after intraventricular administration of 15 U of interleukin-1β (IL-1β).Quantitative studies in which immunoblot technique was used for estimation of GFAP content, and ELISA for estimation of NGF content, have shown that the effects of the lesion and IL-1β administration are not additive, pointing to the suggestion that endogenous IL-1β plays a role in remote astrocytic responses including neurotrophic activity induction. To support this notion are also our other data indicating the increase of endogenous IL-1β both in the septum and hippocampus after a partial interruption of their connections. Remote reactions of astroglia, and especially induction of neurotrophic activity might contribute beneficially to the postlesion reparatory processes.

SESSION II : PATHOMORPHOLOGY

THE ROLE OF ASTROCYTES IN THE TRANSMISSIBLE BRAIN AMYLOIDOSES

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Astrocytosis is regarded as one of the neuropathological hallmarks of the transmissible brain amyloidoses, including kuru, Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker (GSS) syndrome. I report here the unique role of reactive astrocytes in the white matter damage of the panencephalopathic type of CJD. Initially the myelin sheath was separated by cytoplasmic tonques into several concentric bands. Astrocytic processes penetrated between layers of myelin and lifted away the outermost lamella. Then a complicated labyrinth of concentric cellular processes, clearly belonging to either astrocytes or macrophages invested myelinated axons. In terminal stages axons completely denuded of myelin were seen in the center of concentric networks of cellular processes. Myelin remnants were seen within astrocytes and macrophages. Reactive astrocytes overexpressed tumor necrosis factor-α. Furthermore, the intraocular injection of recombinant (r)TNF-α produces lesions in the optic nerve indistinguishable from those reported for the panencephalopathic type of CJD (Liberski, Yanagihara, Nerurkar and Gajdusek, unpublished data). The lesions were patchy and confined to the injected optic nerve. Axons show variable features of degenerations. Numerous vacuoles distended the myelin sheath. Hypertrophic astrocytes were numerous and many active macrophages containing digested myelin debris and lyre-like paracrystalline bodies. At high power, myelinated axons were observed as enveloped by astrocytic processes; formation of labyrinth-like network of such processes around damaged axons were observed. In conclusion, lesions produced by TNF-α mimic those of the panencephalopathic type CJD, in direct support of our previous ultrastructural, immunohistochemical and molecular data on TNF-α involvement in CJD pathogenesis.

GLIAL PARTICIPATION IN CEREBRAL AMYLOIDOSES

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Glia participation in the cascade of events leading to the appearance of amyloid within neuropil was a subject of this study. Seven Creutzfeldt-Jakob (CJD), three progressive supranuclearis palsy (PSP), three Gerstmann-Sträussler syndrome (GSS), ten Alzheimer's disease (AD), ten Parkinson's disease (PD) and

six normal aged cases were examined. Routine stainings (H and E, Klüver-Barrera) and Yamamoto silver impregnation, preceded avidin-biotin immunohistochemistry. The following antibodies were used: 4G8 for A 4, GFAP, RCA 1, LN 1, anti-ferritin, for visualization of astroglia and microglia respectively. Anti - tau 1 and 3. 39 anti - ubiquitin antibodies were chosen to label cytoskeletal alterations. The results showed proliferation of microglia to precede the appearance of diffuse and focal amyloid within areas free of senile plaques (SP) in AD and without PrP deposits in CJD. The first stage of "inflammatory" type was followed by the focal glial proliferation in the vicinity of SP. Almost no glial reaction was noted within areas positive for anti A4 immunohistochemical deposits. The presence of microglia in the center and astroglia in the periphery of SP (A4/PrP) has been repeatedly demonstrated and microglial function in lysosomal phagocytosis and extracellular proteolysis of amyloid is well documented. The latter seems to lead to the appearance of the fibrillar amyloid form. Through interleukin secretion, microglial cells also stimulate the proliferation of astroglia, which create a "scar", isolating the toxic amyloid from "healthy" neuropil.

ASTROCYTIC REACTION IN EXPERIMENTAL COMPLETE CEREBRAL ISCHEMIA INDUCED BY CARDIAC ARREST

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Astrocytes were previously considered as static, supportive elements of the brain. Over the last few years, a physiological role for astrocytes in ion regulation, in neurotransmission and in production of neurotrophic factors was established. Changes of astrocytic functions may play an important role in pathophysiology of cerebral ischemia. Thus, we examined the distribution and time course of the astrocytic reaction in the dentate hilus of the rat hippocampus after 10-min transient global cerebral ischemia (cardiac arrest model). Astrocytes were visualized in brain sections using antibodies against GFAP and S-100 protein following intervals of reperfusion ranging from 3h to 1 year. Increased staining and number of astrocytic cells were detected in the dentate hilus as early as 3h after reperfusion. The strongest reaction was observed 6h after reperfusion when reactive astrocytes were abundant throughout the dentate hilus. Cell hypertrophy and increased GFAP persisted only by 24h, whereas the number and size of GFAP-positive astrocytes returned to near control between 7 and 28 days. The lattice formed by the astrocytic processes was completely disturbed and astrocyte bodies were small and less numerous. Increased GFAP staining was prominent again at 6 months and was associated with hyperplasia. S-100 protein largely followed the pattern of GFAP. In general, postischemic changes were seen as astrocytic hypertrophy and hyperplasia. Hypertrophy was characterized by increased staining and enlargement of astrocytes, but the regular lattice of astrocytes was maintained. During hyperplasia, the pattern of astrocytes became irregular, double figures appeared and their number increased. The lattice appeared again. Glial reaction may be evoked by the damage of hilar neurons sending their processes to the molecular layer.

SESSION III : IONIC HOMEOSTASIS

THE SWOLLEN ASTROCYTE - A PATHOLOGICAL STATE?

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Astrocytes *in vivo* show two fundamental responses to CNS injury. 1) An early response characterized morphologically as a swelling of cell processes and/or the soma resulting in the appearance of an enlarged

and "watery" cytoplasm (1), or 2) a reactive astrogliosis usually occurring at later times and characterized principally as an increased cell size (hypertrophy). Increased density of GFAP(+) intermediate filaments in gliotic astrocytes has also been reported and more rarely an increase in the number of cells (hyperplasia) (2). Astrocytic swelling occurs in response to a wide variety of pathological states such as trauma and ischemia, and often reverses with time (1). While there have been detailed descriptions of the morphology of this response, the functional consequences are unknown. It can be viewed as a pathological extension of more limited and controlled volume changes which are otherwise part of the normal homeostatic functions of astrocytes. Evidence for astrocytic swelling seen in pathological states being deleterious will be presented; in a diffuse head injury model, administration of an anion transport inhibitor, L-644, 711 is associated with inhibition of astrocytic swelling and improved outcome (3,4), and in a rabbit thromboembolic focal ischemia model this same inhibitor causes decreased cerebral infarct size (5). In astrocyte cultures, L-644, 711 inhibits swelling-induced release of aspartate and glutamate due to hypotonic or high K^+ media (6,7), while at higher concentrations it also inhibits glutamate uptake (8). Work in progress using microdialysis with the gerbil ischemia model shows a similar biphasic effect of L-644,711 on glutamate release (Keller, Feustel and Kimelberg, unpublished observations).

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THE ROLE OF GLIA IN IONIC AND VOLUME HOMEOSTASIS DURING DEVELOPMENT, ANOXIA AND INJURY

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Glial cells control ionic, particularly K^+ and pH, homeostasis and ECS diffusion parameters. Activity-related changes in extracellular K^+ concentration ($[K^+]_e$) and pH (pH_e) were studied by means of ion-selective microelectrodes in the rat spinal cord. Concomitantly, the extracellular space (ECS) volume fraction (α), tortuosity (λ) and nonspecific cellular uptake (k'), three parameters affecting the diffusion of substances in CNS were measured. In adult rats electrical nerve stimulation (10 Hz) elicited increases in $[K^+]_e$ by 2.0-2.5 mM and alkaline-acid changes in pH_e with a dominating acid shift. The ECS space in the adult rat occupies about 20% ofthe tissue, α=0.20 ±0.003, λ=1.62±0.02 and k'=4.6±0.4 x $10^{-3}S^{-1}$ (SD, n=39). In pups at P3-6, the $[K^+]_e$ increased during the stimulation by as much as 6.5 mM, i.e. K^+ ceiling level was elevated, and there was a dominating alkaline shift. The decrease in $[K^+]_e$ ceiling level and the acid shift in pH_e at P10-14 were blocked by X-irradiation, the procedure which blocks gliogenesis. The alkaline, but not the acid, shift was blocked by Mg^{2+} and picrotoxin. Acetazolamide and Ba^{2+} enhanced the alkaline but blocked the acid shift. Application of glutamate or GABA evoked an alkaline shift in the pH_e baseline at P3-14 as well as after X-irradiation. The results suggest that the activity-evoked acid shifts in pH_e are related to membrane transport processes in mature glia, while the alkaline shifts have postsy-

naptic origin and are due to activation of ligand-gated ion channels. At P3-6 the ECS volume was almost double of that in adult rats, $\alpha = 0.37 \pm 0.01$ and the $\lambda = 1.78 \pm 0.02$ (n=17). In adult rats electrical stimulation or peripheral injury evoked a shrinkage of the extracellular space by 20-50%, while no significant changes in α were found in P3-6. It is evident that the changes in ECS diffusion parameters are closely related to gliogenesis and to activity-related swelling of glia. Consequently, the ECS diffusion parameters might be impaired during disease states. Indeed, in rats with experimental autoimmune encephalomyelitis or after X-irradiation the ECS volume was about double of that in control rats. During severe hypoxia the α decreased to 0.05 ± 0.006, while the λ increased to 2.00 ± 0.07 (n=12).

EFFECTS OF HEAVY METALS ON ASTROCYTIC HOMEOSTASIS: CORRELATION WITH -SH REACTIVE REAGENTS

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Compelling evidence favors direct astrocytic involvement in the neurotoxicity of several heavy metals. As early as 1966, Oyake suggested that mercury (Hg) preferentially accumulates within astrocytes. Lead (Pb), manganese (Mn) and bismuth (Bi) have also been reported to concentrate in astrocytes. Hg- (both organic and inorganic) treated astrocytes are incapable of maintaining transmembrane K^+ gradients. Furthermore Hg interferes with net uptake and release of L-glutamate and D-aspartate in primary astrocyte cultures. The observation that mercuric chloride (MC)-induced D-aspartate release was fully reversed by dithiothreitol (DTT), a membrane permeable compound, but not at all by the membrane non-permeable compound, glutathione (GSH), is consistent with MC modifying critical -SH groups associated with D-aspartate transport, either within the membranes or on their internal surface. Further information about the location of the -SH groups required for the D-aspartate release mechanisms was obtained with other -SH modifying reagents. The inability of iodoacetamide (IA) to impair D-aspartate release could be attributed to its low membrane penetrability due to its size and charge that restricts its action to -SH groups located at the extracellular membrane surface. N-ethylmaleimide (NEM) and methyl methanethiosulfonate (MMTS), both lipophilic -SH reactive reagents which readily cross through the cell membrane also had no appreciable effect on D-aspartate release in isotonic conditions. Taken together, the correlation between the effects of -SH modifying agents and D-aspartate release suggests that in isotonic conditions the -SH groups essential for D-aspartate release are located within membranous domains or on the internal surface of the astrocytic membrane.

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GLIA-DERIVED PERINEURONAL NETS AS A SPECIALIZED GLIA-NEURON INTERFACE

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The neuronal microenvironment may substantially be determined by glial cell processes and the extracellular matrix. Its high content in polyanionic proteoglycans seem to be especially characteristic for two specialized neuron-glia communication systems: perineuronal nets and nodes of Ranvier. Perineuronal nets composed of glial processes were visualized with the Golgi technique in cortical and subcortical brain regions (1). Similar net-like patterns in the neuronal microenvironment were shown to be formed

by the extracellular matrix components hyaluronectin and hyaluronan (2, 3), chondroitin sulphate proteoglycans (4, 5), and N-acetylgalactosamine (GalNac) containing glycoconjugates (6). It was the aim of our cytochemical studies to demonstrate glial structures forming, together with components of the extracellular matrix, a specialized glia-neuron interface. Using the glial markers GFAP, S100-protein and glutamine synthetase we could show that perineuronal nets were composed of perisynaptic astrocytic processes associated with polyanionic, GalNac-containing matrix material (7). These perineuronal nets were detected in more than 100 brain regions. Dual labelling demonstrated that GalNac-bearing nets are frequently situated around neurons which are parvalbumin-immunoreactive (8, 9). It is concluded that, similar to the ensheathment of nodes of Ranvier, perineuronal nets may provide a special ion buffering capacity in close vicinity to distinct types of neurons which might be required for high frequent or tonic discharge pattern.

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SESSION IV : NEUROTRANSMITTERS AND RECEPTORS

ROLE OF ASTROGLIA IN GLUTAMATE AND GABA HOMEOSTASIS: IMPLICATIONS FOR NEURONAL INJURY AND EPILEPSY

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Studies of uptake and release of the neurotransmitter amino acids glutamate and GABA and of their major precursor glutamine have shown that during neuronal activity there is a net loss of these transmitters from neurons with a corresponding net uptake into astroglial cells. Part of this loss is accounted for by transfer of glutamine from glia to neurons. At the same time there is a transfer of tricarboxylic acid (TCA) constituents as well as alanine from glia to neurons. These transfer processes for precursors of biosynthesis of the neurotransmitter amino acids are necessary due to the fact that neurons lack the enzymes glutamine synthetase and pyruvate carboxylase which are responsible for synthesis of glutamine and TCA constituents, respectively. The astrocytes accordingly are of key importance for the supply to the metabolically handicapped neurons of these precursors. This has been confirmed in co-cultures of neurons and astrocytes using ^{13}C-labelled glucose or acetate and NMR-spectroscopy. Label from acetate which is metabolized exclusively in astrocytes could only be found in neuronally synthesized GABA provided glutamine synthetase was active. Contrary, label from glucose being metabolized also in neurons could always be detected in GABA regardless of inhibition of glutamine synthetase. Using this technique it could also be shown that only astrocytes are able to synthesize and release alanine and the TCA constituent citrate. The transport processes in astrocytes for glutamate and GABA have been shown to be of functional importance for the maintenance of proper extracellular concentrations of the amino acids. The astrocytes accordingly are important for protection of neurons against the cytotoxic action of glutamate and they seem to regulate

the overall inhibitory balance. Manipulation of astrocytic GABA uptake using GABA uptake blockers with preference for astrocytic GABA uptake has been shown to be beneficial for control of seizure activity. One such compound, THPO (4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol) has been shown to protect mice against chemically induced convulsions.

GLUTAMATE RECEPTORS OF BERGMANN GLIAL CELLS

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Bergmann glial cells are a distinct population of astrocytes in the cerebellum and form intimate morphological interactions with Purkinje cells. Using the patch-clamp technique, we have studied Bergmann glial cells in cerebellar slices. We found two distinct types of glutamate receptors activated by kainate and NMDA, respectively. The kainate-type glutamate receptor showed a sigmoid current to voltage relationship and activation of the receptor led to an increase in $[Ca^{++}]_i$. This Ca^{++} elevation was blocked by CNQX and low external Ca^{++} and not mediated by the activation of Ca^{++} channels indicating an influx of Ca^{++} through the kainate receptor itself. The entry of Ca^{++} led to a marked reduction in the resting (passive) K^+ conductance of the cell. This $[Ca^{++}]$ increase and the concommitant blockade of K^+ channels was either triggered by activation of the kainate receptor or by application of a Ca^{++}-ionophore. The specific ligand NMDA also led to intrinsic responses in Bergmann glial cells: NMDA increased the membrane conductance and the responses were blocked by the NMDA antagonist ketamine, but not by the non-NMDA glutamate receptor antagonist CNQX. In contrast to responses in neurons, the current voltage relation of the glial NMDA induced current was linear, reversed at -40 mV, currents were not blocked by Mg^{++} or enhanced by glycine and NMDA did not induce an increase in cytosolic Ca^{++} as recorded with a fura-2 imaging system. These data imply the presence of distinct NMDA and kainate receptors on Bergmann glial cells. Since Purkinje cells with their glutamatergic synapses and Bergmann glial cells are morphologically closely associated, a (yet unknown) functional interaction becomes likely and the presence of these distinct receptors increases the possible complexity of neuron-glia interactions in the cerebellum.

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ASTROCYTES VS NEURONS IN HEPATIC ENCEPHALOPATHY AND HYPERAMMONEMIA

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Hepatic encephalopathy (HE) is a complex neurological disorder related to liver damage and subsequent release into the circulation of numerous neurotoxins, of which ammonia appears to be the main pathogenic factor. Current evidence implicates astrocytes as a primary biochemical and morphological target of ammonia. This presentation describes attempts to test a hypothesis that some of the neurophysiological manifestations of HE and hyperammonemia may be due to changes in the neuromodulatory functions and neurotransmission-related metabolic events in the astrocytes. Evidence in favor of this concept was derived from two different experimental approaches. In our laboratory, we have worked with fractions enriched in astrocytes, neurons and nerve endings derived from rats with hepatotoxin (thioacetamide) - induced HE. These studies revealed marked changes of neurotransmitter and ion transport in the astrocytes, and a relative resistance of both nerve cell compartments. Other groups have analyzed the *in vitro* effects of ammonium salts added to astrocytes cultured *in vitro*, with a roughly similar outcome.

One of the leading hypotheses related to the pathomechanism of HE is that of the failure of excitatory, glutamatergic neurotransmission. Therefore, we have recently focused on the effects of HE and simple hyperammonemia (HA) produced by administration of ammonium acetate on the enzymes involved in the synthesis and turnover of glutamate (GLU), in the synaptic and nonsynaptic, mostly astrocytic mitochondria. Identical results were obtained in both experimental models, emphasizing the leading role of excess ammonia load in HE. We found the enzymes involved in GLU synthesis: the malate-aspartate shuttle enzymes: malate dehydrogenase (MDH), aspartate aminotransferase (AAT), and the NADH-coupled form of glutamate dehydrogenase (G1DH-NADH) to be specifically inhibited in the synaptic mitochondria. The GLU-consuming form of G1DH (G1DH-NAD) was stimulated in synaptic mitochondria, as was 2-oxoglutarate dehydrogenase (2-OGDH)- an enzyme consuming 2-OG, which is a metabolic precursor of GLU. Pyruvate carboxylase (PC), an astroglia-specific anaplerotic enzyme furnishing precursors for synaptic GLU synthesis, was inhibited in nonsynaptic mitochondria. The results allow to ascribe HE-induced inhibition of GLU-ergic transmission to depletion of synaptic GLU resulting from both its decreased synthesis and increased consumption, and point to a concerted contribution of the synaptic and astrocytic compartment to this effect.

CYTOKINE INDUCTION OF NITRIC OXIDE SYNTHASE IN GLIAL CELLS

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Glial cells display both constitutive (astrocytes) and inducible (astrocytes, microglia) nitric oxide synthase (NOS) activity under various conditions in vitro. NOS can be induced in primary rat astrocytes and C6 glioma cells with endotoxin (1), seemingly via cytokine mediators(2). Using a cDNA for the macrophage inducible NOS and northern blotting, mRNA is detected in astrocytes after two hours exposure to a combination of interleukin (IL)-1β and interferon (IFN)γ, and message persists for at least 8 hours. Phorbol myristate acetate (PMA) alone induces NO synthase to a small extent, potentiates the effects of endotoxin, and is as effective as IL-1β in combination with IFNγ. These effects of PMA are not seen either in protein kinase (PK)C-depleted cells or in the presence of the protein kinase inhibitor H7. This does not mean that cytokines activate NOS gene transcription via PKC. Rather, PMA may cause the release of IL-1β or other cytokines, as suggested by experiments with neutralizing antibodies. Induction of NOS in the CNS has been reported recently in animals treated with endotoxin or infected with viruses, and after induction of experimental allergic encephalitis. The ability of astrocytes to release NO in response to cytokines suggests a role for these cells in CNS-immune interactions.

1. Simmons M.L. and Murphy S. (1992) J. Neurochem. 59: 897
2. Simmons M.L. and Murphy S. (1993) Eur. J. Neurosci. 5: in press

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GLUTAMATE-INDUCED GENE EXPRESSION IN GLIAL CULTURES

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L-glutamate acts as a major excitatory neurotransmitter in the mammalian central nervous system. Over the last few years it has been increasingly clear that L-glu can also act as neuromodulator, producing long lasting modifications of functioning of nervous tissue including e.g. plastic changes or neurodegeneration. Besides neurons also glia are endowed with L-glu responsive receptors, providing also targets for L-glu activity. In our studies we have investigated whether L-glu, through interaction with specific subtypes of

its receptors may stimulate gene expression. We decided to focus on immediate early genes encoding transcription factors (TFs) as they provide particularly interesting models. Their mRNA and protein levels are very low in non-stimulated cells and increase dramatically upon cell activation (1) what makes them relatively easy to study, and moreover, their protein products are of particular significance as they are involved in control of gene expression. We have initially found (2) that c-fos mRNA levels are elevated in newborn rat brain astroglia in culture following stimulation with various ligands such as epidermal growth factor (enhancing cell proliferation), ibotenate (leading to decrease in cell cycle level) and isoproterenol (inhibiting cell proliferation and producing morphological differentiation). The fact that ibotenate, L-glu agonist, provoked c-fos mRNA accumulation prompted us to investigate effects of a spectrum of L-glu agonists and antagonists on expression of genes coding for transcription factors. We have found (3) that in cultured astroglia, 100 μM L-glu activates transient elevation of c-fos, c-jun, jun B and zif/268 but not jun D and c-myc mRNAs. Similarly, 100 μM kainate, 100 μM AMPA, 100 μM quisqualate and 100 μM tACPD activated expression of c-fos and zif/268. The L-glu effects were not reversed by MK 801, kynurenate or DNQX. Kainate effects were, however, abolished by kynurenate, and AMPA effects were blocked by DNQX. The tACPD driven c-fos mRNA accumulation was not inhibited, but on the contrary, potentiated by L-AP3. In conclusion, we can say that both metabotropic and ionotropic, but not the NMDA receptors could transmit the gene activating signals in cultured astroglia. Further studies (4) revealed that in result of L-glu treatment the DNA binding AP-1 transcription factor is formed. On the contrary, L-glu have not elevated DNA binding activities of two other TFs, namely, CREB and NFkB.

1. Kaczmarek L., Kamińska B. (1989) Exp.Cell Res., 183: 24-35.
2. Condorelli D.F. et al. (1989) J. Neurosci. Res., 23: 234-239.
3. Condorelli D.F. et al. (1993) J. Neurochem., 60: 877-885.
4. Łukasiuk K. et al. (submitted)

SESSION V: OLIGODENDROGLIA AND MICROGLIA : DEVELOPMENTAL ASPECTS

EFFECTS OF GROWTH FACTORS ON MYELINATION AND REMYELINATION OF AGGREGATING BRAIN CELL CULTURES

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Demyelination was induced in aggregating brain cell cultures using a monoclonal antibody against myelin/oligodendrocyte glycoprotein (MOG) in the presence of complement. De- and remyelination were assessed by measuring myelin basic protein (MBP). 2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP) was used as an oligodendrocyte marker and glutamine synthetase (GS) as an astrocytic marker. MOG mAb concentrations were chosen to obtain, after two days, a reduction of MBP concentration corresponding to 35% of control cultures treated with normal mouse IgG and complement. Seven days after having removed anti-MOG and complement, and restored the normal serum-free chemically defined medium, the cultures fully remyelinated. Experiments using ^{14}C-thymidine incorporation showed that remyelination occurred from preexisting oligodendrocytes. When demyelinated cultures were exposed to PDGF AA, cells proliferated but no remyelination occurred. After PDGF AA removal, newly formed oligodendrocytes differentiated and myelinated. Treatment with arabinoside C destroyed proliferating oligodendrocytes but partial remyelination still occurred indicating that surviving mature, non-dividing oligodendrocytes were able to remyelinate. Under those experimental conditions, GS levels remained stable indicating that PDGF AA affected only oligodendrocyte proliferation.

EXPRESSION OF MYELIN-SPECIFIC PROTEINS AND OLIGODENDROCYTE MATURATION IN HYPOMYELINATING PT RABBIT MUTANT

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Paralytic tremor (pt) is a spontaneous, sex-linked, recessive mutation which affects myelination in rabbit CNS. The neurological symptoms, such as rapid tremor and progressive limbs paresis appear during the second week of life and usually recede with maturation. The life-span of majority of animals is only slightly reduced, although some rabbits develop a severe, progressive disease and expire within 3-6 months. The extent of myelin deficit is variable in the individual cases depending on the severity of symptoms and animal age. The aberrations in myelin sheaths such as their thickness, uncompactness and other irregularities are noticed in all CNS structures together with the morphological and functional signs of oligodendrocyte underdevelopment. This results in the reduction of myelin-specific galactosfingolipids (cerebrosides and sulphatides), unmatured pattern of myelin-basic-protein (MBP) and proteolipid-protein (PLP) isoforms, increased content of monosialoganglioside (GM1) and enhanced calcium-activated-proteinase (CANP-ase) activity. Preliminary experiments showed also increased content of protein kinase C (alfa and beta isoforms) in pt homogenate. Pt mutation affects the developmental expression of most of the major myelin proteins (PLP, MBP, MAG, MOG, CNP-ase) as revealed by Western blots of brain homogenates. However, except of PLP, reduction of these marker proteins mirrors most probably the myelin depletion and oligodendrocyte unmaturation in pt rabbits. On the other hand, the exclusively hard reduction of PLP in pt homogenate and myelin during the entire developmental period studies (from 1 up to 120 days post partum) together with the known mapping of pt trait on X chromosome and synchronised reduction of PLP mRNA expression, strongly suggests the involvement of PLP gene mutation. The mutation, however, seems to affect PLP gene in a less vulnerable point generating comparatively more benign phenotype than in all other PLP mutants. This phenotype is characterised by a transient inhibition of oligodendrocyte differentiation and maturation resulting in delayed and prolonged production of pathological myelin.

IMMUNOHISTOCHEMICAL STUDY OF MICROGLIA DURING THE DEVELOPMENT AND AGING OF HUMAN BRAIN

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Microglia plays a significant role in the normal and particularly in the pathologically-altered brain. Functionally, microglia is the main element involved in several human neurological disorders. The ontogeny and morphology of microglia in the mesencephalon have been the focus of attention of neuropathologists interested in the central nervous system degenerative diseases. To assess the cytogenesis and the structure of these glial elements, we studied mesencephalon in 47 human fetuses at 7-40 weeks of gestation age, and in 18 adult brains from 20 to 70 years old. Microglia was identified and characterized by morphological criteria using immunohistochemistry for ferritin and histochemistry for Ricinus Communic Agglutinin-1 (RCA-1). As early as at 8 weeks of gestation, RCA-1 positive cells (rounded cells without processes - amoeboid microglia) were detected in fetal mesencephalon in the vicinity of germinal matrix, in the perivascular area or in the brain pia. At 16-40 weeks of gestation age, there was a variability in the morphology of microglia. The forms observed in mesencephalon included: amoeboid microglia, ramified

microglia (cells with a few long processes) and perivascular microglia with processes touching the wall of vessels. These three categories of microglia were observed in all adult mesencephalons from 20 to 70 years old. The results show that mesencephalic microglia cells appear very early in the ontogeny and do not show marked pathological changes during brain aging.

POSTERS

SPECIFIC INHIBITION OF OLIGODENDROCYTE PROLIFERATION BY A FACTOR FOUND IN A HUMAN PLACENTA EXTRACT

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Placenta is a potential source of growth factors, among which the best known are: bFGF, EGF, I-LGF, NGF, which play an important role in CNS development. Besides these positive factors, others were found in the placenta which may have a positive, as well a negative effect on CNS cells. These factors include: TGFβ, TNFα and INFα. This study dealt with the effect of human placenta extracts on pure central nervous system cell cultures based on proliferation tests. To test the placenta extracts, pure cultures of astrocytes and oligodendrocytes isolated from the brain hemispheres of newborn rats were used. Secondary cultures of oligodendrocytes were prepared from mixed glial primary cultures. Human Placenta extracts (EAP 1306, Imedex) containing proteins with a molecular weight over 10,000 D were fractioned by Aca 54 (gel filtration chromatography) and a FPLC system using Mono Q and Mono S ion-exchange columns. The obtained fractions were checked using proliferation tests on astroblasts and oligodendroblasts. Proliferation was measured by testing the 24 hour incorporation of ^{125}J-dUrd 16 hours after adding the chromatographic fractions to the cell cultures. It was found, that: (1) Human placenta extract protein is mitogenic for both astroblasts and oligodendroblasts of rats *in vitro*. (2) Division of the placenta extract with Aca 54 filtration gel allows the isolation of chromatographic fractions which stimulate astroblast and oligodendroblast proliferation and fractions which inhibit oligodendroblast proliferation. (3) The FPLC system with the Mono Q and Mono S columns that were used confirms the presence of fractions that act mitogenically on astroblasts and oligodendroblasts. (4) Fractions from the Aca 54 column which specifically inhibit oligodendroblast proliferation block the mitogenic action of bFGF, which was confirmed using the FPLC system.

EFFECT OF NOOTROPICS ON INCORPORATION OF ^{3}H-VALINE AND ATP LEVEL IN RAT ASTROCYTE CULTURE

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Protein synthesis, energy metabolism and cell morphology are parameters which allow to examine the influence of drugs on cells in culture. We used the primary culture of astrocytes for this purpose. The effect of short-term treatment with nootropics (piracetam, oxiracetam, aniracetam, pramiracetam, tenilsetam) as well as Vinca alcaloids (vincamine, vinpocetine) on intracellular content of ATP and ^{3}H-valine incorporation into astrocytes was studied. Astrocytes derived from cerebral hemispheres of neonatal rats were prepared and maintained by the method of Hertz et al. The astrocytes were cultured with or without dibutyric cyclic AMP (db-cAMP). At day 13 of the experiment, astrocytes were exposed to each drug (10 M) for 24h. At the end of the experiment astrocytes were incubated with ^{3}H-valine (3 μCi/dish) for 3h. The in-

corporation of ^{3}H-valine into astrocytes was measured radiometrically, ATP content was determined by bioluminescence method. It was shown that short-term treatment with piracetam and its analogues decreased the ATP content only in astrocytes cultured with db-cAMP. The drugs decreased (excluding oxiracetam) ^{3}H-valine incorporation into astrocytes, too. In astrocytes cultured without db-cAMP the ATP content was increased after treatment with Vinca alkaloids, whereas in astrocytes cultured with db-cAMP only increased the ATP content. Moreover, vinpocetine decreased ^{3}H-valine incorporation into astrocytes. These results indicate that in spite of similar application in clinical practice nootropics and Vinca alkaloids differently affect ATP- content and ^{3}H-valine incorporation into astrocytes *in vitro*.

MORPHOMETRIC ANALYSIS OF CULTURED ASTROCYTES *IN VITRO* AFTER SHORT-TERM TREATMENT WITH NOOTROPICS

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Astrocytes are the most numerous cell type in the mammalian central nervous system. Primary cultures of astrocytes from neonatal rat brains have proven to be an excellent model for studying the effect of drugs and toxins on astrocyte properties. The aim of the present study was to examine the effect of nootropics (piracetam, oxiracetam, aniracetam, pramiracetam and tenilsetam)and Vinca alkaloids (vinpocetine, vincamine) on the morphological changes of astrocytes. Primary astrocyte cultures were preparated from rat cerebral cortex as described by (Hertz et al.). The following morphometric parameters were determined for astrocytes in primary culture: perimeter, area of cells and form factor (as an index of the number of processes per cell). The computerized image analysis system (VIDS IV) was used to measure these parameters. 14-day cultures with or without dibutyryl cyclic AMP (db cAMP) were used in the experiment. After 24-h exposure to nootropics in concentration of $1x10^{-4}$M, astrocytes were stained with toluidine blue. It was shown that nootropics (excluding vincamine) increased area of cells in culture with db cAMP. Perimeter of cells was increased after treatment with piracetam, oxiracetam and decreased after treatment with Vinca alcaloids in culture without db cAMP. In culture with db cAMP, vincamine increased perimeter of cells. Nootropics increased the form factor of astrocytes (excluding piracetam in culture without db cAMP). It has been demonstrated that nootropics alter the morphology of astrocytes in primary cultures.

CHANGES IN CYTOSKELETAL PROTEINS IN RAT BRAIN AFTER TRIMETHYLTIN (TMT) ADMINISTRATION

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Trimethyltin (TMT) is a widely used industrial chemical which causes several neurological disturbances in man. A single, systemic exposure of this neurotoxin to rat produces a specific morphological pattern of brain damage, especially in the hippocampus. In the present study we investigated neuronal and astroglial responses to TMT administration in rat hippocampus monitored by immunocytochemistry for microtubule - associated protein-2 (MAP-2) and glial fibrillary acidic protein (GFAP), respectively. Single staining for MAP-2 and GFAP as well as double staining for both proteins was performed. A monoclonal mouse anti-MAP-2 (Sigma) and a monoclonal mouse anti-GFAP (Boehringer) antibodies were used. We have especially focused on examinations at 3 weeks postexposure. Our results suggest that there is a differential response of MAP-2 to TMT exposure: a loss of MAP-2-IR in CA4/CA3 which most probably reflects massive loss of pyramidal neurons in these areas and an increase in dendritic MAP-2-IR in dentate molecular layer which might be associated with some compensatory mechanisms after neurotoxical dam-

age and/or changes in phosphorylation state of the protein. A strong enhancement of GFAP-IR in CA4/CA3 and CA1 was found which correlates with a decrease in MAP-2-IR in these areas. This may suggest that the density of reactive astrocytes correlates with the sites of neuronal cell injury. Comparison of single staining patterns of MAP-2-IR and GFAP-IR and additionally double-staining for both proteins indicate that after neurotoxin exposure the microtubule protein is probably not involved in the process of reactive gliosis as has been reported for a stab wound model (1).

1. Geisert et al. (1990) PNAS USA, 87: 3967-3971

ARGINASE IN HUMAN BRAIN GLIOMAS

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Arginase (EC 3.5.3.1) from human brain gliomas was characterized and compared with the enzymes in human brain meningiomas and neurinomas. In gliomas, the mean arginase activity was 15.9±8.1 U/g protein or 0.97±0.07 U/g wet tissue, and was significantly higher than in meningiomas (9.2±2.1 U/g protein or 0.65±0.08 U/g tissue) and neurinomas (8.2±0.32 U/g protein or 0.38±0.09 U/g tissue).

Arginase from human gliomas required preincubation with Mn^{2+} at final concentration of 5 mmol/l for 20 min. at 55°C for complete activation. No difference was found in the activation pattern between arginase from gliomas, meningiomas and neurinomas. Rates of activation with Mn^{2+} were 1.9, 2.1 and 1.8, respectively. Glioma arginase was labile on storage losing about 70% of its initial activity in one day at 4°C and about 30% during 7 days at -10°C. In this respect arginases of all human brain tumors tested were similar. The pH optimum of arginase from gliomas as well as meningiomas and neurinomas was 9.8. The K_m of the glioma arginase for L-arginine at pH 9.8 was 4.7±0.6 and did not differ significantly from the values obtained for meningiomas (4.3±0.5) and neurinomas (5.0±0.7). In all gliomas tested three forms of arginase (A_1, A_3 and A_4) were detected by DEAE-cellulose column chromatography. Arginase A_1 (cationic form) represented on the average 24% of the total enzyme activity, and anionic arginase A_3 and A_4, 28% and 48%, respectively. Isoenzymatic pattern of arginase in gliomas was related to the tumor histology. In astrocytoma anaplasticum arginase A_4 was the main form and represented more than 70% of the enzyme activity whereas in glioma mixtum and glioblastoma multiformae 45% and 26%, respectively. Arginase A_4 seems to be characteristic for gliomas, it was never found in other human brain tumors tested.

MERCURIC CHLORIDE LOWERS THE THRESHOLD FOR GLUTAMATE NEUROTOXICITY IN RAT CEREBELLUM *IN VITRO*: AN ASTROGLIA-MEDIATED EFFECT ?

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Separate exposure of organotypic cultures, derived from newborn rat cerebellum, to nontoxic concentration of either 100 µM glutamate (GLU) or 1 µM mercuric chloride (MC), for as long as 3 days, produced no distinct ultrastructural changes in neurons and glial cells. By contrast, simultaneous exposure to both agents resulted, as early as after 30 minutes, in microvacuolar degeneration of neurons and later on in postsynaptic abnormalities, typically accompanying excitotoxic lesions but not heavy metal-induced lesions. The results indicate that MC at low micromolar concentrations lowers the threshold for GLU neurotoxicity, which may reflect MC-induced inhibition of astrocytic GLU uptake.

EFFECT OF ANTIDEPRESSANTS ON INCORPORATION OF ^{3}H-VALINE AND ATP LEVEL IN RAT ASTROCYTE CULTURE

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Primary culture of astrocytes as a model for studying drug actions and toxic effect on cellular level is used. Impairment of protein synthesis, energy metabolism and cell morphology are the parameters usually used to estimate drug action. The aim of the study was to examine the effects of short-term treatment with antidepressants on the intracellular ATP content and ^{3}H-valine incorporation into astrocytes *in vitro*. Imipramine, amitriptiline, clomipramine and doxepine as tricyclic derivatives; mianserin and maprotiline as tetracyclic antidepressants were tested. Astrocytes derived from cerebral hemispheres of neonatal rats were prepared according to method of Hertz et al. Fourteen-day old cultures treated with or without dibutyryl-cAMP (db-cAMP) were used for the experiment. Except maprotiline (10^{-5} M), other antidepressants were added at 10^{-4} M concentration. A 3h incubation with ^{3}H-valine (3 μCi/dish) was performed to estimate protein synthesis. ATP content was measured by bioluminometric method. It was shown that: (1) Short-term exposure to antidepressants caused an increase of ATP level in cultured astrocytes. Maprotiline was the only antidepressant which decreased ATP content in culture with db-cAMP. (2) All examined antidepressants lowered ^{3}H-valine incorporation into astrocytes *in vitro* independently of the presence or absence of db-cAMP.

INFLUENCE OF ANTIDEPRESSANTS ON THE MORPHOLOGY OF ASTROCYTES *IN VITRO*

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Cell culture is a convenient pharmacological tool for testing the cytotoxic effects of drugs. Glial cells are a potential target for antidepressants. Astrocytes derived from cerebral hemispheres of neonatal rats were prepared according to the method of Hertz et al. Two-weeks old cell cultures (with and without dibutyryl-cAMP; db-cAMP) were treated with antidepressants. Changes in cells morphology were evaluated after short-term (24h) incubation with tricyclic (amitriptiline, imipramine, clomipramine, doxepine (all 10^{-4} M) and tetracyclic (mianserin 10^{-4} M, maprotiline 10^{-5} M) antidepressants. Cell perimeter, area and form factor as attributes of morphology were analysed. A computerized image analysis system (VIDS IV) was used to measure those parameters. It was proved that tricyclic compounds (in cultures with and without db-cAMP) diminished the cell area, whereas tetracyclic, produced variable effects. Similar differences in perimeter of cells were observed. Astrocytes treated with amitriptilline (in the absence of db-cAMP) enlarged the value of form factor opposite to mianserin which diminished it. Other antidepressants did not significantly alter the form factor value. Both tricyclic and tetracyclic drugs - in the presence of db-cAMP - caused an extension of this parameter.