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REAKCJA ASTROGLEJU WE WCZESNYM OKRESIE TOKSYCZNOŚCI OŁOWIOWEJ U SZCZURA W ŚWIETLE ZALEŻNOŚCI ASTROCYT-NEURON.

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The protective role of astroglia in the early period of experimental lead toxicity in the rat

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Abstract. Although the clinical manifestations of lead (Pb) neurotoxicity are documented, the subcellular mechanisms of its action are still an open question. The purpose of this study was to assess the function of nerve ending particles after acute lead exposure and to investigate whether it exerts a toxic effect on astroglial functions. The studies were performed using the rodent model of acute lead toxicity. Cellular fractions were used in biochemical measurements – synaptosomes and glial plasmalemmal vesicles (GPV). Since a procedure for the isolation of the fraction of astroglial origin has been developed, it becomes possible to investigate lead-astroglia interactions after *in vivo* exposure. It is of importance because most of the studies concerning lead toxicity were performed using astroglial culture systems. It was found that the uptake of glutamate (Glu) to the synaptosomes was lowered and KCl-dependent release was increased, suggesting the impairment of glutamatergic transmission leading to the elevation of extracellular amino acid concentration. In contrast, glutamate uptake to the GPV fraction was significantly elevated. The activity of the marker enzyme – glutamine synthetase (GS) was also significantly increased in the GPV fraction. The activation of glial functions suggest a regulatory role for these cells in the early period of acute lead toxicity.

Key words: acute lead toxicity, astroglia, glutamine synthetase, glutamate uptake

INTRODUCTION

Lead (Pb) – a nonphysiological metal and environmental pollutant – is presented to most of the general human population below the level of toxic effect. However, its cumulative properties, which may lead to the exceeding of the "safe level", were demonstrated (Bercovitz and Laufer 1991, Mushak 1993). Despite a long history of scientific investigation, Pb toxicity remains a significant health problem of both industrial workers and those exposed environmentally (especially children). The collective data from *in vivo* experiments (Holtzman et al.1984) and from culture systems (Tiffany-Castiglioni et al. 1986, Holtzman 1987, Aschner and LoPachin 1993) suggest that astroglial cells posses the ability to sequester heavy metals, including lead. Holtzman (1984) proposed that astroglia serve as a "lead-sink" to protect neurons which are more sensitive to the toxic Pb effect. On the other hand, the glial Pb store may constitute a reservoir for its continuous release and thereby contribute to the toxicity of adjacent neurons (Holtzman 1987). Thus, it is of interest to assess to what extent astroglia can adapt to and tolerate the presence of intracellular lead.

The crucial role of astroglia in regulating the environment of adjacent neurons is beyond argument. One of most important roles is the regulation of glutamate (Glu) concentration. It is known that astrocytes are involved in the modulation of synaptic activity by the regulation of the extraneuronal level of released Glu (Vernadakis 1988). Glutamate, the predominant excitatory amino acid neurotransmitter in vertebrate brain, is taken up by glial cells after release from neurons and amidated to the non-neuroactive glutamine by the enzyme glutamine synthetase (GS), which is specific to astrocytes (Martinez--Hernandez et al. 1977, Norenberg, 1977). Thus, when that regulative mechanism cannot act properly, it may lead to excitotoxic damage of neurons. Taking into account that the ratio of astrocytes to neurons is 10:1 and approximately 30% of brain volume consists of these cells (Aschner and LoPachin 1993), it is probable that they may actively participate in brain injuries, also in that of toxic origin. So it becomes interesting, if not necessary, to assess the glia involvement in brain function after acute *in vivo* lead exposure.

Previous studies on glial toxicity by lead were mainly performed using cultured glial cells. In contrast to synaptosomes, which are a good model for nerve ending studies, there were no preparations available for the study of glial functions after *in vivo* exposure, until recently.

The present studies were undertaken to estimate the possible alterations in glial cells (as measured with the glia-derived fraction) that may occur in adult rat brain after acute lead exposure. Synaptosomal glutamate uptake and release, together with the response of the glial fraction, were investigated.

METHODS

Animal treatment

Male Wistar rats were used in the experiments of acute lead toxicity. Animals were injected intraperitoneally with lead acetate at a concentration of 25 mg/kg b.w. for 3 days. Controls were treated with distilled water. Pb doses and distilled water doses were administered at a constant small volume of 100 µl. During the time of experiments, animals had unrestricted access to the standard laboratory chow. In the group of lead-exposed rats we did not notice either incidents of death, or symptoms of severe lead toxicity like seizures.

Preparation of GPV fraction

A Percoll density gradient centrifugation technique by Daniels and Vickroy (1998), with slight modifications according to Nakamura and coworkers (Nakamura et al. 1993), was used to isolate glial plasmalemmal vesicles termed GPV. Briefly, forebrains from 2 rats were homogenized in 30 ml medium (0.32 M sucrose and 1 mM EDTA) and centrifuged at 1,000 g for 10 min using a fixed-angle rotor. The supernatant was diluted using a SEDH solution containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM dithiothreitol and 20 mM HEPES (pH 7.4) and centrifuged at 5,000 g for 15 min. Thereafter, the resultant supernatant was saved and the pellet resuspended in a new portion of SEDH solution and centrifuged at 1,000 g for 10 min. Both supernatants were combined into one and centrifuged at 33,500 g (20 min). Pellets, suspended in SEDH, were centrifuged in a three--step discontinuous Percoll gradient (20% : 10% : 6%) at 33,500 g for 10 min. The layer between 0% and 6% Percoll was collected and centrifuged 1,000 g (20 min) after dilution in SEDH. The supernatant was centrifuged at 33,500 g for 20 min to obtain the pellet – glial plasmalemmal vesicle fraction (GPV). The resultant pellet was suspended in SEDH solution and used for glutamate uptake measurements or for determining glutamine synthetase activity.

Preparation of synaptosomal fraction

The synaptosomes were prepared according to the Booth and Clark method (1978). Rats were decapitated and forebrains homogenized in sucrose buffer (0.32 M sucrose, 1mM EDTA, 10 mM TRIS-HCl, pH 7.4). Samples were centrifuged at 1,300 g for 10 min and subsequently 17,000 g for 10 min. Then, the pellet was centrifuged in a discontinuous Ficoll gradient (7%/12%) 99,000 g for 30 min. Each fraction for further examination was obtained from one rat.

Glutamine synthetase assay

The enzyme activity in GPV samples was determined according to the method of Pishak and Philips (1979). A reaction mixture contained equal amounts (5 µl) of 500 mM imidazole-HCl, 150 mM $MgCl_2$, 100 mM ATP, 40 mM NH_4Cl, 10 mM dithioerithritol (DTE) and L-[G-^{3}H] glutamic acid (specific activity 1.06 mCi/mmol) mixed with unlabeled glutamic acid to a conc. of 75 mM. After the addition of 20 µl of GPV fraction, the reaction mixture was incubated for 30 min. at 37°C. The reaction was stopped with 1 ml of cold water and transferred to two stacked ion exchange columns (Dowex-1-H+ on top of Amberlite CG-50, H+) to separate the reaction product (^{3}H-glutamine) from other labeled compounds. Each column was eluted with 3 ml of cold water. The eluent was collected and radioactivity measured. Results are expressed as specific activity in nmoles glutamine per min per mg protein.

[^{3}H]-glutamate uptake

The GPV and synaptosomal fractions were used for glutamate uptake according to the filtration method described by Divac (Divac et al. 1977). For measurements of glutamate uptake, aliquots (50 µl) of fractions (0.8-1 mg protein/ml) were added to 400 µl of buffer (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 20 mM HEPES, pH 7,4). Isomolar concentrations of choline chloride were used instead of NaCl to measure sodium-independent uptake. Assay duplicates were preincubated in a shaking water bath at 30°C for 5 min. After the addition of 50 µl of radioactive glutamate (f.c. 5 µM, specific activity 45 Ci/mmol), the reaction was stopped by filtration under vacuum through a Whatman GF/B glass filter at the several time points. Tubes and filters were washed 3 times with 2 ml of cold normal or sodium-deficient buffer, respectively, and the activity counted after soaking the filters in 1 ml of 10% Triton X-100 for 10 min. Radioactivity trapped on the filters was measured by a liquid scintillation spectrometer. Sodium--dependent uptake was assessed as the difference between uptake in the two above mentioned buffers. The time course of glutamate uptake was done and the maximum was found to be in 4th min (synaptosomes) and 2nd min (GPV). In the case of synaptosomal fractions, the KCl-dependent release of taken up [^{3}H]-glutamate was measured after adding 50 mM KCl in the 4th min of the uptake process.

Measurement of lead content

The lead levels in blood and brain homogenate were measured by an atomic spectrometer with graphite furnace.

Protein measurement

The amount of protein in GPV and synaptosomal fractions was determined by the method of Lowry and coworkers (Lowry et al. 1951) using bovine serum albumin as a standard.

Data analysis

All values are expressed as a mean ± SEM from the number of experiments stated in legends to the figures. The number of experiments corresponds with either the number of animals (Table I) or the number of separately and freshly isolated fractions (Figs. 1-3). Single fraction used for one measurement was obtained either from 1 (in the case of synaptosomes) or from 2 (in the case of GPV) experimental animals and their respective controls. Statistical significance was determined using the Student's *t* test and accepted at a level of $P<0.05$.

RESULTS

The lead level

The lead dose received by the exposed rats produced blood levels of metal within the high concentration range, as observed in acute lead poisoning (Goyer and Chisolm 1972). Blood lead levels were much higher in lead-exposed rats than controls and reached 97.2 µg/dl. A significant penetration of lead from blood to brain was noticed. The amount of lead in the forebrain homogenates of exposed rats was 1.8 µg/g w.w. (Table I).

TABLE I

Lead levels in blood and brain homogenates of the control and lead-treated rats in the acute model of toxicity

Sample	Lead level	
	Control group	Lead-exposed group
Blood (μg/dl)	2.0 ± 0.2	97.2 ± 3.0
Brain homogenate (μg/g w.w.)	<0.019*	1.8 ± 0.4

Data expressed the means ± SD of 4 independent measurements performed on samples derived from 4 animals in each group. *Below the sensitivity of applied method.

Uptake and release of GLU in synaptosomes

Uptake of glutamate to the synaptosomes was impaired in a fraction obtained from Pb-treated rats. The decrease (calculated in maximum of uptake curve i.e. 4 min.) reached 25%. The KCl-dependent release of glutamate from the synaptosomal fraction was found to be enhanced in Pb-treated rats by about 20% (Fig. 1).

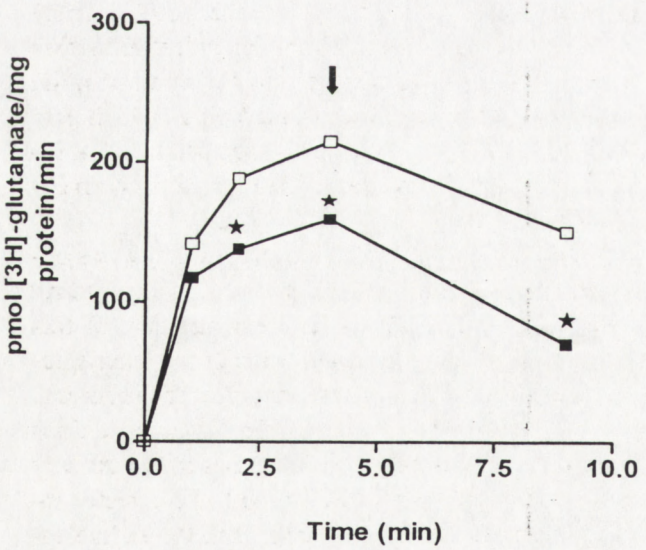

Fig. 1. Na^+-dependent uptake and release of $[^3H]$-glutamate in synaptosomal fractions obtained from control (open square) and Pb-treated (filled square) rats. The release was measured after adding 50 mM KCl in the 4th min of the uptake curve (arrow). Points are means ± SEM of 6 independent experiments, each performed on 6 separately prepared fractions in both control and experimental group. SEM was less than 5%. *The values are significantly different at the level of $P<0.05$.

GLU uptake to GPV

The major component of astrocytic glutamate uptake (high affinity transport system) is Na^+-dependent (Schousboe et al. 1977). It was determined as the difference between total and Na^+-independent uptake. Total glutamate uptake was determined in a buffer system with Na^+, Cl^- and Ca^{2+} ions, all of them identified to be necessary in that process (Flott and Seifert 1991). Na^+-independent uptake was determined with a buffer lacking sodium ions.

That astroglial high affinity glutamate uptake measured in the GPV fraction obtained from rats after Pb exposure, was found to be enhanced. The increase reached 45% in the maximum (2nd min) of uptake (Fig. 2).

Glutamine synthetase activity

Glutamine synthetase amidated glutamate released by neurons to nontoxic glutamine. A marked activation of specific activity of the enzyme was noticed as the result

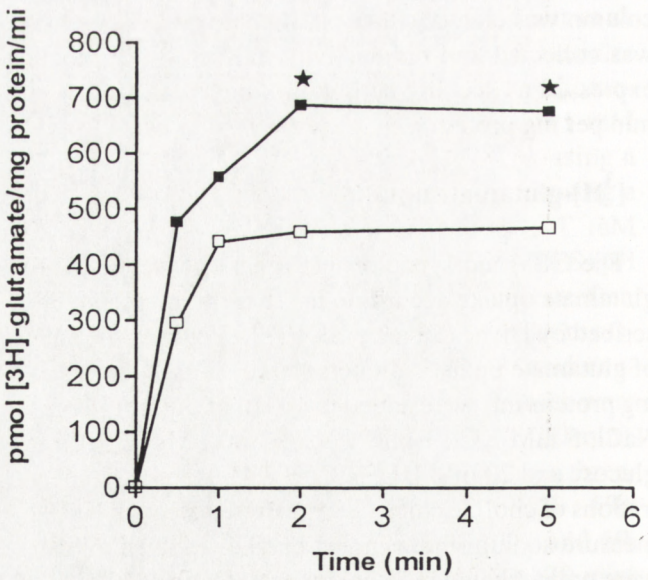

Fig. 2. Na^+-dependent uptake of $[^3H]$ glutamate to the GPV fraction obtained from control (open square) and Pb-treated (filled square) rats. Points are means ± SEM of 4 independent experiments each performed on separately prepared fraction. SEM was less than 5%. * The values differ significantly at the level of $P<0.05$.

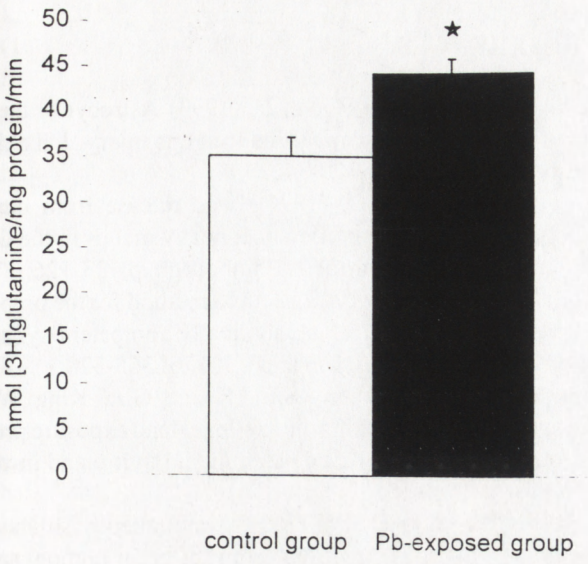

Fig. 3. The activity of glutamine synthetase in GPV fractions obtained from control and Pb-treated rats. The values represent the means ± SEM of 7 independent measurements in both groups, each performed on 7 separately prepared fractions. *$P<0.05$.

of acute lead exposure. The activity of GS in the glial fraction obtained from lead-treated rats was found to be significantly ($P<0.05$) higher than in controls (Fig. 3). The increase reached about 20%.

DISCUSSION

Lead - an ubiquitous environmental pollutant - may be dangerous not only for those who are occupationally or accidentally exposed. The greater population may also suffer from Pb toxicity due to its cumulative properties which may lead to the exceeding of the "safe level" and to various resulting consequences, including neurotoxicity. It is likely enough that the absorption of lead and its storage in the brain occurs at any level of blood lead, therefore man can not estimate the threshold (Niklowitz 1977). Unquestionably, neurotoxicity is one of the most serious toxicological events, since damage to even a small number of neurons can have consequences for the whole organism. Therefore, the necessity to study the subcellular mechanisms of neurotoxic action of lead exists.

Until recently, no biochemical method was available for the study of lead and glia interactions, so the attention was focused on cultured cells (Holtzman et al. 1987, Tiffany-Castiglioni 1993). Experimental procedures using culture cell systems may be vigorously standardized, the toxic exposure is continuous and easily quantitated. However, the culturing process may alter some cellular properties and consequently develop an altered response to the tested agent that limits the range of applications for this method (Veronesi 1992). Additionally, it is known that pure astrocytic cultures do not respond in the same manner as cocultures (Veronesi 1992, Heidinger et al. 1999), so the question arises to what extent cultured cells reflect the functions of the cells in living tissue.

The GPV fraction obtained by the method of discontinuous density-gradient centrifugation by Daniels and Vickroy (1998) was verified by the authors for the structural integrity and analyzed for glial markers and functional specificity. The studies provide solid evidence for the highly-enriched glial nature of the GPV fraction.

Morphological examination performed for the present studies revealed a large number of membrane-encapsulated vesicles that could be classified as small spherical structures or large irregularly shaped structures (data not shown) (Strużyńska et al. 1999). The morphological characteristics of the GPV fraction is consistent with observations published in the original method.

Glutamine synthetase (GS) is an enzyme primarily localized to astroglia with a key role in glutamate and ammonia metabolism in the brain (Norenberg 1997). There are data about the dose- and time- dependent reduction of this enzyme activity, measured in cultured astroglial cells after lead exposure (Engle and Volpe 1990, Sierra and Tiffany-Castiglioni 1991). It is suggested that the reduced GS activity is a very sensitive indicator of Pb exposure in astroglia (Tiffany-Castiglioni 1993).

On the other hand, the induction of GS activity was observed *in vivo* in brain homogenates of lead-exposed rat pups (Cookman et al. 1988). The enhancement of this enzyme activity may be the expression of the intense activity of the astroglial cells (gliosis) as the result of neuronal damage of a different origin, including toxic conditions (Norenberg 1996). Glu-induced increases in GS activity were observed, showing this enzyme to be directly involved in neuroprotection (Heidinger et al. 1999). Interestingly, the results of the present experiments also show an increased GS activity. From our experiments it may be concluded that GS elevated activity signals a disruption of glutamate metabolism in neurons. The lowered uptake and enhanced potassium-evoked release of glutamate from the synaptosomal fraction after acute lead exposure suggest the impairment of glutamatergic transmission which may cause the excess of extracellular glutamate and in consequences lead to glutamate-induced neuronal injury.

The function of GS in the brain is related to the synaptic roles of glutamate and is a critical factor in the phenomenon of glutamate compartmentation. There is evidence that signaling from neurons influences glutamate uptake by astrocytes (Mennerick et al. 1996, Heidinger et al. 1999).

Data presented in the literature show that other astroglial functions, especially high affinity glutamate uptake, were impaired in astroglial primary cultures in the presence of lead (Rönnbäck and Hansson 1992). The results of the present studies are surprisingly different. We observed the enhancement of Na^+-dependent glutamate uptake in glia-derived fraction obtained from lead-treated rats. The data suggest that the astroglial response induced by Pb may differ in different developmental stages and especially in different models of studies. The animal model of studies presented herein is closer to the *in vivo* situation after lead exposure than experiments on cell cultures, especially one-line cell cultures. It is evident that in pure astroglial cultures the deprivation of signals derived from neurons may significantly change the astroglial response.

In these studies the enhanced glutamate uptake by the glia-derived fraction, together with an increased GS activity, may reflect the activation of the astroglial glutamate/glutamine system which is instrumental for the astroglial homeostatic function, i.e. controlling of glutamate concentration.

Since increases in GS levels are associated with the formation of reactive astrocytes (or with stimulated metabolism), the findings of lead-induced increases in that enzyme activity in the present experiments indicate that the glial compartment of the adult rat brain is actively responding to the effects of lead.

In conclusion, the present experiments demonstrate for the first time the induction of functional activity of the astroglia-derived fraction obtained from adult rat brain after acute lead exposure. All the results, taken together, may reflect the protective role of astroglia in the early period of lead toxicity in the rodent model of acute exposure. It does not exclude that a delayed response (in chronic, prolonged lead exposure) may be markedly different.

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Astroglial reaction during the early phase of acute lead toxicity in the adult rat brain

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Abstract

The developing nervous system is susceptible to lead (Pb) exposure but less is known about the effect of this toxic agent in adult rat brain. Since astrocytes serve as a cellular Pb deposition site, it is of importance to investigate the response of astroglial cells in the adult rat brain in a model of acute lead exposure (25 mg kg b.w. of lead acetate, i.p. for 3 days). An increased immunoreactivity of glial fibrillary acidic protein (GFAP) on Western blots was noticeable in fractions of astroglial origin-glial plasmalemmal vesicles (GPV) and in homogenates from the hippocampus and cerebral cortex but not in the cerebellum. The features of enhanced astrocytic reactivity (i.e. large accumulation of mitochondria, activated Golgi apparatus and increment of gliofilaments) were observed in electron microscopy studies in the same tissues. Total glutathione levels increased both in GPV fractions and in brain homogenates—in the cerebellum (120% above control) and in hippocampus (30% above control). The results of current studies indicate that acute lead exposure is accompanied by astrocyte activation connected with the presence of the enhanced expression of GFAP. It may indicate lead-induced neuronal injury. At the same time, a regional enhancement of detoxicative mechanisms (GSH) was noticed, suggesting activation of astrocyte-mediated neuroprotection against toxic Pb action.

Keywords: Acute lead toxicity; Astroglia; GFAP; Glutathione

1. Introduction

It is known that functional and structural abnormalities of the brain accompany acute and chronic lead intoxication (Niklowitz, 1977; Rönnbäck and Hannson, 1992). However, the specific cellular and molecular mechanisms underlying Pb-induced neurotoxicity remain obscure.

Several studies have shown that astroglia can accumulate and store Pb (Holtzman et al., 1984; Tiffany-Castiglioni et al., 1986), suggesting the role of a 'Pb sink' in the brain. Although these cells may be the target for the toxic Pb effect, as stated using cell culture studies (Tiffany-Castiglioni et al., 1986; Holtzman et al., 1987), they

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are more resistant to the toxic Pb effect than other cells in the central nervous system (CNS) (Tiffany-Castiglioni, 1993). These findings suggest that astrocytes may possess compensatory mechanisms that allow the cells to adapt to Pb. One of the most common features of astrocytes is the reaction to CNS damage with reactive gliosis connected with some morphological changes inside the cell and an increased synthesis of many proteins, among others, glial fibrillary acidic protein (GFAP) (Norton et al., 1992; Norenberg, 1996). Such an astrocytic response is induced by many neurotoxic agents (O'Callaghan, 1988), including Pb (Stoltenburg-Didinger et al., 1996; Van Den Berg et al., 1996).

There is much evidence that glutathione (GSH) functions in the protection of mammalian cells against the effects of oxidative damage and certain toxic compounds of endo- and exogenous origin (Meister, 1988; Bains and Shaw, 1997). It is present in high concentrations in both astrocytes and neurones (Langeveld et al., 1996) but there is evidence that neurones are dependent on astrocytes for the delivery of precursors for GSH synthesis inducing a rise in neuronal GSH content (Sagara et al., 1993; Dringen et al., 1999). The depletion of astrocytic GSH is associated with total loss of neuroprotective action (Drukarch et al., 1997), so that the molecule plays an important role in the maintenance of regular astrocyte-neuron interactions.

As is known from many studies, due to the important role of astrocytes in brain maturation (Janzer and Raff, 1987; Rosenbluth, 1988), a toxic Pb insult to these cells in the early period of development could impair both glial and neuronal function (Holtzman et al., 1982; Stoltenburg-Didinger et al., 1996; Zawia and Harry, 1996). Exposure to Pb in the critical period of brain development results in selective interference with glial cells in the cerebellum (Zawia and Harry, 1996) and hippocampus (Slomianka et al., 1989; Selvin-Testa et al., 1991). Up to now, there is little information about the lead effect on astroglia in adult brain. Based on the above mentioned data concerning Pb gliatoxicity following developmental period and reported data about the toxicity in astroglia cultures (Holtzman et al., 1987; Tiffany-Castiglioni, 1993), it was of interest to assess the nature of alterations in astrocytic cells after acute in vivo lead intoxication in adult rat brain. The attention was focused on several brain regions (forebrain cortex, hippocampus and cerebellum) as there is evidence about specific targets of lead neurotoxicity (Collins et al., 1982; Van Den Berg et al., 1996).

In our previous report (Strużyñska, 2000), features of astrocytic reactivity, such as an enhanced uptake of glutamate and increased activity of glutamine synthetase, were observed in the rat model of acute lead toxicity. The present studies were undertaken to follow further the response of astrocytic cells. To check whether astrocytes were reactive, combined immunochemical and electron microscopic studies were performed. The detoxificative ability of astroglia was assessed by measuring total GSH levels.

The use of cellular fractions of astroglial origin (i.e. glial plasmalemmal vesicles—GPV) in biochemical procedures gave the possibility to investigate lead-astroglia interactions post in vivo exposure.

2. Methods

2.1. Animal treatment

The study was performed on 7-week-old male Wistar rats (supplied by the Medical Research Centre, Poland), weighing about 200 g. All procedures were done in accordance with ethical guidelines for care and use of laboratory animals and were approved by the local Care of Experimental Animals Committee. Animals were exposed to lead by injecting lead acetate solution (25 mg kg b.w.) intraperitoneally for 3 days. In order to prevent the precipitation of lead, the solution was prepared in distilled water and, in connection with that, controls were treated with distilled water as well. Pb doses and distilled water doses were administered in a constant small volume of 100 μl. During the time of experiments, animals had free access to standard laboratory diet R-Z V 1324 (SSNIFF, Germany) and tap water. Body weight was recorded daily but no significant differences

Table 1
Lead levels in blood and brain homogenates of control and lead-treated rats

Sample	Lead level	
	Control group	Lead-exposed group
Blood (μg dl)	1.7 ± 0.2	85.2 ± 2.5*
Brain homogenate (μg g wet weight)	<0.019[a]	2.4 ± 0.3

Data expressed the means ± S.D. of four separate samples in both groups. Each sample derived from one animal.
[a] Below the detection limit of the applied method.
* Significantly different from the control group ($P<0.05$) (Student's t-test).

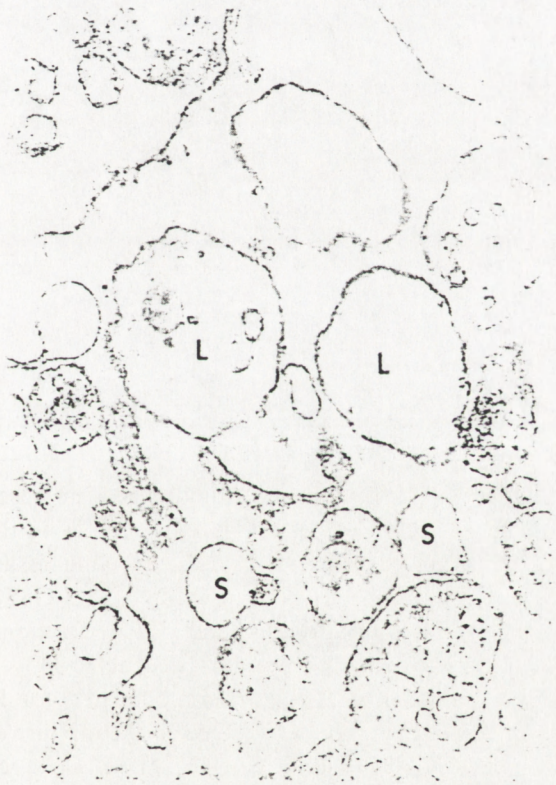

Fig. 1. Representative electron micrograph of GPV fraction. Two types of vesicles—small spherical (S) and large irregular-shaped (L) are visible. Magnification × 50 000. The purity of the fraction (10% of synaptosome-derived particles and mitochondria) was comparable to that obtained in the original method.

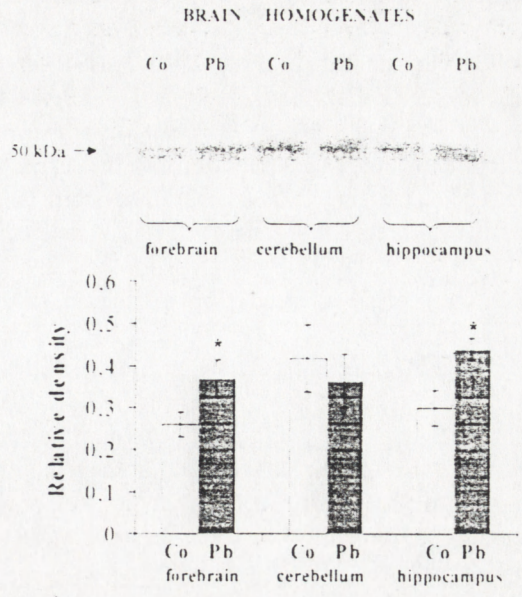

Fig. 2. Western blot analysis for GFAP immunoreactivity in brain homogenates. Immunoreactive bands near 50 kDa in each lane represent GFAP. Densitometric measurements in homogenates are given in the lower part of the figure. Bars represents means ± S.E.M. from four different immunoblots, each done using homogenates obtained from the respective parts of one rat brain. * A statistically significant difference between Pb-treated (Pb) and control (Co) rats ($P < 0.05$) (one-way ANOVA test with post-hock Tukey test).

were observed between the groups. In the group of lead-exposed rats, we did not notice either incidents of death, or symptoms of severe lead intoxication such as seizures. Rats were sacrificed 24 h after the final injection.

2.2. Preparation of homogenates and glial fractions

After decapitation, brains were quickly removed and placed in ice-cold sucrose medium. To obtain homogenates, brains were dissected into three regions: cerebellum, hippocampus and forebrain cortex (remaining after hippocampus isolation). Each part of the brain was homogenised separately in sucrose medium (0.32 M sucrose, 1 mM EDTA) and centrifuged at 1000 × g for 10 min. The supernatants were used for further examination.

The glial plasmalemmal vesicles fraction (GPV) was obtained from freshly isolated brains as described earlier (Strużyñska, 2000) based on the method of Daniels and Vickroy (1998). As the yield of the method is relatively low, and it was difficult to obtain GPV fractions of the respective parts of the brain, GPVs were made from whole brain. Homogenates from two whole rat brains (including cerebella) were fractionated by the multi step procedure with both differential and discontinuous density gradient centrifugation in Percoll. The resultant pellet was used for further biochemical examination. A parallel sample of the GPV fraction was fixed in a mixture of glutaraldehyde and paraformaldehyde buffered in sodium cacodylate and, after a routine method of preparation, used for morphological examination by electron microscopy.

In all further biochemical procedures, except Western blots, freshly isolated material was used. In the case of immunoblotting analysis, homogenates and GPV fractions were frozen.

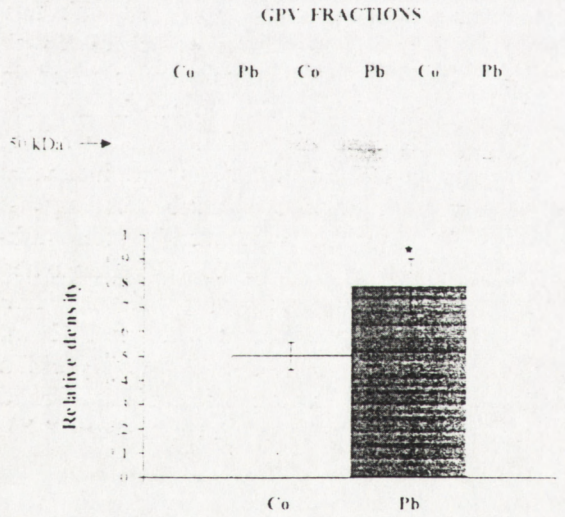

Fig. 3. Western blot analysis for GFAP immunoreactivity in GPV fraction obtained of control (Co) and Pb-treated (Pb) rats. Strongly positive immunostaining to a single band near 50 kDa in each lane represents GFAP. Densitometric analysis was performed on seven different pairs of GPVs (control vs. Pb-treated). Each fraction was obtained from whole brains of two rats. * Significantly different from controls ($P < 0.05$) (one-way ANOVA test).

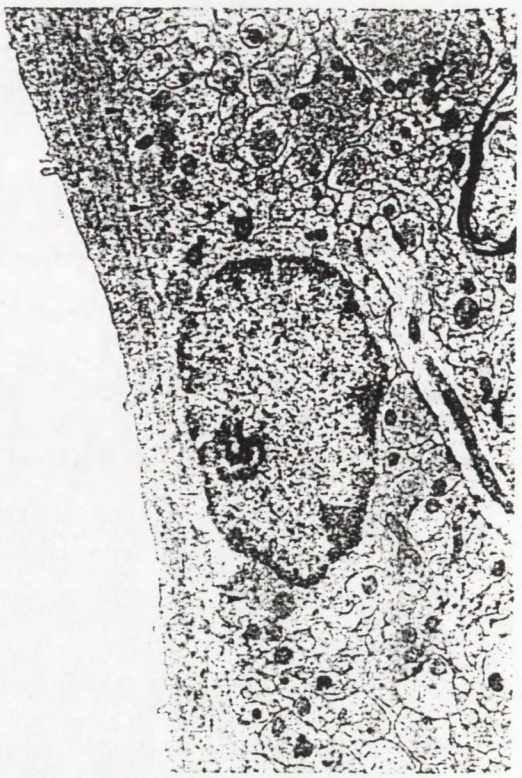

Fig. 4. Micrograph of individual astrocytes in the section of the hippocampus obtained from control rat brain (× 12 000). In the perinuclear cytoplasm, a small amount of short intermediate filaments is present (arrowheads).

2.3. Measurement of total GSH content

The level of total GSH was measured in brain homogenates and GPV fractions from controls and lead-treated rats. Determinations were based on the method of Tietze (Tietze, 1969) using the GSH disulfide reductase DTNB recycling assay. Briefly, 5% PCA was used to extract GSH GSSG from brain homogenates and GPV fractions. Then, the samples were centrifuged at 35 000 × g (GPV fraction) or 3000 × g (homogenates) for 15 min. The supernatant was added to the mixture of 0.3 mM NADPH and 6 mM DTNB in stock buffer (125 mM Na_2HPO_4, 6.3 mM NaEDTA, pH 7.5) and the absorbance was measured at 412 nm (A_1). Then, 5 μl of GSH reductase (173 U mg protein) was added and the A_{412} was monitored continuously until it reached 2.0 (A_2). The total

GSH concentration was calculated from the difference between A_2 and A_1, using a standard curve as a reference, and expressed as ng/mg protein.

2.4. Electron microscopy procedure

For electron microscopic studies, animals were anaesthetised with ketamine hydrochloride (20 mg/kg) and perfused through the heart with fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 cacodylate buffer pH 7.4). Before fixation, a brief rinse of the circulatory system with 0.9% NaCl was performed. Two hours after the perfusion, brains were removed and small specimens of cortex, cerebellum and hippocampus taken. After additional fixation in the above mentioned fixative, the material was rinsed in 0.1 M cacodylate buffer, pH 7.4 and postfixed in 1.5% osmium tetroxide. Thereafter, samples were dehydrated in graded ethanol and propylene oxide. Embedded in Spurr resin, blocks were cut into ultrathin sections, stained with uranyl acetate and lead citrate and examined in a JEM 1200 EX electron microscope.

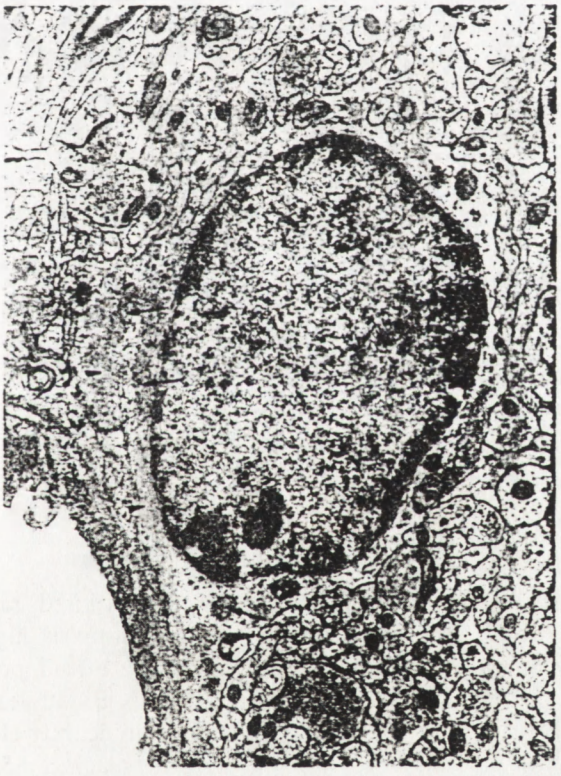

Fig. 5. Micrograph of individual astrocytes in the section of the cerebral cortex obtained from control rat brain (× 14 000). In the perinuclear cytoplasm, a small amount of short intermediate filaments is present (arrowheads) and not numerous mitochondria are visible (arrows).

2.5. Western blot analysis

Brain homogenates and GPV fractions were analysed for glial-specific protein—GFAP. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1976). Samples containing 20 μg protein were combined with Laemmli buffer (1:1) and subjected to 10% SDS-polyacrylamide gel electrophoresis (80–130 V; mini-protean II™, Bio-Rad). Subsequently, proteins were transferred onto nitrocellulose membranes (Hybond-C extra, Amersham) using a Bio-Rad Mini-Trans Blot system according to the instruction manual. Blots were washed with TBS buffer for 10 min. The transfer was checked with Ponceau-S (Sigma). The blots were blocked in TBS buffer containing 0.1% Tween-20 and 5% non-fat milk (TTBS) for 1.5 h. After washing (3 × 10 min) in TTBS buffer, blots were incubated with primary monoclonal antibodies against GFAP (1:1000) for 2 h. Subsequently, after washing with TTBS (3 × 10 min), blots were incubated with secondary antibodies conjugated with HRP (1:4000). Bands were detected with an ECL kit (Amersham) and exposed 1–5 min to Hyperfilm ECL (Amersham). Densitometric analysis of band patterns was performed using an UltraScan™ XL (Pharmacia).

2.6. Measurement of lead content

The lead levels in blood and brain homogenate were measured by atomic absorption spectrophotometry (Perkin Elmer 1100 B). The concentration of lead in each sample was calculated by the analysis of the calibration standards (Yeager et al., 1971).

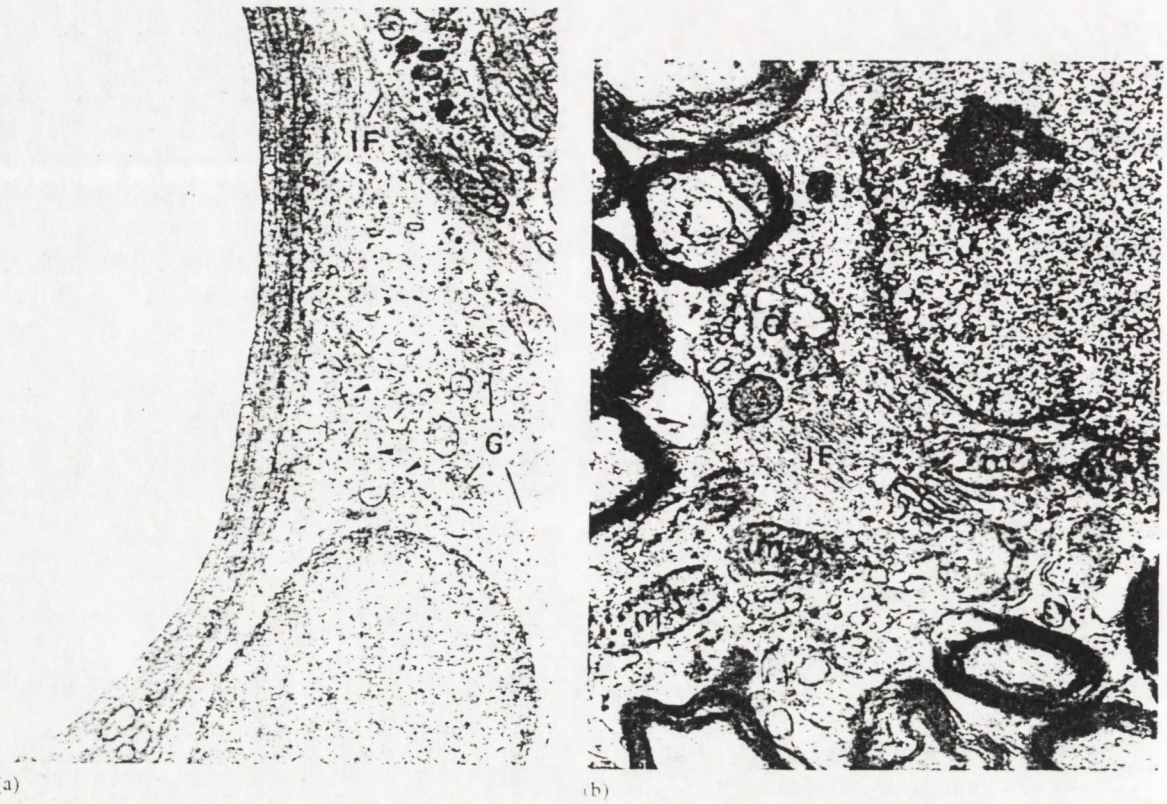

Fig. 6. (A) Astrocytes in a hippocampus section of lead-treated rat. Numerous mitochondria (arrowheads), enlarged Golgi complexes (G) and abnormal accumulation of intermediate filaments (IF) are visible (× 15 000). (B) Astrocytes in a hippocampus section of lead-treated rat. Thick bundles of intermediate filaments (IF) surrounded by mitochondria (m) and abnormally enlarged Golgi complexes (G) are visible (× 30 000).

2.7. Protein measurement

The amount of protein in GPV and homogenates was determined by the method of Lowry and coworkers (Lowry et al., 1951) using bovine serum albumin as a standard.

2.8. Data analysis

All values are expressed as means ± S.E.M. from the number of experiments stated. Statistical analyses were performed by one-way ANOVA with post-hoc Tukey-test to compare regional differences in brain homogenates and assays done with GPV fractions.

In the case of Pb level measurements, values are means ± S.D. and control and experimental groups were compared by Student's t-test. $P < 0.05$ was considered significant.

3. Results

3.1. Lead levels

The lead dose received by the exposed rats produced blood levels of metal within a high concentration range observed in acute lead poisoning (Goyer and Chisolm, 1972). Blood lead levels in the exposed group were significantly elevated over those of controls and reached 85.2 μg dl. A significant penetration of lead from blood to brain was noticed. The amount of lead in brain homogenates of exposed rats was 2.4 μg g wet weight (Table 1).

3.2. The GPV fraction

Morphological examination of the GPV fraction revealed a large number of membrane-encapsulated vesicles. The vesicles could be classified as small spherical structures or large irregularly shaped structures (Fig. 1). The morphological characteristics of the GPV fraction are consistent with observations published in the original paper (Daniels and Vickroy, 1998).

3.3. GFAP immunoreactivity

Glial fibrillary acidic protein is classically used as a marker of astrocyte activation. Western blot experiments were conducted to evaluate changes in GFAP content in brain homogenates and GPV fractions. There was strong positive immunostaining in a single band near 50 kDa of each lane (Figs. 2 and 3). A significant ($P < 0.05$) elevation of GFAP immunoreactivity was seen in brain homogenates obtained from Pb-exposed rats, in hippocampus and forebrain (Fig. 2). No differences were observed between both groups in the case of cerebellum. Densitometric analysis of bands revealed the enhancement of GFAP immunoreactivity in GPV fractions obtained from lead-treated rats. Statistical comparison indicated a significant ($P < 0.05$) increase in this protein after lead treatment (30% when compared to controls) (Fig. 3).

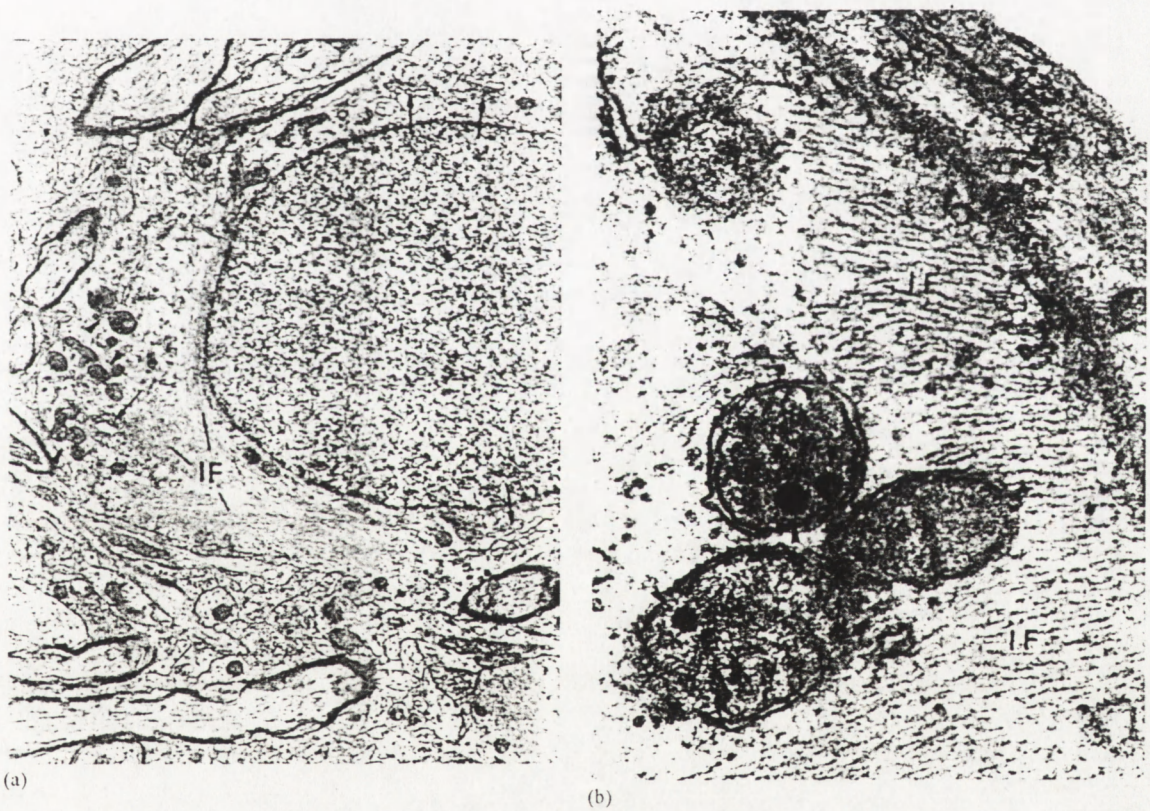

Fig. 7. (A) Astrocytes in a cerebral cortex section of lead-treated rat. Numerous mitochondria (arrowheads), enlarged Golgi complexes (arrows) and abnormal accumulation of intermediate filaments (IF) are visible (× 14 000). (B) Astrocytes in a cerebral cortex section of lead-treated rat. Inclusion bodies in mitochondria (arrowheads) and accumulation of intermediate filaments (IF) are visible (× 80 000).

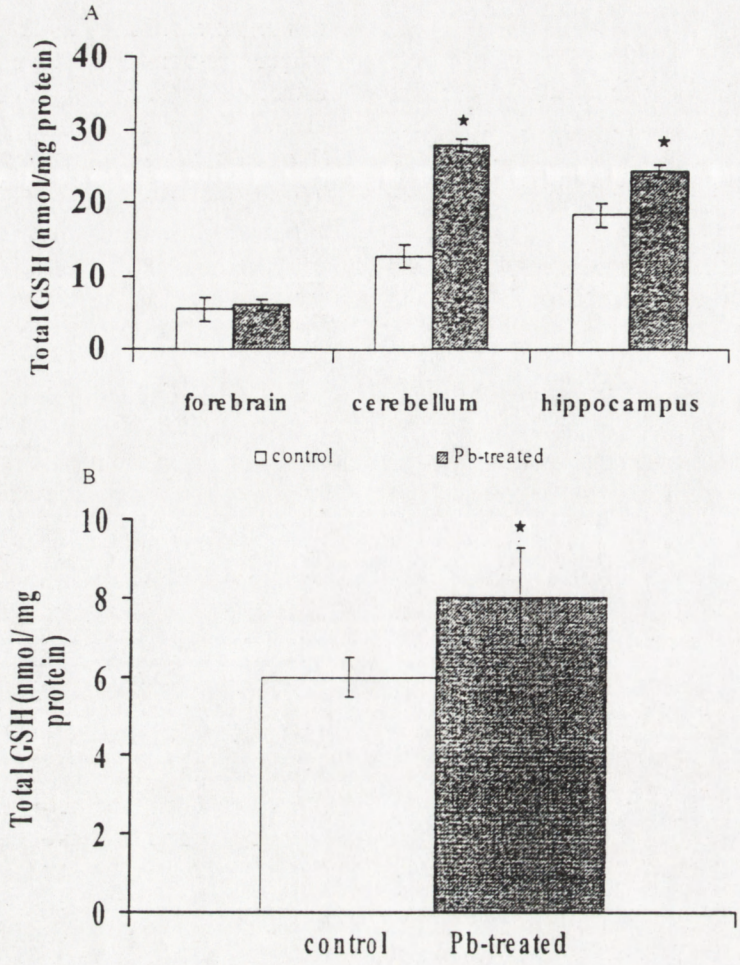

Fig. 8. Total glutathione levels in brain homogenates (A) and GPV fractions (B) from control and lead-treated rats. The results are means ± S.E.M. from four (A) and five (B) independent experiments. Each experiment was conducted using freshly isolated material obtained from one (A) or two (B) rats. * Significantly different from the control group ($P < 0.05$) (one-way ANOVA test: for comparing pairs of means between three brain regions post-hock Tukey test was used).

3.4. Electron microscopy

The presence of small amounts of gliofilaments were found in the perinuclear cytoplasm of astrocytes, in sections of control rat hippocampus (Fig. 4) and in the cerebral cortex (Fig. 5). After acute lead administration, features of enhanced cell reactivity were observed in most of the astrocytic cells. Reactive astrocytes were found in the cortex and hippocampus but not in the cerebellum of lead-treated rats. They were distributed in the neuropil and mostly in the vicinity of endothelial cells. The cytoplasm showed an abnormally large accumulation of organelles, especially mitochondria (Fig. 6A; Fig. 7A). More active Golgi apparatus (Fig. 6A.B; Fig. 7A) and an increment in gliofilaments (Fig. 6A.B; Fig. 7A.B) were constant findings. Thick bundles of gliofilaments were present in the perinuclear cytoplasm and in the cell processes of astrocytes. An interesting observation was the presence of bundles of gliofilaments surrounded by numerous mitochondria (Fig. 6B). In many mitochondria, electron-dense inclusion bodies were detected (Fig. 7B). Local destructive lesions or edema were not observed.

3.5. GSH levels

The amounts of GSH in forebrain, cerebellum and hippocampus of control and lead-treated rat brains are shown in Fig. 8A. Only in the hippocampus and cerebellum were increases in GSH levels detected in Pb-exposed groups of rats. The difference was 32% in hippocampus, and in cerebellar homogenates the GSH level was doubled (120% of control).

In GPV fractions, the level of GSH after Pb treatment was about 30% higher than in controls (Fig. 8B).

4. Discussion

Enhanced expression of GFAP is generally accepted as a marker of neurotoxicity (O'Callaghan, 1988). However, prenatal and early postnatal exposures to Pb caused a decreased expression of GFAP mRNA in the cerebellum (Zawia and Harry, 1996) and hippocampus (Stoltenburg-Didinger et al., 1996) as the result of altered glia integrity and function. When Pb exposure began after the critical period of brain development, an elevation of GFAP expression (Selvin-Testa et al., 1991; Harry et al., 1996) or mRNA for GFAP (Stoltenburg-Didinger et al., 1996) was observed in the hippocampus of young, chronically-exposed rats. In the present studies, during acute exposure of adult rats, enhanced GFAP protein expression was detected in hippocampus and cerebral cortex but not in cerebellum. Such an observation of GFAP increases suggests a selective Pb effect in specific brain regions and supports earlier studies concerning adult rats (Van Den Berg et al., 1996). GFAP is not exclusively located in astrocytes, but is also present in Bergman glia and oligodendrocytes (Dahl, 1981; Laundry et al., 1990). Thus, except in brain homogenates, we detected the immunoreaction of GFAP in the subcellular fraction of astrocytic origin, where we also found it to be enhanced. To our knowledge these studies are novel.

Electron microscopy studies revealed features of activated astrocytes, a large number of astrocytic filaments (Figs. 6 and 7A,B) corresponding to the increased immunoreactivity for GFAP. Astroglial response, evidenced in the rat brain as astrocyte hypertrophy with enhancement of GFAP reactivity, had a regional character (hippocampus and cortex) and was not observed in the cerebellum. The enhanced expression of GFAP in this model of Pb toxicity may not be a primary reaction of astrocytes to the toxin because in cultures these cells do not express such a feature after Pb treatment (Holtzman et al., 1987; Tiffany-Castiglioni, 1993). It may rather reflect the formation of reactive astrocytes following neuronal damage caused by lead. In our previous studies on Pb toxicity using the same model, we observed severe disturbances in the transport and receptor binding of dopamine (Sulkowski et al., 1999) and in the energy metabolism of isolated nerve-ending fractions (Strużyñska et al., 1997).

GSH is proposed to protect the cell against metal toxicity through a chelation mechanism, removal of generated reactive species and maintenance of the redox state of other thiols (Romero et al., 1990; Meister, 1991). Experimental studies on Pb toxicity with different species, methods and doses of Pb delivery, have reported both increases (McGowan and Donaldson, 1987) and decreases (Legare et al., 1993) in tissue GSH content, also depending on the tissue examined. Elevated GSH content may be a compensatory mechanism against Pb toxicity, since Pb was found to stimulate the biosynthetic pathways of GSH (Hsu, 1981) and accumulation of Pb in astrocytic culture was observed to be significantly reduced in the presence of GSH (Lindahl et al., 1999). The elevation of GSH content observed in the current studies may suggest a direct reaction of toxic metal (stimulation of de novo synthesis), or an indirect reaction through the elevation of extracellular glutamate concentration, since GSH is also considered to provide neuroprotection against amino acid excitotoxicity (Bains and Shaw, 1997). It is in good agreement with our earlier studies on this model of Pb toxicity (Strużyñska, 2000), where we found alterations in uptake-release processes, suggesting the elevation of glutamate concentration in the extraneuronal space.

Cerebellar granule cells, unlike other neuronal cell types, also contain significant levels of GSH

and GSH-related enzymes and, what is more, this pool is more resistant to GSH-depleting agents than the astrocytic pool (Huang and Philbert, 1995). Our results indicate the highest increase in GSH level in the homogenate from cerebellum together with the lack of enhanced GFAP immunoreactivity in cerebellar homogenate and absence of features of reactivity in astrocytic cells under electron microscopic examination. It is also possible that other cells than the astrocytic pool of GSH participated in such an increase in GSH content. The exact mechanisms remain to be investigated. Different subcellular distributions of GSH in neural cells may be the basis for selective cellular vulnerability to neurotoxic agents, including Pb.

It has been proposed that astrocytes protect neurones against toxic manifestations of Pb by its accumulation and sequestering in intracellular compartments (Holtzman et al., 1984). It seems, however, that the astrocytic reaction on the presence of Pb may be more complex. It may be connected with the activation of some cellular processes and specific enzymes (glutamate uptake, glutamine synthetase activity), as observed before (Strużyńska, 2000), and with an increased level of antioxidant agent—GSH.

Acute lead toxicity in adult rat brain leads to reactive astrocytosis demonstrating neuroprotective features in the initial stages after toxic insult.

Acknowledgements

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Lead stimulates the glutathione system in selective regions of rat brain

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Lead (Pb) is a highly neurotoxic agent that causes functional and structural abnormalities in the brain. Glutathione (GSH) is a main molecule involved in the protection mechanisms against Pb itself and against reactive oxygen species generated by the metal. This study was carried out to investigate the effect of Pb on the glutathione system in several regions of adult rat brain. In the model of Pb toxicity, adult Wistar rats were exposed to 25 mg of lead acetate/kg b.w. for 3 days. Glutathione and the related enzymes i.e. γ-glutamylcysteine synthetase (γ-GCS), glutathione reductase (GR) and glutathione S-transferases (GSTs) were examined in the hippocampus, cerebellum and forebrain. In the cytosolic fraction the concentration of total GSH and the activity of GR increased only in the cerebellum of Pb-exposed rats. Higher increases of both parameters were observed in mitochondrial fraction obtained from the cerebellum and hippocampus. The activity of cytosolic enzyme – γ-GCS was enhanced only in the forebrain. Regarding cytosolic GSTs, changes in the activity together with enhanced relative density of enzyme protein were found in the cerebellum and hippocampus. Generally, activation of the glutathione system observed shortly after Pb treatment suggests the protective response of the brain to toxic insult. Regional differences in the pattern of these changes may coincide with different susceptibility to Pb. Present results suggest also that mitochondrial mechanisms might account for lead toxicity.

key words: lead neurotoxicity, glutathione system, glutathione-related enzymes

INTRODUCTION

Lead (Pb) is a ubiquitous environmental toxin that induces a broad range of dysfunctions. Exposure to lead has been shown to disrupt many processes in the brain and to result in impaired brain function [29]. Several molecular mechanisms of lead neurotoxicity are thought to exist. One possibility is the disruption of the prooxidant/antioxidant balance [1], which can lead to brain injury. The main molecule, which is of importance in cell defence against oxidative damage, is glutathione (GSH) [3, 27]. It functions in the protection of cells against toxic compounds in general (including heavy metals). Several mechanisms of this protection take place: from metal chelation and direct interaction of the SH groups with reactive oxygen species generated by the metal [34] until the maintenance of the availability of the metal as a nucleophile for the peroxidase system [11].

GSH is synthesised intracellularly from its constituent amino acids (glutamate, cysteine, glycine) by two enzymes: γ-glutamylcysteine synthetase (γ-GCS), which is the rate-limiting enzyme [26], and GSH synthetase. Detoxification of free radicals leads to the formation of disulphide form (GSSG), which is rapidly reduced by another component of the antioxidant defence system – glutathione reductase (GR). Maintenance of a highly

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reduced GSH pool is physiologically important to the brain. Inhibition of GSH synthesis leads to the increased free radical formation and decreased mitochondrial function [16]. Most of the cellular GSH is found in the cytosolic compartment, however a small part is present also in the mitochondria [13] where it is probably imported from the cytosol by the specific transport systems [24]. It was revealed [39] that in the brain GSH is predominantly localised in astroglial cells and it is suggested that astrocytes play a special role in its metabolism, providing GSH (and cysteine for GSH synthesis) to the neurones.

The cellular concentration of GSH is related to the efficiency of GSSG reduction by glutathione reductase and GSH *de novo* synthesis [26]. Thus, we intended to examine total glutathione concentration together with the activity of the most important enzymes involved in GSH metabolism i.e. γ-GCS and GR. Among the enzymes that utilise the glutathione molecule during the catalytic activity are glutathione-S transferases (GSTs), which have been shown to function as metabolic detoxicants [19]. GSTs are the family of enzymes that catalyse the conjugation of a variety of electrophilic xenobiotics with GSH to form less toxic GSH-S-conjugates. New data suggest that GSTs play an important role in regulation of the intracellular concentration of the lipid peroxidation products and thus are involved in the mechanisms of protection against oxidative stress in the brain [36, 44].

In the case of Pb toxicity, there are contradictory data concerning GSH changes [22, 42] derived from studies with different models and tissues.

To our knowledge, neither GSH level nor its related enzymes have been studied extensively in the animal model of Pb toxicity. Preliminary experiments [40] resulted in the observations about enhancement of glutathione and glutathione reductase, especially in whole brain mitochondrial fraction.

Based on the above considerations, this study was carried out to investigate the effects of lead exposure on the brain glutathione system in adult rats. Our intention was to compare the reaction of the GSH system to Pb toxicity in cell compartments (cytosolic and mitochondrial). As there is evidence about the specific targets of lead neurotoxicity [6, 43], we focused our attention on several parts of the brain to evaluate possible regional differences.

The concentration of GSH and the activities of the relevant enzymes (i.e. γ-glutamylcysteine synthetase, glutathione reductase, glutathione S-transferases) were evaluated in cytosolic and mitochondrial fractions obtained from three parts of the rat brain: cerebellum, hippocampus and forebrain.

MATERIAL AND METHODS

Animal treatment

Twenty adult male Wistar rats, weighing about 200 g, were used throughout this study. Animals were arranged into two groups. The experimental group was injected intraperitoneally with lead acetate solution (25 mg/kg b.w.) for 3 days, controls were treated with distilled water. During the time of experiments, animals were fed with standard laboratory chow and tap water *ad libitum*. Body weights were controlled before and after 3-day-period of Pb treatment. Rats were sacrificed 24 h after the final injection. All procedures of animal treatment were done in accordance with ethical guidelines for care and use of laboratory animals and were approved by the local Care of Experimental Animals Committee.

Preparation of tissue

Rats were decapitated quickly and the brains were removed rapidly. Brains were dissected into three parts: cerebellum, hippocampus and forebrain (remaining after hippocampus preparation). From each part of the brain the cytosolic or mitochondrial fraction was obtained. For cytosolic fraction, tissue was homogenised in 10 mM Tris-HCl buffer, pH = 7.4, containing 0.32 M sucrose and 1 mM EDTA or 10 mM EDTA, 2 mM EGTA and 2 mM DTT (in the case of GSTs assay). The homogenate was centrifuged at 36,000 × g for 30 min and the supernatant was used for GSH and enzyme assays.

The mitochondrial fraction was obtained from respective parts of the brain according to the method of Ouyang [31] after homogenisation in ice-cold 15 mM TRIS-HCl buffer (0.25 M sucrose, 1 mM DTT, 1 mM EGTA, 1 mM $MgCl_2$, 0.5 mM PMSF, 1.25 μg/ml pepstatin, 10 mg/ml leupeptin, pH = 7.6). Homogenates were centrifuged initially at 1,000 × g for 10 min with subsequent centrifugation of supernatants at 17,000 × g for 20 min. The crude mitochondrial fraction found in the pellet was further centrifuged 99,000 × g for 30 min in 12% Ficoll to remove cytosolic contamination. After washing in isolation medium, mitochondrial pellet was used for biochemical assays

Measurement of total GSH content

Determination was based on the method of Tietze [41] using the glutathione disulphide reductase/DTNB recycling assay. Briefly, 5% PCA was added to the samples of cytosol or mitochondria before centrifugation at 10,000 × g for 15 min. The supernatant was added to the mixture of – 0.3 mM NADPH and 6 mM DTNB in stock buffer (125 mM Na_2HPO_4, 6.3 mM NaEDTA, pH = 7.5)

and the absorbance was read at 412 nm. Thereafter 5 μl of glutathione reductase (130 units/mg protein) was added and the absorbance was monitored continuously until it reached the value of 2.000. The total GSH concentration was calculated from the difference between absorbances as μg/mg protein using standard curve.

Enzyme assays

γ-glutamylcysteine synthetase (γ-GCS) activity was measured spectrophotometrically at 340 nm, using a coupled enzymatic assay leading to NADH utilisation [37]. The reaction mixture contained 100 mM TRIS-HCl buffer (pH 8.2) and substrates for consequent reactions (10 mM glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 5 mM ATP, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 5 units pyruvate kinase and 10 units lactate dehydrogenase). One unit of enzyme activity is defined as the amount that catalyses the formation of 1 nmol of product per hour per mg protein.

Glutathione reductase (GR) activity was measured spectrophotometrically according to the method of Carlberg and Mannervik [5]. The reaction mixture contained 1 mM oxidised glutathione (GSSG) as a substrate, 2 mM NADPH and protein sample in phosphate buffer (pH 7.0). The decrease in absorbance at 340 nm in terms of NADPH oxidation was measured. One unit of enzyme activity is defined as 1 nmol of NADPH oxidised in 1 hour per mg protein.

Glutathione-S-transferase (GST) activity was determined spectrophotometrically with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as an agent conjugating with 1 mM GSH in 0.1 M potassium buffer (pH 6.5) according to the method of Habig and Jacoby [15]. Increase in absorbance at 340 nm was measured for 2 min. One unit of GSTs activity is defined as the amount of enzyme that conjugates 1 μM CDNB/min/mg protein.

Western blot analysis

Brain cytosolic fractions obtained from three regions of control and lead-treated rats were first subjected to 10% SDS-PAGE electrophoresis. Samples (20 μg protein combined with Laemli buffer 1:1) were placed in each lane. After electrophoresis, the proteins were then transblotted onto nitrocellulose membrane. After blocking with 5% milk in TTBS buffer, blots were incubated with commercially available primary monoclonal antibodies against total GSTs diluted 1:250 (Sigma) for 2 hr. Subsequently, after washing with TTBS, blots were incubated with secondary antibodies conjugated with HRP (1:4000). Bands were detected with the ECL kit (Amersham) and exposed for 10 min to Hyperfilm ECL (Amersham). Densitometric analysis of band patterns was performed using UltroScan^{tm} XL (Pharmacia).

Determination of lead content

The lead levels in blood samples were measured by atomic absorption spectrophotometry (Perkin Elmer 1100B). Certified reference solutions were used to generate standard curves.

Protein measurement

Protein concentration was measured in cytosolic and mitochondrial fractions according to the method of Lowry [23], using bovine albumin as a standard.

Statistical analysis

All results (except Pb level) are expressed as means ± SD from the number of experiments stated in legends to the figures. Data were statistically assessed using analysis of variance to determine differences between control and experimental group (ANOVA) followed by the Tukey post-hoc test for multiple comparisons. In the case of Pb measurements means were compared by Student's *t*-test. In all cases the *p* value was accepted at a level of 0.05.

RESULTS

The mean blood Pb levels were 79.0 ± 3.0 μg/dl for the Pb-exposed group and 3.5 ± 0.9 for control group, respectively. Difference between means was statistically significant at the p level $\leq$ 0.05 (Student's *t*-test). The Pb regimen used in the present experiments caused elevation of metal in blood to the high concentration range characteristic for acute type of poisoning [12]. However, Pb exposure did not affect body weight of rats. We did not notice incidents of death, either.

Effect of lead on GSH

The effect of Pb on total GSH concentration in both examined cellular fractions in the three brain regions of interest is shown in Figure 1A, B. In cytosolic fraction the level of total glutathione was slightly elevated after Pb treatment only in the cerebellum (20%). In mitochondrial fraction, however, statistical analysis revealed significant increases of GSH levels in the cerebellum (70%) and hippocampus (60%) of Pb-treated rat brains compared with respective controls.

Effect of lead on γ-GCS and GR activity

In cytosolic fraction the level of γ-GCS rose significantly above control value only in the forebrain (Fig. 2). In contrast, elevation of cytosolic GR activity was ob-

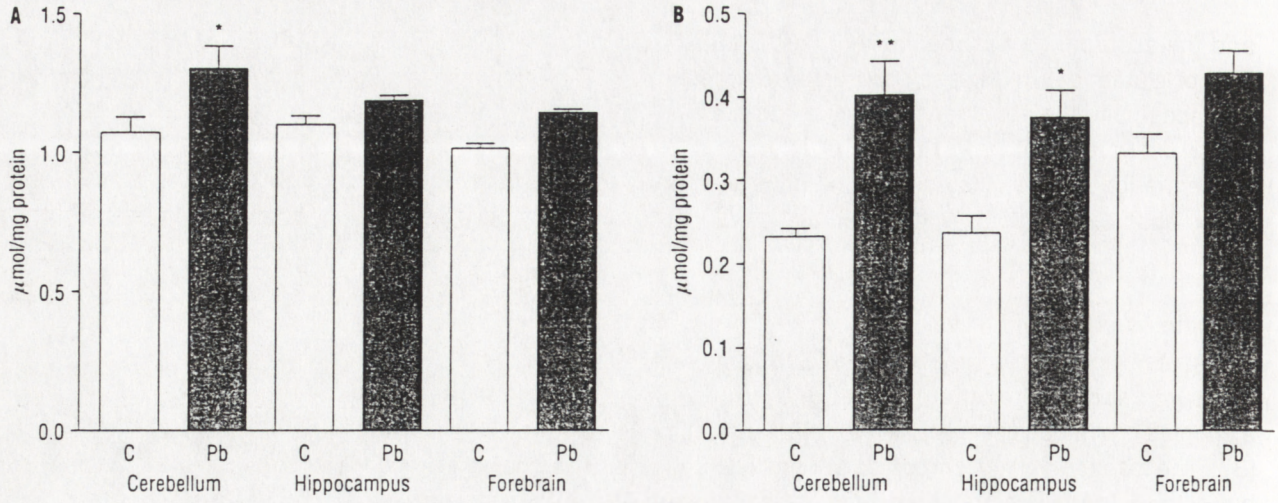

Figure 1. Total glutathione levels in cytosolic (**A**) and mitochondrial (**B**) fractions obtained from the respective parts of control (C) and Pb-treated (Pb) rat brains. The results are means ± SD from 5 independent experiments, each conducted using freshly isolated fractions obtained from one dissected rat brain. Difference between groups statistically significant at *$p < 0.05$ or **$p < 0.01$ (one-way ANOVA with post-hoc Tukey test).

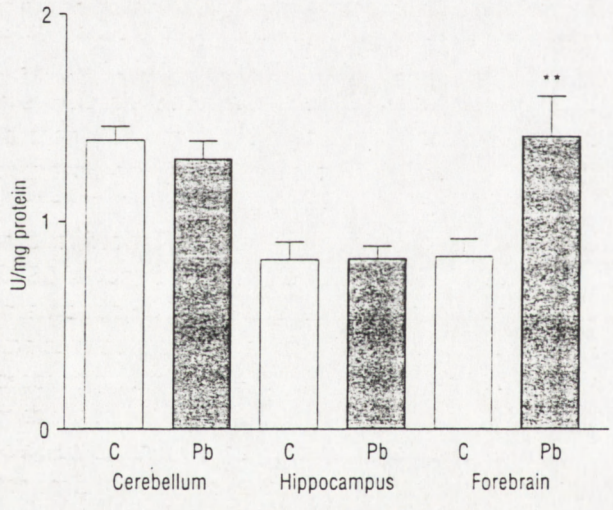

Figure 2. Specific activity of γ-GCS in cytosolic fractions obtained from the respective parts of control (C) and Pb-treated (Pb) rat brains. The results are means ± SD from 7 independent experiments, each conducted using fraction freshly isolated from one rat; **$p < 0.01$ (one-way ANOVA with post-hoc Tukey test).

served only in the cerebellum. In other examined regions a tendency towards enhanced activity was observed but it was statistically insignificant (Fig. 3A). The largest increases of GR activity occurred in mitochondrial fraction obtained from the cerebellum and hippocampus of Pb-treated rats. In both cases the activities of the enzyme were almost doubled when compared with controls (Fig. 3B).

Effect of lead on GSTs

Regarding GSTs' activity, Pb-exposed rats presented higher activities of this cytosolic enzyme in the cerebellum and hippocampus. The values were found to exceed respective controls by about 30 and 25%. In the forebrain, however, enzyme activity was not significantly different from controls (Fig. 4). Western blot analyses of cytosolic fractions with anti-total GSTs antisera revealed two bands with molecular weight between 24–26 kDa. The most intense staining was observed after Pb treatment in bands derived from the cerebellum (Fig. 5). Densitometric analysis of 4 different Western blots, each done using cytosolic fraction obtained from the respective parts of one rat brain, showed a 40% increase in the intensity of immunoreaction in the case of the cerebellum and about 20% increase in the hippocampus compared with controls. In the case of the forebrain no significant differences in the intensity of bands were detected in both groups of rats.

DISCUSSION

Whereas previous studies on the glutathione system after Pb treatment concerned only GSH concentration and activities of selected enzymes examined in whole brain fraction [2, 28], the present report regards also differences in two cellular compartments of GSH and its related enzymes (if they are present in both compartments).

We observed the enhanced concentration of total glutathione in two of the examined brain regions i.e. in the cerebellum and hippocampus. It is worth stressing that

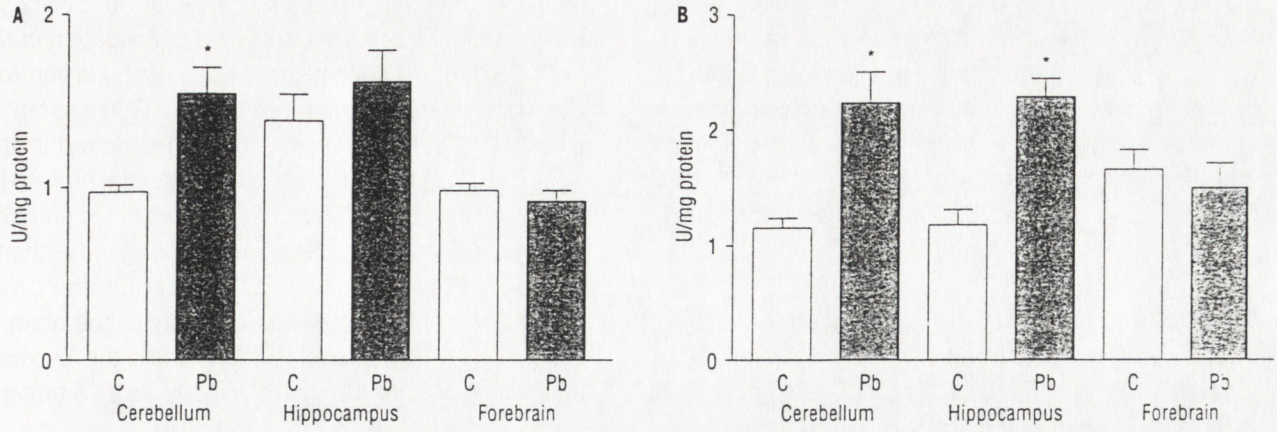

Figure 3. Specific activity of GR in cytosolic (**A**) and mitochondrial (**B**) fractions obtained from the respective parts of control (C) and Pb-treated (Pb) rat brains. The results are means ± SD from 4 experiments done using freshly isolated fractions each derived from one dissected rat brain; *p < 0.05 (one-way ANOVA with post-hoc Tukey test).

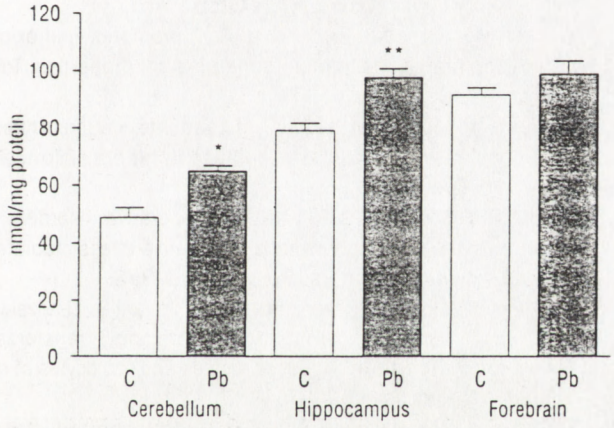

Figure 4. Specific activity of GSTs in cytosolic fraction obtained from the respective parts of control (C) and Pb-treated (Pb) rat brains. The results represent means ± SD from 4 independent experiments, each conducted using fraction freshly isolated from one dissected rat brain; *p < 0.05, **p < 0.01 (one-way ANOVA with post-hoc Tukey test).

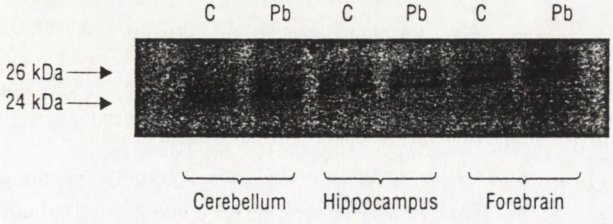

Figure 5. Representative Western blot analysis for total GSTs' immunoreactivity in cytosolic fraction obtained from three parts of control (C) and Pb-treated (Pb) rat brains. Bands between 24–26 kDa represent GSTs. Statistical analysis (one-way ANOVA with post-hoc Tukey test) of densitometric results revealed significant differences between groups in the case of the cerebellum and hippocampus at the p level < 0.05.

evidently the highest increases were observed in both cases in mitochondrial fraction. The amounts of total glutathione measured in the cytosolic fractions considerably exceeded levels observed in mitochondrial ones. This is in agreement with the data that most cellular GSH is in the cytosolic compartment [33]. The mitochondrial pool does not exceed 20% but is presumably very important in defence against reactive oxygen species [16] and the integrity of mitochondria in general [18]. The cellular concentration of GSH is related to the efficiency of GSSG reduction by glutathione reductase and GSH *de novo* synthesis [26]. The higher activity of GR was presented previously in whole rat brain homogenates after high doses of lead [2]. In contrast, examination of the antioxidant system status in three brain regions (hypothalamus. hippocampus and striatum) of rats exposed to Pb does not reveal changes in the activities of antioxidant enzymes including glutathione reductase [28]. We show that the large elevation of total GSH level in mitochondrial fraction is accompanied by an increase of GR activity. In the case of both parameters, differences between control and treated groups were mostly pronounced in the cerebellum and less in the hippocampus. However, we found the activity of cytosolic γ-GCS to be increased only in the forebrain, where GSH was not augmented. It suggests that *de novo* synthesis of GSH after Pb exposure occurs, at the moment of studies, only in this region. The lack of such increases of γ-GCS activity in both other structures may be explained by the fact that this enzyme is feedback inhibited by the reaction product, GSH, which regulates its own synthesis [7]. Probably, the cerebellum and hippocampus are more susceptible to Pb and react immediately after exposure. In the forebrain the reaction is weak or delayed.

The primary observations of the current studies concerning the activity of GSTs lead to the conclusion that exposure to Pb results in the enhancement of total activity of the enzyme and the increased relative density of the enzyme protein in two of the examined brain regions – cerebellum and hippocampus. It has been shown earlier that exposure of rats to inorganic lead may result in increases of GST izoenzymes [30] and GSH [8] in the kidney, whereas the liver exhibited a decrease in total GST activity and total GSH levels [8]. Since we have used commercially available antibodies against total GST, it is impossible to make detailed conclusions regarding the fact that the brain exhibits a complicated pattern of GST expression. But, evidently, enhanced total activity of enzyme(s) (measured chemically) together with results of immunoblotting, indicate the GSTs' role in detoxification of a heavy metal – lead. The concentrated location of many GSTs was found in the astroglia [4, 38]. Based on the above information and the reports about the relatively high level of multiple GST isoenzymes in Müller cells of the retina [25] and in Bergmann cells in the cerebellum [20], the primary role of astroglial cells in detoxification processes may be considered [7]. As is known astroglia is also the main cell compartment of cerebral glutathione [35, 39]. That suggests the capability of these cells to protect neurones from toxic insult by conjugating toxicants with glutathione through the action of GSTs. The cerebellum is the exclusive site in the brain where neuronal cells, both granule cells and Purkinje cells, possess high levels of GSH [32]. Moreover, there are data showing that Pb accumulates predominantly in astrocytes in cerebral tissue but is distributed in both astrocytes and neurones in the cerebellum [17]. These facts may help to explain our results, in which we observed the most expressed response after Pb exposure just in this brain structure. This is of clinical importance because of the well-known susceptibility of this structure to degeneration after toxic or metabolic insult [20]. Similarly, the hippocampus is considered as a target for many toxic agents, including lead [14, 21].

The colocalisation of GSH and GSTs in the astrocytic pool of neural cells suggests that astrocytes may protect neurones against oxidative stress (or toxic compounds generally) via a GSH/GST system. Moreover, astroglial GSTs highly reacted with the compounds containing the C = C double bond [36] susceptible to the peroxidation. It is known that astrocytes have a lower rate of aerobic metabolism than neurones, thus are more resistant to oxidative stress [10]. Although now we found alterations in some indicators of oxidative stress (i.e. total GSH, GR and possibly GSTs), in our earlier work concerning the same model of Pb toxicity, we did not observe direct signs of lipid peroxidation in the brain (TBARS and conjugated dienes levels) in contrast with the liver [9]. The enhanced levels of GSH and higher activities of glutathione-related enzymes found in this study may reflect a protective response of the brain to Pb shortly after treatment. Protection is connected probably with the astroglial pool of cells. The pattern of these changes, observed in three examined parts of rat brain, may coincide with the regional susceptibility of the brain to toxic insult. Moreover, cellular differences in the response to Pb action may indicate mitochondria as a Pb target.

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Relationships between glutamine, glutamate, and GABA in nerve endings under Pb-toxicity conditions

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Abstract

Glutamine (Gln), glutamate (Glu) and γ-amino butyric acid (GABA) are essential amino acids for brain metabolism and function. Astrocytic-derived glutamine is the precursor of the two most important neurotransmitters: glutamate, an excitatory neurotransmitter, and GABA, an inhibitory neurotransmitter. In addition to their roles in neurotransmission these neurotransmitters act as alternative metabolic substrates that enable metabolic coupling between astrocytes and neurons. The relationships between Gln, Glu and GABA were studied under lead (Pb) toxicity conditions using synaptosomal fractions obtained from adult rat brains to investigate the cause of Pb neurotoxicity-induced seizures. We have found that diminished transport of [^{14}C]GABA occurs after Pb treatment. Both uptake and depolarization-evoked release decrease by 40% and 30%, respectively, relative to controls. Lower expression of glutamate decarboxylase (GAD), the GABA synthesizing enzyme, is also observed. In contrast to impaired synaptosomal GABA function, the GABA transporter GAT-1 protein is overexpressed (possibly as a compensative mechanism). Furthermore, similar decreases in synaptosomal uptake of radioactive glutamine and glutamate are observed. However, the K^+-evoked release of Glu increases by 20% over control values and the quantity of neuronal EAAC1 transporter for glutamate reaches remarkably higher levels after Pb treatment. In addition, Pb induces decreased activity of phosphate-activated glutaminase (PAG), which plays a role in glutamate metabolism. Most noteworthy is that the overexpression and reversed action of the EAAC1 transporter may be the cause of the elevated extracellular glutamate levels. In addition to the impairment of synaptosomal processes of glutamatergic and GABAergic transport, the results indicate perturbed relationships between Gln, Glu and GABA that may be the cause of altered neuronal–astrocytic interactions under conditions of Pb neurotoxicity.

Keywords: Glutamine; Glutamate; GAbA; Lead; Pb toxicity; PAG; GAD; EAAC1; GAT-1

1. Introduction

Glutamate, glutamine, and the related amino acid GABA, are central components in brain metabolism and function [1]. Glutamine, produced from glutamate in the astrocytic compartment of the mammalian brain via a highly active glutamine synthetase pathway, is subsequently transferred to the neuronal compartment and metabolised in the glutamate–glutamine cycle [2]. Neurons use glial glutamine as a main substrate for glutamate and GABA synthesis [3] and exhibit high levels of phosphate activated glutaminase (L-glutamine aminohydrolase EC 3.5.1.2; PAG) activity and glutamate decarboxylase (L-glutamate 1-carboxy-lyase; EC 4.1.1.15; GAD) activity. Although glutamine has no neurotransmitter action, it is a main precursor of the excitatory neurotransmitter glutamate in glutamatergic neurons [4,5], and a main precursor of the inhibitory neurotransmitter GABA in GABAergic synapses [1]. There is evidence that the pool of glutamine used in production of neuronal glutamate is of astrocytic origin [6,7].

Metabolism of glutamine by synaptosomes proceeds primarily through the action of mitochondrial PAG. Synaptic terminals possess abundant PAG activity and this pathway is present in both glutamatergic and

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GABAergic terminals [8], whereas glutamate decarboxylase is involved in the regulation of mammalian brain excitability through the synthesis of the main inhibitory neurotransmitter GABA [9]. In addition to their roles in neurotransmission, Gln, Glu and GABA act as alternative metabolic substrates to glucose in astrocytes and neurons after oxidation [10,11]. Thus, these neurotransmitters also play roles in the coupling of the metabolic action of astrocytes and neurons.

Lead (Pb), is a non-physiological metal and environmental pollutant that is exposed to most of the general human population below levels known to cause clinical effects of toxicity. However, the cumulative effects of low-level exposure may result in toxic effects not anticipated for exposure at "safe levels" [12]. Despite a long history of scientific investigation, Pb toxicity remains a significant health problem for both industrial workers and those exposed to Pb in the environment. Toxic effects of Pb are manifested in the central nervous system, where encephalopathy, seizures and irritability are the most severe symptoms observed [13,14].

Behavioural seizures may be the consequence of either excess glutamatergic neurotransmission or inadequate GABAergic inhibitory action. It is known that Pb exerts toxic effects on neurotransmission (including GABAergic and glutamatergic neurotransmission) [15,16]. As shown in patch-clamp experiments, Pb has two effects on neurotransmitter release: enhancement of spontaneous transmitter release and impairment of stimulated, voltage-dependent release [17]. A doubly detrimental effect is the result of interference with extra- and intra-cellular sites of Ca^{2+} action [18].

However, the data are often contradictory and depend upon experimental models (animal/cell culture) as well as the model of toxicity used in the study. Moreover, characterization of the relationships between several targets for Pb toxicity remains very limited and investigations tend to focus mainly on young organisms due to a very high susceptibility of immature brain to Pb toxicity [19].

Thus, there is a need for research to improve our understanding of the complex mechanisms of toxic Pb effects in brain, including adult brain which is also susceptible. The aim of the present work is to investigate of the effects of Pb on transport of amino acids glutamine, glutamate and GABA into nerve endings and the activity of PAG and GAD – elements involved in the chain of critical metabolic importance in the brain.

2. Materials and methods

2.1. Animal treatment

Male Wistar rats weighing 200–220 g (supplied by Medical Research Centre, Poland) were used throughout the study. Animals were arranged into two groups, one-injected intraperitoneally with lead acetate in conc. of 25 mg/kg b.w. for three days and a control group which was treated with distilled water. Pb and distilled water doses were administered at a constant small volume of 0.1 ml. During the experiment, animals were fed with standard laboratory diet R-Z V 1324 (SSNIFF, Germany). Body weight was recorded twice: before the initial injection of lead acetate and at the end of experiment on the third day. Rats were decapitated 24 h after the last injection and brains were prepared for assays. All procedures were carried out in accordance with ethical guidelines for care and use of laboratory animals and were approved by the local Care of Experimental Animals Committee.

2.2. Preparation of synaptosomes

Synaptosomes were isolated according to the method of Booth and Clark [20] with centrifugation in a discontinuous Ficoll gradient (7%/12%) at 99,000 g. The final pellet was suspended in Krebs–Ringer buffer (140 mM NaCl, 5 mM KCl, 10 mM Tris–HCl, 1.3 mM $MgSO_4$, and 1 mM Na_2HPO_4, pH 7.4) and used for further experiments. Synaptosomes obtained by this method are free from non-synaptic mitochondria and exhibit a high purity and well-maintained energy metabolism [21].

2.3. Transport of amino acid neurotransmitters in brain synaptosomes

2.3.1. GABA

Since it is known that uptake of GABA is strongly Na^+-dependent [22], the measurements were performed only in sodium containing buffer as described below according to Troeger et al. [23]. Synaptosomes suspended at about 1 mg of protein/ml were preincubated in Krebs–Ringer buffer pH 7.4 with 2.5 mM $CaCl_2$ and 10 mM glucose at room temperature for 5 min. The incubation mixture contained 0.2 mM AOAA in order to inhibit enzymatic degradation of GABA. The reaction was started by adding [^{14}C]GABA (f.c. 2 μM; specific activity 22.6 mCi/mmol). Samples of incubation mixture were withdrawn at the desired time intervals and centrifuged through silicon oil (specific gravity 1.03, General Electric) on Beckman microfuge. Pellets were solubilized with NCS tissue solubilizer and the radioactivity was determined using Bray scintillation fluid.

For release studies, in the maximum of the uptake curve at 6 min, 50 mM KCl was added to the incubated synaptosomes and the sample of mixture was centrifuged as above after 6 min.

2.3.2. Glutamate

Synaptosomal fractions were used for radioactive glutamate uptake accumulation according to the filtra-

tion method described by Divac et al. [24]. Aliquots of fraction were added to the buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 20 mM HEPES, pH 7.4. Isomolar concentrations of choline chloride were used instead of NaCl to measure Na^+-independent uptake. The assay was initiated by addition of [^{3}H]glutamate (f.c. 5 μM; 45 Ci/mmol). Uptake was quenched by filtration under vacuum through glass filters (Whatmann GF/B) at several time points. Filters were washed in ice-cold buffer and soaked in 1 ml of 10% Triton X-100 for 10 min. Radioactivity trapped on filters was then measured. Sodium-dependent uptake was calculated as the difference between values obtained in the two above-mentioned buffers. In the case of release, 50 mM KCl was used at the maximum of the uptake curves (4 min) and radioactivity was assayed after 6 min.

In order to prevent the conversion of glutamate to α-ketoglutarate, AOAA was used as an inhibitor of AAT (aspartate aminotransferase) [25,26].

2.3.3. Glutamine

Glutamine transport to the synaptosomal fractions was assayed in the incubation mixture containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM $NaHPO_4$, 10 mM glucose and 20 mM HEPES, pH 7.4, and 50 μl of synaptosomal fraction. After incubating the samples at 37 °C for 5 min, uptake was initiated by adding 50 μM of radioactive glutamine (31.9 mCi/mmol [^{3}H] glutamine). At varying time intervals, samples were filtered throughout the glass fibre filters using the vacuum–filtration system. Filters were washed out with two portions of ice-cold buffer and placed for 20 min in Triton X-100. Radioactivity trapped on filters was counted in scintillation Bray fluid. Only total uptake of glutamine was measured, without distinguishing the types of Gln entry.

2.4. PAG activity measurement

The activity of the enzyme was measured according to the method of Shapiro et al. [27]. The standard assay was performed at 37 °C with 60 μl of a solution containing 0.1 μM of radioactive glutamine – L-[3,4-^{3}H(N)] (specific activity 31.9 Ci/mmol) in a buffer containing150 mM potassium phosphate, 0.2 mM EDTA, 20 mM Tris–HCl, and 20 mM glutamine, pH 8.6. The reaction was initiated by adding 10 μl of synaptosomal fraction (subjected earlier for fractionating with Triton X-100). After 10 min of incubation, the samples were thawed by adding 1 ml of 20 mM imidazole buffer, pH 7.0, with 30 mM glutamine and 3 mM glutamate and applied to the columns with Dowex AG 1-X2 for separating. The resting glutamine radioactivity was then washed out with four portions of imidazole buffer containing 30 mM glutamine. The PAG reaction product – [^{3}H]glutamate – was eluted with 2 ml of 30 mM HCl and collected in the scintillation vials with Bray scintillation fluid for counting.

2.5. Western blot analysis for GAD

Synaptosomal fractions (40 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis [28], and the proteins were transferred electrophoretically onto nitrocellulose membrane. After appropriate blocking steps, blots were immunostained with primary monoclonal antibodies against GAD (Sigma) diluted 1:1000 and subsequently with secondary antibodies conjugated with HRP (1:4000). Bands were visualized with ECL kit. To quantify the staining, densitometric analysis was performed using an UltraScan^{TM} XL.

2.6. Western blot analysis for Glu and GABA transporters

Forty micrograms of synaptosomal protein was subjected to electrophoresis according to the procedure described above. Primary goat polyclonal antibodies against human excitatory amino acid transporter EAAT3 (Santa Cruz Biotechnology) were applied in dilution of 1:250 and the secondary anti-goat HRP-conjugated antibodies were applied at a dilution of 1:4000 (Sigma).

Anti-EAAT3 Abs were raised against a peptide mapping to the carboxyl terminus of the transporter, which is identical to the corresponding rat sequence of EAAC1. In the case of the GABA transporter, primary rabbit polyclonal abs against C-terminus of GAT-1 (Chemicon International) were diluted 1:200 and secondary anti-rabbit HRP-conjugated abs were diluted 1:12,000 (Sigma). The immunoblotting step was performed as described for according GAD.

2.7. Determination of lead content

Blood lead levels were estimated by atomic absorption spectrophotometry (Perkin–Elmer 1100B). A certified reference solution was used to generate standard curves.

2.8. Protein assay

Protein concentration in synaptosomes was measured according to the method of Lowry et al. [29], using bovine albumin as a standard.

2.9. Statistical analysis

The results are expressed as mean ± SD from three to six experiments as stated in the figure legends. The Student's *t*-test was used to compare differences between two groups. $P < 0.05$ was considered significant.

3. Results

3.1. Animals in the model of Pb poisoning

During the three-day experiments, rats were housed in plastic cages and behavioural consequences of Pb exposure were observed. On the first day, all rats initially were quiescent followed by occasional hyperkinetic episodes. On the third day of the experiment, exposed animals expressed symptoms of hyperactivity relative to controls. Other symptoms, characteristic of febrile seizures [30] such as tremor, clonic movements, loss of posture and tonic spasm, were not observed. The Pb regimen used in the present experiment did not affect the body weight of animals.

The mean blood lead levels were 65 ± 5.8 and 2.8 ± 0.8 μg/dl for the exposed group and the control group, respectively. These levels were within the range of average concentrations observed in the model of Pb poisoning [31].

3.2. Transport of GABA after Pb exposure

Impaired activity of nerve endings was observed after Pb treatment. Uptake of GABA into synaptosomes was decreased by 40% (measured at the maximum of the uptake curve at 6 min) (Fig. 1). K^+-dependent release of previously accumulated radioactive GABA was diminished by 30% below control values (Fig. 2).

3.3. Transport of glutamate after Pb exposure

Uptake of glutamate into synaptosomes obtained from Pb-treated rats was reduced (similar to the GABA) to 75% of control (Fig. 3). Levels were calculated from the maximum of the uptake curve at 4 min. The pattern of Glu release was not similar to that of GABA. In contrast, the K^+-dependent release was enhanced by about 20% (Fig. 4).

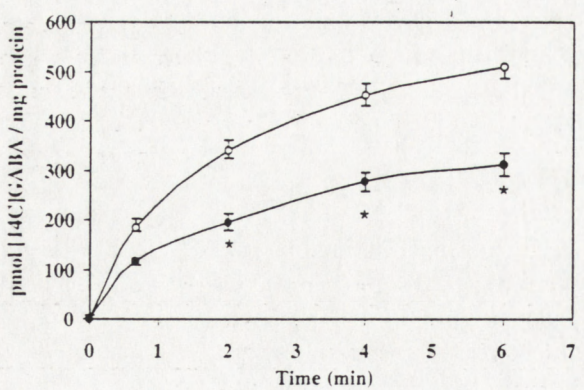

Fig. 1. Na^+-dependent uptake of [^{14}C]GABA to the synaptosomal fraction obtained from control (○) and Pb-treated (●) rats. Results are means ± SD of four independent experiments, each done with freshly isolated synaptosomes. *$P < 0.05$ (Student's t-test).

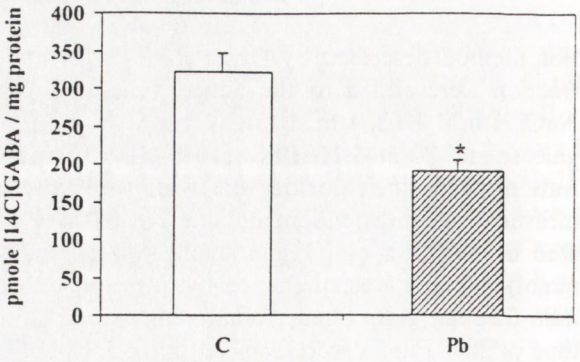

Fig. 2. Depolarization-evoked release (50 mM KCl) of [^{14}C]GABA from the synaptosomal fractions. Bars express the difference between the amount of radioactivity measured in the maximum of uptake's curve and after depolarizing stimulus (for details see Section 2). Results are means ± SD of four experiments each done with freshly isolated synaptosomes obtained from control (C) and Pb-treated (Pb) rats. *$P < 0.05$ (Student's t-test).

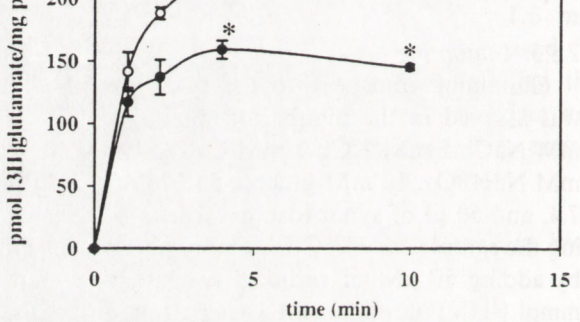

Fig. 3. Na^+-dependent uptake of [^{3}H]glutamate to the synaptosomal fraction obtained from control (○) and Pb-treated (●) rats. Curves express differences between values measured in NaCl- or choline chloride-containing buffers (see Section 2). Results are means ± SD of four experiments each done with freshly isolated synaptosomes. *$P < 0.05$ (Student's t-test).

3.4. Accumulation of glutamine by synaptosomes after Pb exposure

During the first 2–3 min, the synaptosomal content of labelled glutamine increased sharply to 25–30 nmol/mg protein. Thereafter the accumulation continued and reached a maximum after the fifth minute of incubation and declined subsequently. The control rate of uptake was in the range observed earlier [32]. Impaired glutamine accumulation was noted in synaptosomes obtained from Pb-treated rats. The rate of amino acid uptake was reduced 30% below control values (Fig. 5).

3.5. Effect of Pb on GAD expression

GAD is used as a classic marker of GABAergic neurons. For GAD immunoblotting we used monoclonal antiserum that recognizes both molecular forms of

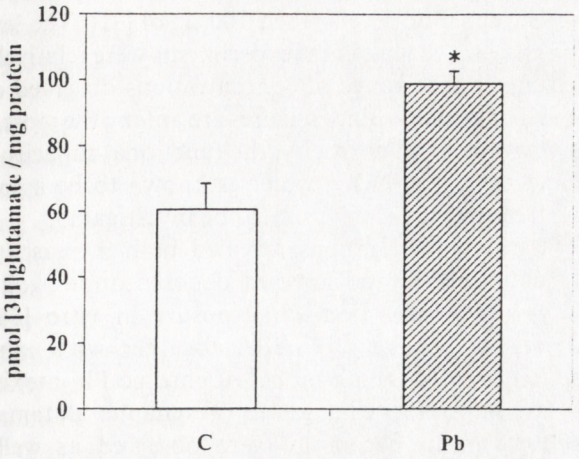

Fig. 4. K^+-dependent release of [^{3}H]glutamate from the synaptosomal fraction obtained from control (C) and Pb-treated (Pb) rats. Bars express the difference between the amount of radioactivity measured in the maximum of uptake's curve and after depolarizing stimulus (for details see Section 2). Results are means ± SD of four independent experiments with freshly isolated synaptosomes. *$P < 0.05$ (Student's t-test).

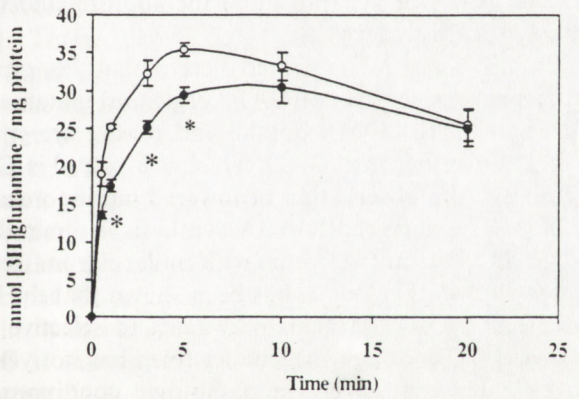

Fig. 5. Total accumulation of [^{3}H]glutamine in the synaptosomal fraction obtained from control (○) and Pb-treated (●) rats. Results are means ± SD of three experiments each done with freshly isolated synaptosomes. *$P < 0.05$ (Student's t-test).

the enzyme. Experiments with anti-GAD antibodies in the synaptosomal fraction demonstrated one band corresponding to the molecular weight of smaller PAG (about 63–64 kDa). No other immunopositive bands were observed. Visual inspection of several blots ($n = 6$) showed that the staining intensity of bands obtained for Pb-treated rats was decreased (Fig. 6). Densitometric analysis confirmed these observations showing a significant reduction to 65% of control bands intensity ($P < 0.02$).

3.6. Effect of Pb on PAG activity

When synaptosomes were incubated with labelled glutamine, glutamate was produced by the PAG reaction and the amount of the newly created glutamate was then calculated after elution of incubation mixture

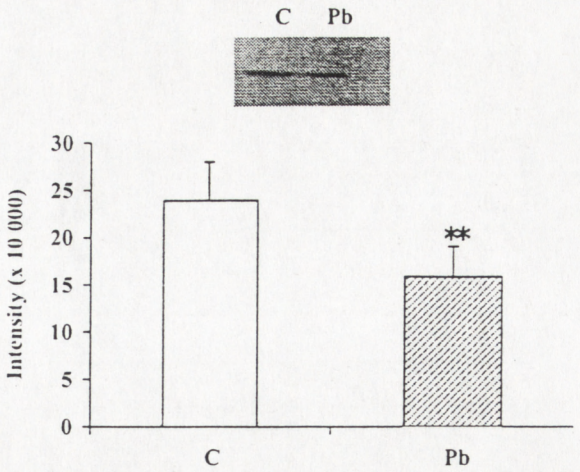

Fig. 6. Representative immunoblot for GAD in synaptosomal fraction obtained from control (C) and Pb-treated (Pb) rat brains. The graph presents the results of densitometric measurements performed for six independent immunoblots, each done with synaptosomal fraction from six distinct rat brains. **$P < 0.02$ (Student's t-test). The single band visible on Western blot represents the "small" GAD of nerve endings' origin (64 kDa).

through the Dowex column. The specific activity of [^{3}H]glutamate in the synaptosomal fraction obtained after Pb treatment was markedly lower than that of the controls (37%) indicating decreased enzyme activity (Table 1).

3.7. Effect of Pb on Glu and GABA transporters expression

Excitatory Amino Acid Transporter (EAAT3 in human/EAAC1 in rat) is a neuronally localized membrane-bound protein that transports the excitatory neurotransmitters, aspartate and glutamate. This process is essential for terminating the postsynaptosomal action of Glu by its reuptake. The results of immunoblots clearly show the enhanced immunoreactivity of the band corresponding to synaptosomal material obtained from Pb-treated rat brains. Densitometric analysis revealed a statistically significant difference between both groups that reached about 35%, suggesting enhanced expression of EAAT3 protein (Fig. 7). In the case of the GABA transporter GAT-1 a similar effect of increased protein

Table 1
Specific activity of PAG in controls and after Pb exposure

Activity of PAG in synaptosomes from rat brains (μmol [^{3}H]glutamate/min/mg protein)	
Control	0.96 ± 0.01
Pb-treated	0.60 ± 0.03[a]

Data expressed as means ±SD of five independent experiments, each done using freshly isolated material.

[a] Significantly different from the control group at the $P < 0.05$ (Student's t-test).

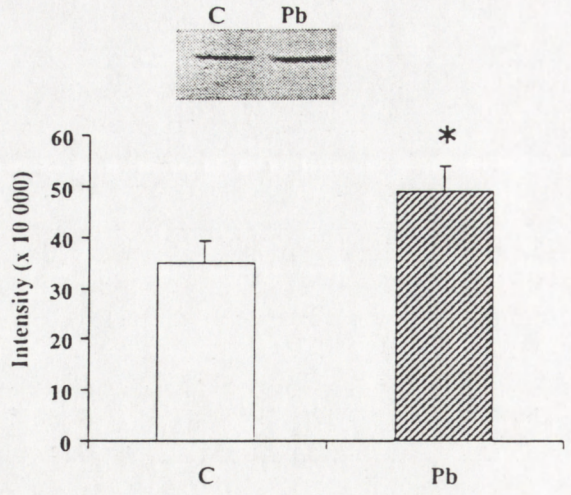

Fig. 7. Representative immunoblot for neuronal transporter for glutamate (EAAC1) in synaptosomal fractions obtained from control (C) and Pb-treated (Pb) rat brains. Results of densitometric analysis of immunoblots ($n = 3$), each done using synaptosomal fraction from distinct rat brain are presented on the graph. $^*P < 0.05$ (Student's t-test).

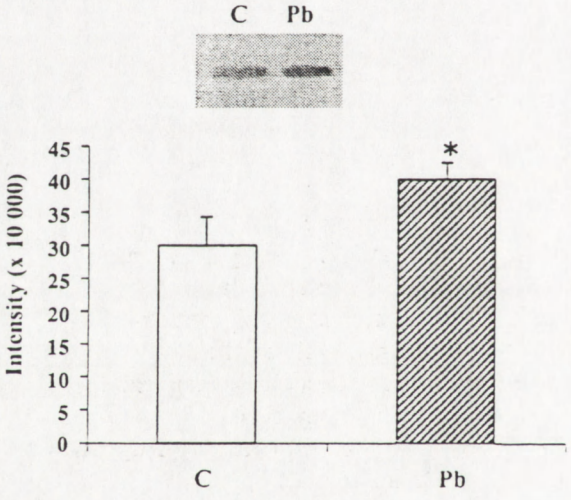

Fig. 8. Representative immunoblot for neuronal type of GABA transporter (GAT-1) in synaptosomal fraction obtained from control (C) and Pb-treated (Pb) rat brains. The graph represents the results of densitometric analysis of four immunoblots, each done using synaptosomal fraction from distinct rat brain. $^*P < 0.05$ (Student's t-test).

quantity (about 30% as compared to controls) was observed (Fig. 8).

4. Discussion

The magnitude of exposure to Pb is accurately indicated by the metal concentration in blood. As the biological half-life of Pb in blood is several weeks, the blood lead (PbB) reflects recent exposure and the measurement of this parameter is useful for monitoring acute, short-term poisonings [33]. PbB levels over 60 μg/dl are known to cause mild impairment of brain function and severe brain impairment to the point of lead encephalopathy is seen when PbB levels reach 100 μg/dl [31]. The levels of metal observed in our rat experiments were relatively high but not in the range of concentrations observed during encephalopathy. Since seizures are among the most severe symptoms of lead toxicity, the functional capacity of the GABAergic pathway, which is known to be associated with brain excitability, should be investigated.

Earlier investigations revealed both increased spontaneous release and lowered depolarisation-evoked release of GABA after Pb exposure in vitro [34] and decreased uptake of GABA together with enhanced activity of GAD in a model of chronic Pb intoxication of rat pups [15]. Changes in presynaptic glutamatergic activity in hippocampus were observed as well [35]. However, due to apparent contradictions of the data, we chose further investigate the effects of Pb on neurotransmission and determine the Pb effect on amino acids which are of significance to brain metabolism.

These investigations of glutamine, glutamate and GABA enable evaluation of the effectiveness of their transport and indicate the potential availability of these amino acids for synaptosomal metabolism under conditions of Pb toxicity.

It has been demonstrated herein that Pb exposure affects several aspects of GABAergic and glutamatergic function. Both GABA uptake and release are strongly affected together with GAD, which is involved in GABA synthesis (the observation of lowered immunoreactivity of GAD suggests that GABA synthesis is diminished).

GAD exists in two forms with molecular masses that differ by 2–4 kDa [36]. It has been shown that the larger of the two GAD proteins may react to selective brain lesions [37], although the smaller form has not yet been investigated extensively in pathologic conditions. Immunocytochemical studies indicated that the smaller GAD protein is predominately localized in cell bodies and in the neurites and terminals [38]. Our immunoblotting experiments with nerve-ending particles (synaptosomes) resulted in observation of a single band of immunoreactivity. This observation is consistent with the results of previous investigations.

The activity of the enzyme form bound to nerve endings appears to be regulated by its interaction with the cofactor pyridoxal P and is not decreased by GABA [38]. The selective feedback control of the level of GAD by GABA was observed only for the 66 kDa form of the enzyme [39]. It is very likely that diminished expression of GAD protein observed in the present study is connected with its lowered activity and consequently with the observation of a decreased rate of GABA synthesis. These results indicate a close relationship between decreased expression of GAD protein and the lowered activity of GABA transport. It has been well established that GAD regulates brain excitability through the synthesis of GABA [40]. Therefore, our results suggest that

a deficiency in GABA transport and inhibition of GAD, may be related to the observed hyperactivity. However, the overexpression of GAT-1 protein suggests that the lower activity of the transporter may be compensated by increased expression of its protein. This mechanism should be regarded as an indirect Pb effect rather than being due to the direct action of Pb on GAT-1.

It has been demonstrated that glutamine and PAG are critical intermediates in the glutamate synthesis pathway [5]. We observed decreased accumulation of glutamine after Pb treatment. Additionally, the specific activity of [^{3}H]glutamate in the synaptosomal fraction obtained after Pb treatment decreased significantly below that of controls, indicating the decreased enzyme activity. Among physiological inhibitors of this enzyme, glutamate is the most potent and has been demonstrated to affect synaptosomal form of PAG [41]. Moreover, since the concentration of the inhibitor glutamate is largely determined by the concentration of the substrate glutamine, synaptosomal PAG indirectly may be controlled by this amino acid.

PAG is an internally localized mitochondrial enzyme [42]. Thus, glutamine must be transported through the inner membrane. Whether or not the activity of the enzyme is limited by the supply of glutamine is the subject of some controversy. However, the possibility must be considered that the decreased activity of PAG following Pb treatment may be a result of impaired mitochondrial transport of glutamine. An opposing explanation for the inhibition of PAG activity is a direct effect of Pb on the enzyme or mitochondria [43] (or both). It has been shown that the synaptosomal form of PAG is susceptible to reaction with sulfhydryl group reagents [44]. Pb is known to react with SH groups, which is a cause of the conditions of toxicity investigated in the present work [45].

Since it is accepted that PAG is mainly responsible for the production of neurotransmitter glutamate [42] and glutamate is the potent inhibitor of PAG, the decreased activity of the enzyme may be feedback regulated by an increased level of glutamate. Another explanation is that the tendency toward enhanced release of glutamate is the result of a direct effect of Pb on glutamatergic transmitter, a possibility which seems plausible in light of our immunoblotting results.

The role of Glu transporter family is to clear extracellular space of released Glu and attenuate excitation [46]. The reuptake of Glu by glutamate transporters has been shown to protect neurons from excitotoxicity caused by its accumulation [47]. Lowered uptake and augmented release of Glu observed in Pb-toxicity condition, leads to the suggestion that the action of synaptosomal transporter is reversed. This together with simultaneous overexpression of transporter protein may result in elevation of extracellular Glu – an important factor in neuronal damage.

The electrophysiological research of neurotransmitter transporters has proven their tendency toward dynamic equilibrium and their ability to work in both directions [48]. Since uptake of Glu is Na^+-dependent, transporter reversal has been recognized to occur in response to pathological stimuli leading to energy demand. However, a large number of factors have been shown to affect the activity and expression of Glu transporters [47].

Collectively, our observations indicate that decreased efficiency of GABA and reversed Glu transport may be related to the observed hyperactivity of rats. This supports the hypothesis that the hyperactivity or seizures after Pb exposure are the endpoint of a continuum of specific neurochemical alterations [15].

Since astrocytes supply neurons with glutamine [49], disturbances in the three amino acids functions in nerve endings may, in consequence, influence interactions between neurons and astrocytes resulting in a change in the astrocytic processes of GABA and glutamate uptake [50] and glutamine release. Further mechanisms of these interactions must be investigated.

5. Abbreviations

Glu	glutamate
Gln	glutamine
GABA	γ-aminobutyric acid
PAG	phosphate-activated glutaminase
GAD	glutamate decarboxylase
HEPES	*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethane-sulfonic acid)
EDTA	ethylenediaminetetraacetic acid
HRP	horseradish peroxidase
ECL	Western blotting detection reagents
NCS	tissue solubilizer
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
AOAA	aminooxyacetic acid
Abs	antibodies

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The role of astroglia in Pb-exposed adult rat brain with respect to glutamate toxicity

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Abstract

Astrocytes maintain neuronal homeostasis in brain and controlling of the released glutamate is one of the most important functions. Since it is suggested that glutamatergic component underlies lead-induced neurotoxic effects and simultaneously, astrocytes serve as a cellular lead (Pb) deposition site, it was of interest to investigate the functioning of astroglia in adult rat brain after short-term exposure to Pb. We examined the expression of main astrocytic glutamate/aspartate transporters—GLAST and GLT-1, which regulate extracellular glutamate concentration. Molecular evidence is provided which indicates overexpression of GLAST mRNA and protein. Simultaneously, decreased expression of GLT-1 mRNA and protein was observed, indicating that of the two glial transporters, GLT-1 is more susceptible to the toxic Pb effect. Protein expression of glutamine synthetase (GS), which converts toxic glutamate to non-toxic glutamine, was doubly enhanced. Moreover, Na^+-dependent transport of radioactive glutamine to astroglia-derived fraction was affected in Pb-exposed rats. Both the rate of accumulation and the efflux of amino acid were diminished. Additionally, we observed enhanced expression of glutathione–protein complexes after Pb treatment what suggests activation of *S*-glutathionylation processes.

The results of current studies indicate that lead toxicity in adult rat brain activates astrocytic processes connected with the controlling of glutamate homeostasis. The response of astroglia is rather of neuroprotective character however, downexpression of GLT-1 glutamate transporter and activation of *S*-glutathionylation processes lead to the question about their significance in Pb-induced neurotoxicity.

Keywords: Pb neurotoxicity; GLAST; GLT-1; Glutamine synthetase; Glutathionylation; Glutamine

1. Introduction

The main function of astroglial cells is maintenance of the neuronal environment and the glial–neuronal

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interactions may be considered as the basic for the central nervous system functioning. Controlling of glutamate homeostasis is most important due to the contribution of this amino acid to normal brain function (neurodevelopment, synaptic plasticity) but also to pathological processes (excitotoxic cell death). Astrocytes occupy strategically important positions surrounding the synapse (Ventura and Harris, 1999) and express glutamate transporters and glutamine synthetase (GS) activity. The concentration of glutamate in the synaptic cleft is influenced by the rate of its release and diffusion and regulated mostly by glutamate transporters. Extracellularly released glutamate is rapidly taken up into astrocytes. Five distinct high-affinity glutamate transporters have been cloned (review: Danbolt, 2001). Two of them, glutamate/aspartate transporters GLAST/EAAT1 and GLT-1/EAAT2 (rat/human, respectively) are astrocytic and play a crucial role in glutamate clearance in the adult brain (Rothstein et al., 1996).

Glutamate accumulated into astrocytes is then converted to glutamine by the enzyme—glutamine synthetase specifically located in astrocytes, but not in neurons (Norenberg and Martinez-Hernandez, 1979). Glutamine synthesis and its export from astrocytes to neurons are essential for resynthesis of glutamate and gamma-amino butyric acid (GABA) in neurons (the glutamate–glutamine–glutamate cycle) (Peng et al., 1993). A substantial amount of glial glutamine, which has no neurotransmitter action, is released from astrocytes. Supply of glutamine as a precursor for neurotransmitters glutamate and GABA in glutamatergic and GABAergic neurons depends on metabolic processes in astrocytes and their functional activity.

Astrocytes play a special role in the metabolism of glutathione (GSH). It was revealed that cerebral GSH is mainly localised in astrocytic pool of cells (Slivka et al., 1987). Moreover, neurons depend on astrocytes for delivery of precursors for its own GSH synthesis (Dringen et al., 1999). GSH is a defensive reagent against toxic xenobiotics (drugs, pollutants, carcinogens) and effective antioxidant. It protects cell against oxidative stress under different pathological conditions, including glutamate toxicity (Bain and Shaw, 1997). It can also regulate numerous cellular events like gene expression, DNA and protein synthesis, cell proliferation and apoptosis, signal transduction, cytokine production, and *S*-glutathionylation (Wu et al., 2004). *S*-Glutathionylation is recognized as a form of modulation of the activities of redox-sensitive thiol proteins where GSH forms disulfide bonds with cysteine residues of proteins. Astrocytic GSH was shown to be associated with neuroprotective action of these cells (Drukarch et al., 1997), providing neuroprotection against glutamate excitotoxicity (Bain and Shaw, 1997), so this molecule plays an important role in the maintenance of regular astrocytes–neuron interactions.

Lead (Pb) is a common environmental neurotoxicant that still remains as a significant health problem, although many efforts were done to minimize its levels in gasoline, paints and printing inks. It was recognized as a potent neurodevelopmental toxin, even at low levels of exposure (Canfield et al., 2003). The common opinion exists that adult organisms are less vulnerable to Pb intoxication therefore, scarce information of Pb effects on adult brain is available. However, there are several reports which demonstrated that exposure to Pb in adult experimental animals may influence mechanisms in which glutamate signaling plays a crucial role like learning and memory (Garcia-Arenaz et al., 2004; Vazquez and Pena de Ortiz, 2004) and apoptotic cell death (Sharifi et al., 2002). All this suggests the contribution of glutamatergic component to lead-induced neurotoxicity. Additionally, Pb has been shown to preferentially accumulate in astroglia (Holtzman et al., 1984; Tiffany-Castiglioni and Qian, 2001), where it may impair cell function and perturb glial–neuronal interactions (Bressler et al., 1999). Taking into account the role of astroglial cells in the maintaining of neurons homeostasis (especially glutamate concentration), it was of interest to assess the function of these cells in adult rat brain under Pb-toxicity conditions.

The present investigation was conducted to test the functional activity of astroglia with respect to the function of controlling glutamate metabolism.

2. Materials and methods

2.1. Animal treatment

Fourteen male adult Wistar rats weighing 200–220 g were used throughout the study. All procedures were carried out in accordance with ethical guidelines for

care and use of laboratory animals and were approved by the Local Care of Experimental Animals Committee. Animals were exposed to Pb by injecting Pb acetate solution (25 mg/kg b.w./day) intraperitoneally for 3 days at a constant small volume of 0.1 ml. A control group was treated with distilled water. Such way of Pb administration (i.p. injection) is the method commonly used in short-term exposures of acute nature (Chetty et al., 1996; Zhang et al., 2004). During the experiment, animals were fed a standard laboratory diet R-Z V 1324 (SSNIFF, Germany). Rats were decapitated 24 h after the last injection, on the morning of fourth day, and brains were rapidly removed and frozen in liquid nitrogen and stored at −70 °C for further experiments or homogenates and glial fractions were prepared.

2.2. Determination of Pb content

Blood and brain lead levels were estimated by atomic absorption spectrophotometry with graphite furnace (AA Scan 1 Thermo Jarrell Ash). A certified reference solution of Pb (Merck) was used to generate standard curve. The detection limit for the applied method is (0.002 μg/g).

2.3. Preparation of homogenates and glial fractions

After decapitation, brains were quickly removed and placed in ice-cold sucrose medium. To obtain homogenates, forebrains were homogenized in 50 mM phosphate buffer (pH 7.4) containing 10 mM EGTA, 10 mM EDTA, 0.1 mM PMSF, 100 mM NaCl in the presence of a protease inhibitor cocktail (1 μg/ml leupeptin, 0.1 μg/ml pepstatin, 1 μg/ml aprotinin) and centrifuged at 1000 × *g* for 10 min. The supernatants were used for Western blots.

GPV—the glial plasmalemmal vesicles fraction was obtained from freshly isolated brains, based on the method of Daniels and Vickroy (1998). Morphological characteristic of obtained GPV fraction was described earlier (Strużyńska et al., 2001) and was consistent with observations published in the original paper (Daniels and Vickroy, 1998).

Homogenates from the rat brains were fractionated by the multi step procedure with both differential and discontinuous density gradient centrifugation in Percoll. The resultant pellet was used for further biochemical examination. In further procedure (glutamine transport), freshly isolated material was used.

Protein concentration in homogenates and fractions was measured according to the method of Lowry et al. (1951) using bovine albumin as a standard.

2.4. [^{3}H]Glutamine transport in GPV fraction

Glutamine transport in the astroglia-enriched fraction was assayed in the incubation mixture containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ 1 mM $MgCl_2$, 1.2 mM $NaHPO_4$, and 20 mM HEPES, pH 7.4, and 50 μl of GPV fraction (0.8–1 mg protein/ml). Isomolar concentrations of choline chloride were used instead of NaCl to measure sodium-independent transport. Fractions were initially preloaded for 10 min with 50 μM of radioactive glutamine (31.9 mCi/mmol [^{3}H]glutamine). Efflux was initiated by dilution of samples with respective buffer containing 0.1 mM glutamine at room temperature. In time intervals samples were filtered throughout the glass fibre filters using the vacuum-filtration system. Tubes and filters were washed out with two portions of ice-cold normal or sodium-deficient buffer and placed for 20 min in Triton X-100. Radioactivity trapped on filters was counted in scintillation Bray fluid by a scintillation spectrometer. Sodium-dependent uptake was assessed as the difference between uptakes in the two abovementioned buffers. Total net (Na^+-dependent and Na^+-independent) transport of glutamine was measured, without distinguishing the types of Gln transporters.

2.5. Western blot analysis for glutamine synthetase, GSH and glutamate transporters

Forty microgram samples of protein from forebrain homogenates were mixed with loading buffer, subjected to SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane. After appropriate blocking steps, blots were immunostained with primary antibodies: goat polyclonal antibodies against glutamine synthetase (Santa Cruz Biotechnology, Inc.), mouse monoclonal antibodies for glutathione–protein complexes (ViroGen, USA), and goat polyclonal against excitatory amino acid transporters EAAT1 and EAAT2 (Santa Cruz Biotechnology Inc.). EAAT1 and EAAT2 are antibodies

raised against peptides mapping to the amino terminus (N-19) of EAAT1 and EAAT2 of human origin, which are almost identical to corresponding rat sequences of GLAST and GLT-1. Primary antibodies were applied in dilution of 1:300, 1:500 and 1:300, respectively. The secondary anti-goat HRP-conjugated antibodies were applied at a dilution of 1:4000 (Sigma). Bands were visualized using the ECL kit. To quantify the staining, densitometric analyses were performed using NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

2.6. Determination of glutamate transporters' mRNA by RT-PCR method

Total RNA was extracted from brains of control and Pb-exposed rats. Isolation was performed using Sigma TRI-reagent according to the method of Chomczyński and Sacchi (1987). RT-PCR reactions were carried out using Enhanced Avian HS RT-PCR kit (Sigma) according to manufacturer's manual.

Reverse transcription of 5 μg of total RNA was performed in a final volume 20 μl using 20 units of AMV reverse transcriptase, 20 unit RNase inhibitor, 3.5 μM oligo(dT)$_5$ as a primer and 0.5 mM each dNTP in one cycle: 42 °C for 1 h and 99 °C for 5 min with subsequent cooling to 4 °C.

Polymerase chain reaction of 5 μl of (cDNA) RT product was carried out according to the manufacturer's manual in total volume of 50 μl using JumpStart AccuTaq and 20 pmol of each primer. The primers for all glutamate transporters were designed by Oligo.pl® (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland) based on the previously reported sequences: for GLT-1—sense: 5′-ACCAGATTCGTCCTCCCAGTC-3′ and antisense: 5′-CCAAGGTTCTTCCTCAACACT-3′ (Pines et al., 1992); for GLAST—sense: 5′-CCATTTTCATCGCTCAAGTTA-3′ and antisense: 5′-GCTGTCTGCCACGGGTTTCTC-3′ (Storck et al., 1992). The primer sequences amplified the PCR products at 555 and 388 base pairs (bp) for GLT-1 and GLAST, respectively. Rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal positive control. The primer sequences used for GAPDH are: sense: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, antisense: 5′-CATGTGGGCCATGAGGTCCACCAC-3′ which amplify a 980 bp fragment.

PCR was performed with 35 cycles of amplification for GLT-1 cDNA (annealing at 65 °C), GLAST cDNA (annealing at 65 °C) or 25 cycles of amplification for GAPDH cDNA (annealing at 52 °C). Each cycle included a 1 min denaturation step at 94 °C, a 1 min annealing step at the indicated temperature, and a 2 min extension step at 72 °C. A 7 min extension at 72 °C was carried out at the end of the final cycle. The samples were then cooled to 4 °C.

Fifteen microliters of PCR product was loaded onto one lane with 3 μl of sample buffer and subjected to electrophoresis at 70 V through 2% agarose gel containing 200 μg/l ethidium bromide. The RT-PCR product bands and a 100 bp ladder molecular weight marker (BioRad, Wiena, Austria) were visualized using ethidium bromide staining. The quantity of GAPDH mRNA (internal standard) and mRNA of transporters were estimated by densitometric analysis of the gel in UV light using NucleoVision apparatus and GelExpert 4.0 software from NucleoTech. The relative intensity of each transporter mRNA level was normalized by dividing the signal by that of GAPDH.

2.7. Statistical analysis

The results are expressed as means ± S.D. from four experiments. The Student's *t*-test was used to compare differences between control and exposed groups. In the case of [^{3}H]glutamine transport inter-group comparisons were made using the one-way analysis of variance (ANOVA) followed by post hoc Tukey test. $P < 0.05$ was considered significant.

3. Results

3.1. Animals

Body weight of rats was recorded daily but it did not change significantly during experiment. In Pb-exposed group there were no incidents of seizures or death. The Pb regimen used in the present experiments caused elevation of metal in blood to the range of average concentrations characteristic of short-term Pb exposure (Goyer and Chisolm, 1972). The mean blood Pb levels (±S.D.) in controls and in exposed group were 2.8 ± 0.8 and 59 ± 11.8 μg/dl, respectively.

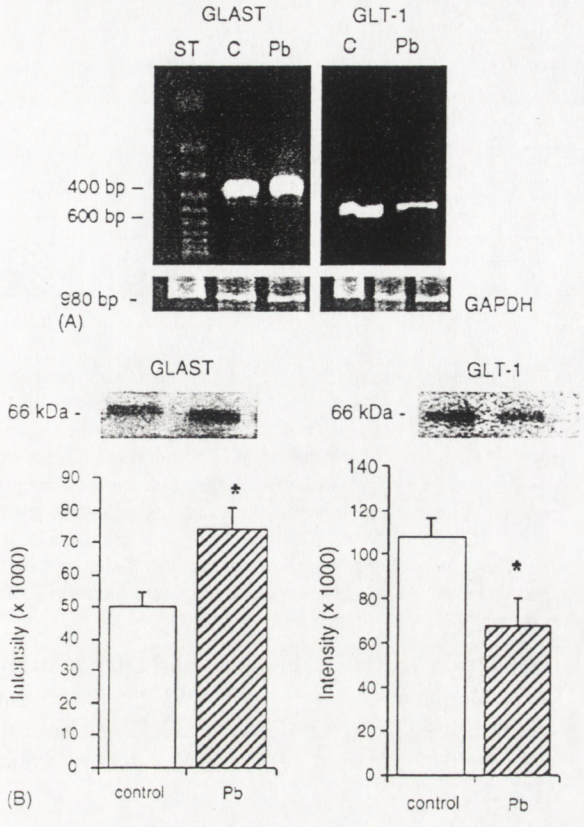

Fig. 1. (A) Expression of GLAST, GLT-1 and internal standard (GAPDH) mRNAs in control (C) and Pb-treated (Pb) rat brain; mean results of relative density of bands measured against GAPDH and statistical analysis are shown in Table 1. (B) Representative immunoblots demonstrating the expression of GLAST and GLT-1 proteins in control and Pb-treated rat brains. The graphs present the summary of results from four independent immunoblots, each done from distinct brain. Data are the means ± S.D. $^{*}P < 0.05$ compared with respective control (Student's *t*-test).

Differences were statistically significant at the P level < 0.05 ($n = 4$; Student's *t*-test). The levels of Pb in brain homogenates obtained from control animals were below the detection limit for applied method (<0.002 μg/g) and in Pb-treated group the mean was 1.8 μg/g ± 0.4 ($n = 4$).

3.2. Expression of astroglial glutamate transporters GLAST and GLT-1

Primers based on the published rat GLAST and GLT-1 sequences (see Section 2) were designed to amplify fragments of 388 and 555 bp, respectively. A single PCR product of the predicted size was obtained in both cases (Fig. 1A). It could be seen that mRNA expression of GLAST was significantly enhanced in brains of Pb-exposed rats (almost two-fold compared to the control) (Fig. 1A). On the other hand, expression level of GLT-1 mRNA was found to be significantly decreased. The relative density of bands calculated as a ratio between density of bands for respective transporters and density of bands for the internal standard GAPDH, and statistical analysis of differences between control and Pb-treated groups are presented in Table 1.

Table 1
Relative expression of astroglial glutamate transporters' mRNA in control and Pb-exposed rat brains exhibited as a ratio between densities of transporter band and internal standard band

	Control	Pb-treated
GLAST	2.75 ± 0.18	5.52 ± 0.53[a]
GLT-1	3.56 ± 0.33	1.48 ± 0.30[a]

Data are the means ± S.D. of densitometric analysis done on independent gels: $n = 3$ (GLAST) and $n = 4$ (GLT-1). Results are presented as a ratio of respective transporter's and GAPDH signal.
[a] $P < 0.05$ (Student's *t*-test).

The immunoreactivity of bands at 66 kDa was observed in immunoblots with anti-GLAST and anti-GLT-1 antibodies. Under Pb-toxicity conditions the increased expression of GLAST protein was found, whereas immunoreactivity of GLT-1 was lowered (Fig. 1B). Densitometric analysis performed to quantify the changes in immunoreactivity showed that protein expression increased relative to controls by about 35% and decreased by about 40% for GLAST and GLT-1, respectively. The pattern of protein expression was consistent with the simultaneously observed changes in mRNA expression in the case of both transporters.

3.3. Glutamine synthetase

Glutamine synthetase catalyses the reaction of amidation of actively taken-up glutamate to form glutamine in astrocytic cells. In Western blots done with brain homogenates obtained from two groups of rats a single band of immunoreactivity was seen at about 45 kDa what correlate with a known molecular weight of the enzyme. The relative density of bands in samples from Pb-exposed rats was enhanced when

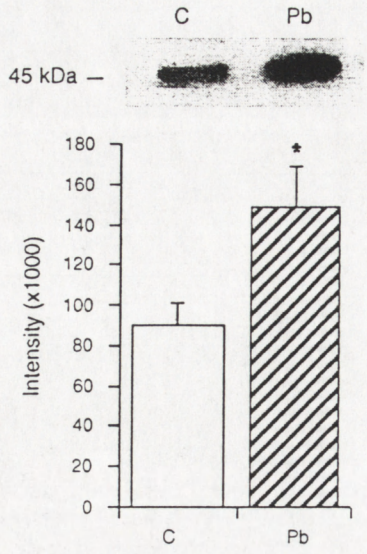

Fig. 2. Representative immunoblot showing the expression of glutamine synthetase protein in control and Pb-treated rat brains. The graph presents the results of densitometric measurements of four independent immunoblots, each done from distinct brain. $^{*}P<0.05$ compared with respective control (Student's *t*-test).

measured densitometrically. Expression of GS protein exceeded almost two-fold control levels (Fig. 2).

3.4. Functional activity of astroglia under Pb-toxicity conditions—transport of glutamine

Several different mechanisms of glutamine efflux were identified in cultured astrocytes—operating either Na^+-enriched or in Na^+-deficient medium (Speciale et al., 1989; Brookes, 1992a). The Na^+-dependent components were identified functionally as system A, N and ASC with the latter being most probable route for efflux (Nagaraja and Brookes, 1996; Dolińska et al., 2004). The efflux of glutamine was measured after the initial uptake during an incubation period of 10 min when uptake is still proportional to time. Most of the glutamine uptake was Na^+-dependent what is in agreement with the previous reports (Bröer et al., 1999). Significant differences between both groups were observable only in Na^+-enriched medium. Under these conditions, the amount of labelled glutamine taken up during the time of incubation was significantly lower in Pb-exposed group than in controls. The difference between respective values reached 40%. A rapid efflux of preloaded glutamine was seen after dilution with buffer containing 0.1 mM glutamine—one of the most potent substrates preferred by ASC system (Bröer et al., 1999). Net efflux of glutamine after Pb treatment was doubly reduced (Fig. 3).

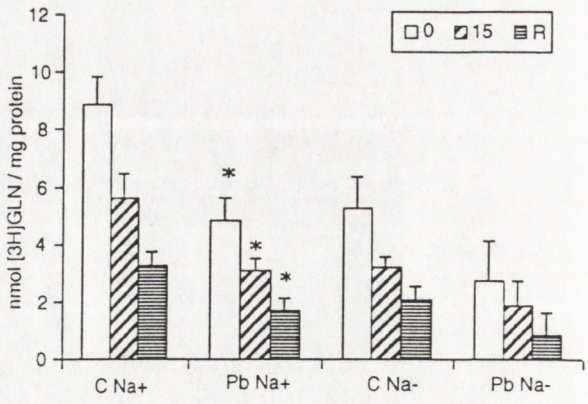

Fig. 3. Na^+-dependent and Na^+-independent net transport of [^{3}H]glutamine in fraction of astroglial origin (GPV) in control (C) and Pb-treated (Pb) rat brain. Bar "0" corresponds to the amount of glutamine loaded to the fraction during 10 min preincubation (time 0). Bar "15" corresponds to the amount of glutamine remaining after 15 min efflux. Bar "R" indicates the amount of glutamine released from GPV fractions during 15 min. Results are means ± S.D. from three independent experiments. In the case of Na^+-dependent transport Pb-treated group significantly different from control at $P<0.05$ (one-way ANOVA with post hoc Tukey test).

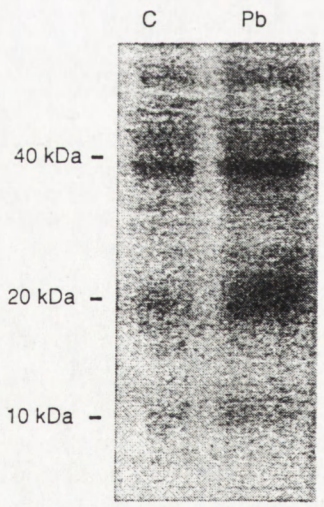

Fig. 4. Representative immunoblot showing the expression of GSH–protein comlexes in control and Pb-treated rat brains. Most intense immunoreactive bands are seen in lanes near 40, 20 and 10 kDa and represent GSH–protein(s) complexes. In Pb-exposed (Pb) rat brain see the enhancement of immunoreactivity.

3.5. Glutatathionylation of proteins under Pb-toxicity conditions

Anti-glutathione antibodies applied in the present study are used to determine complexes, which are created by binding GSH to proteins. Glutathionylation is known to occur in mammalian cells, although mechanisms of this process are not fully understood. The representative immunoblots (Fig. 4) shows several bands, which were obtainable with the applied anti-GSH antibodies. The most intense immunoreactivity was at the level of ~40, ~20, and ~10 kDa where the enhanced expression of protein-bound GSH was seen.

4. Discussion

4.1. The protective mechanisms in astroglia under Pb-toxicity conditions

Glutamine synthetase is a marker for astrocytes, which serves a primary site of conversion of potentially toxic glutamate to non-toxic glutamine. Reduction of the enzyme activity in cultured astroglia after Pb treatment was reported suggesting that it is a very sensitive indicator of Pb exposure (Sierra and Tiffany-Castiglioni, 1991). Interestingly, after in vivo exposure to this metal, the induction of GS activity was observed (Cookman et al., 1988; Strużyńska, 2000). It is consistent with the results of the present study, where we observed the enhanced expression of the enzyme's protein. We suggest that it is a signal of elevated concentration of extracellular glutamate. In support of this view the induction of this enzyme was shown upon astrocytes contact with neurons (Vadimon et al., 1988) and has been shown to protect neurons from degeneration due to excessive extracellular glutamate concentration (Gorovits et al., 1997). The function of GS in the brain is related to the synaptic roles of glutamate and is a critical factor in the phenomenon of glutamate compartmentation. The lack of astrocyte–neuron contact may contribute to the observed discrepancies between in vitro (reduction of GS activity) and in vivo (stimulating of GS activity) effects of Pb on this enzyme activity.

Signaling from neurons influences also glutamate uptake by astrocytes and is required for the expression of glutamate transporters in astrocytes (Gegelashvili et al., 1997). Moreover, it was suggested that soluble neuronal factors differentially regulate the expression of GLT-1 and GLAST (Gegelashvili et al., 1997).

We here demonstrate that astrocytic glutamate transporters—GLAST is overexpressed under conditions of Pb toxicity. Both the mRNA and protein levels of GLAST were found to be enhanced in Pb-exposed rat brains. This may reflect the protective activity of the astroglial transporter against elevated glutamate. Indeed, astroglial GLAST expression was found to be correlated with neuronal glutamatergic activity and glutamate has been shown to increase GLAST gene expression (Gegelashvili et al., 1996). It is possible that activation of GLAST glutamate transporter modulates glutamate neurotoxicity during Pb treatment.

However, the RT-PCR and Western blot assays revealed that GLT-1 mRNA and protein expression tend to decrease after Pb exposure. The astrocytic GLT-1 is the quantitatively dominant form of Glu transporter in brain (Tanaka et al., 1997). Thus, decreased expression of GLT-1 may lead to the impairment of glutamate clearance (and enhanced risk for excitotoxic cell damage). The simultaneously noticed overexpression of GLAST may be considered as the compensative mechanism to decreased expression of GLT-1.

It is known that Pb exerts its effect on neurotransmitters' release through the interactions with calcium ions and thus increasing spontaneous but decreasing evoked release (Marchetti, 2003). If so, this mechanism cannot be rather the only one involved in the enhancing of extracellular glutamate level suggested in the present work. However, the extracellular level of neurotransmitter (glutamate) depends not only on the amount released but is also related to the action of glutamate transporters which clear it from synaptic cleft. The excessive concentration of extracellular glutamate under conditions of Pb exposure may be the result of reversed action of neuronal glutamate transporters (EAAC1) as it was suggested previously (Strużyńska and Sulkowski, 2004).

We also found the function of glutamine transport in astroglia-derived fraction to be affected after Pb exposure. Both the uptake of labelled glutamine and the efflux were significantly diminished. One explanation is that there is a direct effect of Pb on glutamine transporter(s) activity. Alternatively, there is an effect of glutamate-dependent regulation of glutamine flux that represent the process by which a metabolic precursor

is controlling both glutamine release from astrocytes and glutamine uptake by neurons (Tamarappoo et al., 1997). This hypothesis is supported by the known accumulation of intracellular glutamine with little net export generating by the induction of glutamine synthetase activity in astrocytes (Brookes, 1992b). This correlates well with the observed enhancement of GS expression in Pb-exposed rats.

4.2. Dual role of glutathionylation

Mechanisms of glutathionylation are currently under study and it is still little known about the proteins, which may undergo that process in the cell. Reversible *S*-glutathionylation is likely to be the mode of redox signal transduction (Shelton et al., 2005). Evidence accumulated recently that *S*-glutathionylation occurs in a number of physiologically relevant situations, where it can modulate proteins function. However, glutathionylated proteins and their subsequent functional impairment was observed under oxidative and nitrosative stress thus, they are of interest as a possible biomarkers of human diseases associated with that pathology (Giustarini et al., 2004; Wang et al., 2001).

Arrays of proteins have been identified whose cysteine residues are modified in response to oxidants being involved in regulation by redox signaling (glutathionylation). Actin, protein tyrosine phosphatase-1B, and Ras are the best examples (Shelton et al., 2005).

Both astrocytes transporters, GLT-1 and GLAST contain functional cysteine residues that are sensitive to oxidative formation of cystine bridges (Trotti et al., 1998) thus being the potential target for glutathionylation.

Binding of divalent cations of metals like Zn(II) and Ca(II) to some proteins strongly activates glutathione-related *S*-nitrosylation resulting in a global alteration of protein structure (Zhukova et al., 2004). It is known that cationic structure of Pb is very similar to that of Ca and Pb may interfere with normal calcium signaling in numerous intracellular processes (Marcovac and Goldstein, 1988; Zhang et al., 2002; Westerink et al., 2002).

In the reflex of above information, the enhanced expression of GSH–protein complexes observed in the present work in Pb-exposed rat brain, may be doubly interpreted. It may be the protecting mechanism that reflexes elevated GSH level, which is known to act as an antioxidant and as a protectant against heavy metal toxicity, including Pb (Hsu, 1981). That is consistent with previous results (McGowan and Donaldson, 1987; Strużyńska et al., 2001) obtained in experimental Pb-treated animals. On the other hand, enhanced expression of glutathionylated proteins may be the result of inhibited deglutathionylation catalysed by glutaredoxin. Persistent changes in the structure and function of glutathionylated proteins may result in the impairment of cellular mechanisms in which they are involved. Indeed, inactivation of glutaredoxin leading to the inhibition of deglutathionylation and subsequent apoptosis was observed in H9 and Jurkat cells after acute cadmium exposure in vitro (Chrestensen et al., 2000).

What kind of proteins may hide in complexes and are target of GSH action under condition of Pb toxicity, undoubtedly needs further study.

The results of present study illustrate well that in Pb-exposed adult rat brain mechanisms controlling glutamate homeostasis are activated. Activation is connected with the protective role of astroglia towards neurons. However, it should be emphasized that this protection may be insufficient considering decreased expression of GLT-1 transporter and the unknown role of the increased level of glutathionylated proteins. Thus, a possible chain of pathological events resulting from glutamate toxicity in adults under Pb-toxicity conditions should be considered. Moreover, the action of Pb on astrocytic function during development may be not of the same nature as in the case of adult animals. Regarding the fact that young organisms are extremely susceptible to Pb and the levels of Pb in immature brain are much more higher than in adult (exposed to the equal doses of Pb) we suppose that astroglia may react in a different, unnecessary neuroprotective way.

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Changes in expression of neuronal and glial glutamate transporters in lead-exposed adult rat brain

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Abstract

Excitatory amino acid transporters (EAATs) are membrane-bound proteins localized in glial and neuronal cells which transport glutamate (Glu) in a process essential for terminating its action and protecting neurons from excitotoxic damage. Since Pb-induced neurotoxicity has a glutamatergic component and astrocytes serve as a cellular Pb deposition site, it was of interest to investigate the response of main glutamate transporters to short-term lead exposure in the adult rat brain (25 mg/kg b.w. of lead acetate, i.p. for 3 days). We examined the expression of mRNA and protein of GLAST, GLT-1 and EAAC1 in homogenates obtained from cerebellum, hippocampus and forebrain. Molecular evidence is provided which indicates that, of the two glial transporters, GLT-1 is more susceptible than GLAST to the neurotoxic effect arising from Pb. RT-PCR analysis revealed highly decreased expression of GLT-1 mRNA in forebrain and hippocampus. In contrast, GLAST was overexpressed in forebrain and in cerebellum. In the case of EAAC1, the enhanced expression of mRNA and protein of transporter was observed only in forebrain. The results demonstrate regional differences in the expression of glutamate transporters after short-term exposure to Pb. In forebrain, downregulation of GLT-1 is compensated by enhanced expression of GLAST, while in hippocampus, the expression of both is lowered. This observation suggests that under conditions of Pb toxicity in adult rat brain, the hippocampus is most vulnerable to the excitotoxic cell damage arising from impaired clearance of the released glutamate.

Keywords: Pb neurotoxicity; GLAST; GLT-1; EAAC1; Excitatory amino acid transporters

1. Introduction

Glutamate (Glu) is the primary excitatory amino acid neurotransmitter in the central nervous system (CNS) (Fonnum, 1984). It is released into the synaptic cleft and binds to the glutamate receptors which provide signalling critical for normal synaptic transmission, synaptic plasticity and brain development (Mayer and Westbrook, 1987). The post-synaptic response is related to the concentration of the released glutamate as a function of time and is carefully regulated by glutamate transporters. Glutamate released extracellularly is inactivated by uptake into glia and neurons in a process mediated by a family of high-affinity transporters (GLAST/EAAT1, GLT-1/EAAT2, EAAC1/EAAT3, EAAT4 and EAAT5). GLT-1 and GLAST, localized in astrocytes, are the most important transporters for limiting glutamate signalling. Accumulation of glutamate in the extracellular space may be excitotoxic to neurons, thus, optimal function of transporters is essential for maintaining extrasynaptic glutamate levels sufficiently low to restrict overstimulation of glutamatergic receptors and prevent neuronal damage. Dysfunction of these transporters may be the initiating event or part of the cascade leading to the cellular dysfunction and cell death. Many investigations have revealed a significant role for these proteins in pathologic conditions, such as neurodegenerative diseases, epilepsy, stroke and tumour formation

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(Campiani et al., 2003; Gegelashvili et al., 2001; Vemuganti et al., 2001). Selective loss of the glial transporter GLT-1 has also been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS) (Maragakis and Rothstein, 2001).

Lead (Pb) is a ubiquitous environmental pollutant. Excessive exposure to Pb in human populations still persists, despite efforts to reduce Pb levels in ecosystem. Toxic effects of this metal are principally manifested in the central nervous system. A broad range of studies revealed that pre- and early postnatal exposure to Pb results in long-term potentiation (LTP) decrease and cognitive deficits with related behavioral disturbances (Bellinger et al., 1992; Canfield et al., 2003; Mendola et al., 2002; Tong et al., 2000). Further investigated are the effects of selective blockade of ionotropic glutamatergic receptors (NMDA) in hippocampal neurons leading to the modification of NMDAR-mediated calcium signalling (Guilarte, 1997; Guilarte and McGlothan, 1998; Marchetti, 2003). In the adult brain, the LTP impairment that occurs following Pb exposure is not as clear as that observed in immature brain and contradictory data exist (Costa et al., 2004; Gilbert et al., 1999; Gilbert and Rice, 1987). However, investigations using magnetic resonance spectroscopy correlated Pb-induced impairment in cognitive function in adults with neuronal loss (Weisskopf et al., 2004). Moreover, adult Pb exposure produced retinal degeneration due to the apoptotic cell death (Fox et al., 1997). It has been also suggested that Pb may induce cell death in the hippocampus partly due to apoptosis (Sharifi et al., 2002). A glutamatergic component is undoubtedly involved in Pb-induced neurotoxicity (Lasley and Gilbert, 2000), although underlying mechanisms may differ according to extent of exposure and maturity of exposed organism.

The collective data from in vivo and in vitro experiments (Aschner and LoPachin, 1993; Tiffany-Castiglioni et al., 1996) have shown that astroglia can accumulate and store Pb, thus protecting neurons which are more sensitive to the toxic Pb effect. On the other hand, this store of glial Pb may constitute a reservoir for its continuous release and thereby contribute to the toxicity of adjacent neurons (Holtzman et al., 1987). Although effects of Pb on glutamatergic receptors have been proposed as a one of mechanisms of Pb neurotoxicity, the participation of glutamate transporters in Pb toxicity conditions has yet to be investigated. Based on the previously discussed data concerning both the effects of Pb on glutamatergic functions and its gliatoxicity, it is of interest to assess the nature of alterations in glutamatergic transporters in adult rat brain after short periods of lead exposure. In the present study, the expression of the neuronal transporter EAAC1 and two astroglial transporters—GLAST and GLT-1 are investigated with an emphasis on forebrain cortex, hippocampus and cerebellum since evidence exists that some of brain regions are specific targets of lead neurotoxicity (Collins et al., 1982; Van den Berg et al., 1996).

2. Experimental procedures

2.1. Animal treatment and material

Male Wistar rats weighing 200–220 g were used throughout the study. All procedures were carried out in accordance with ethical guidelines for care and use of laboratory animals and were approved by the Local Care of Experimental Animals Committee. Animals were exposed to Pb by injecting Pb acetate solution (25 mg/kg b.w.) intraperitoneally for 3 days at a constant small volume of 0.1 ml. A control group was treated with distilled water. During the experiment, animals were fed a standard laboratory diet R-Z V 1324 (SSNIFF, Germany). Rats were decapitated 24 h after the last injection and brains were rapidly removed. After dividing into parts, cerebellum, hippocampus and forebrain, tissues were then frozen in liquid nitrogen and stored at −70 °C for further experiments. For Western blots, homogenates from respective parts were prepared in 50 mM phosphate buffer (pH 7.4) containing 10 mM EGTA, 10 mM EDTA, 0.1 mM PMSF and 100 mM NaCl in the presence of a protease inhibitor cocktail (1 μg/ml leupeptin, 0.1 μg/ml pepstatin and 1 μg/ml aprotinin).

2.2. Western blot analysis for glutamate transporters

Forty microgram samples of protein from brain homogenates (forebrain, hippocampus and cerebellum) were mixed with loading buffer, subjected to SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane. After appropriate blocking steps, blots were immunostained with primary goat polyclonal antibodies against excitatory amino acid transporters (EAAT1, EAAT2 and EAAT3) (Santa Cruz Biotechnology Inc.). EAAT1 and EAAT2 are antibodies raised against peptides mapping to the amino terminus (N-19) of EAAT1 and EAAT2 of human origin, which are almost identical to corresponding rat sequences of GLAST and GLT-1. Anti-EAAT3 antibodies were raised against a peptide mapping to the carboxyl terminus of the transporter identical to the corresponding rat sequence of EAAC1. Primary antibodies were applied in dilution of 1:300. The secondary anti-goat HRP-conjugated antibodies (Sigma) were used at 1:4000 dilution. Bands were visualized using the ECL kit. To quantify the staining, densitometric analyses were performed using NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

2.3. Determination of glutamate transporters' mRNA by RT-PCR method

Total RNA was extracted from cerebellum, hippocampus and forebrain of control and Pb-exposed rats. Isolation was performed using Sigma TRI-reagent according to the method of Chomczyński and Sacchi (1987). RT-PCR

reactions were carried out using Enhanced Avian HS RT-PCR kit (Sigma) according to manufacturer's manual.

Reverse transcription of 5 μg of total RNA was performed in a final volume 20 μl using 20 units of AMV reverse transcriptase, 20 units RNase inhibitor, 3.5 μM oligo(dT)$_5$ as a primer and 0.5 mM each dNTP in one cycle: 42 °C for 1 h and 99 °C for 5 min with subsequent cooling to 4 °C.

Polymerase chain reaction of 5 μl of (cDNA) RT product was carried out according to the manufacturer's manual in total volume of 50 μl using JumpStart AccuTaq and 20 pmol of each primer. The primers for all glutamate transporters were designed by Oligo.pl® (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland) based on the previously reported sequences: for GLT-1, sense: 5′-ACCAGATTCGTCCTCCCAGTC-3′ and antisense: 5′-CCAAGGTTCTTCCTCAACACT-3′ (Pines et al., 1992); for GLAST, sense: 5′-CCATTTTCATCGCTCAAGTTA-3′ and antisense: 5′-GCTGTCTGCCACGGGTTTCTC-3′ (Storck et al., 1992); for EAAC1, sense: 5′-GACAGATTCTGGTGGATTTC-3′ and antisense: 5′-TGTGACGCTGATAGTGATGA-3′ (Li et al., 1994). The primer sequences amplified the PCR products at 555, 388 and 503 base pairs (bp) for GLT-1, GLAST and EAAC1, respectively. Rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal positive control. The primer sequences used for GAPDH are: sense: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, antisense: 5′-CATGTGGGCCATGAGGTCCACCAC-3′ which amplify a 980 bp fragment.

PCR was performed with 35 cycles of amplification for GLT1 cDNA (annealing at 65 °C), GLAST cDNA (annealing at 65 °C), EAAC1 cDNA (annealing at 49.5 °C) or 25 cycles of amplification for GAPDH cDNA (annealing at 52 °C). Each cycle included a 1 min denaturation step at 94 °C, a 1 min annealing step at the indicated temperature and a 2 min extension step at 72 °C. A 7 min extension at 72 °C was carried out at the end of the final cycle. The samples were then cooled to 4 °C. Fifteen microliters of PCR product was loaded onto one lane with 3 μl of sample buffer and subjected to electrophoresis at 70 V through 2% agarose gel containing 200 μg/l ethidium bromide. The RT-PCR product bands and a 100 bp ladder molecular weight marker (BioRad, Wiena, Austria) were visualized using ethidium bromide staining. The quantity of GAPDH mRNA (positive control), GLT1 mRNA, GLAST mRNA and EAAC1 mRNA were estimated by densitometric analysis of the gel in UV light using NucleoVision apparatus and GelExpert 4.0 software from NucleoTech. The relative intensity of each transporter mRNA level was normalized by dividing the signal by that of GAPDH.

2.4. Determination of Pb content

Blood lead levels were estimated by atomic absorption spectrophotometry with graphite furnace (AA Scan 1 Thermo Jarrell Ash). A certified reference solution of Pb (Merck) was used to generate standard curve.

2.5. Protein assay

Protein concentration in homogenates was measured according to the method of Lowry (Lowry et al., 1951) using bovine albumin as a standard.

2.6. Statistical analysis

The results are expressed as mean ± S.D. from four experiments. Inter-group comparisons were made using the one-way analysis of variance (ANOVA) followed by post hoc Tuckey test. $P < 0.05$ was considered significant. In the case of lead measurements, the Student's t-test was used to compare differences between control and exposed groups.

3. Results

3.1. Animals in the applied model of Pb toxicity

The mean blood Pb levels (PbB) and the levels of Pb in brain homogenates obtained from control and Pb-treated animals are presented in Table 1. The Pb regimen used in the present experiments caused elevation of metal in blood to the range of average concentrations characteristic of short-term Pb exposure (Goyer and Chisolm, 1972). Lead level rises in blood rapidly within hours and remains elevated for several weeks. When administered, Pb is then redistributed in organs according to the rate of blood supply and Pb concentration in soft tissues is in equilibrium with Pb in blood (Marcus, 1979). The blood lead level is the best indicator of recent elevated lead absorption of acute (but not chronic) type (Mushak, 1993).

Body weight of rats did not change during experiment and there were no incidents of seizures or death.

3.2. Expression of neuronal transporter EAAC1

PCR performed with primers designed to amplify the EAAC1 gene according to previously published data (see

Table 1
Lead levels in blood and brain homogenates of the control and Pb-exposed rats

Sample	Lead level	
	Control group	Lead-exposed group
Blood (μg/dl)	2.8 ± 0.8	59 ± 11.8*
Brain homogenate (μg/g b.w.)	<0.002[a]	1.8 ± 0.4

Data expressed are the means ± S.D. of four independent measurements performed on samples derived from four animals in each group.

* Significantly different from the respective control; $P < 0.05$ (Student's t-test).

[a] Below the sensitivity of applied method.

Section 2) yielded a single product of predicted size (503 bp).

In the forebrain tissue of Pb-exposed rats, the relative expression of EAAC1 mRNA normalized to the GAPDH signal was two-fold higher than that of the control (Table 2). In hippocampal tissue, statistically insignificant lowered expression of mRNA was observed, whereas in cerebellum, no differences in expression were noticed (Fig. 1A).

Immunoreactivity of bands in Western blots was significantly different compared to the respective control only in forebrain homogenates (Fig. 1B) and reached about 40%. The pattern of protein expression change in all examined brain structures was comparable to the alteration of mRNA expression.

3.3. Expression of astroglial transporters GLAST and GLT-1

Primers based on the published rat GLAST and GLT-1 sequences (see Section 2) were designed to amplify fragments of 388 and 555 bp, respectively. A single PCR product of the predicted size was obtained in both cases in all examined brain structures (Figs. 2A and 3A). In control rat brain, GLAST mRNA was expressed at much higher levels in the cerebellum and hippocampus than in forebrain, whereas the opposite pattern was noted for GLT-1 mRNA expression. This was an expected result since it is known that GLAST and GLT-1 are expressed at its highest level in cerebellum (Storck et al., 1992; Rothstein et al., 1994) and forebrain (Pines et al., 1992; Robinson, 1999), respectively.

It was noted that mRNA expression of GLAST was significantly enhanced in Pb-exposed rats in cerebellum and especially in forebrain (Fig. 2A). On the other hand, expression levels of GLT-1 mRNA were found to be significantly decreased in forebrain (over three-fold compared to control) and in hippocampus, whereas they were enhanced in cerebellum (Fig. 3A). The relative intensity of bands obtained in the examined regions of brain and statistical analysis of differences between all groups are presented in Table 2..

The immunoreactivity of the band at 66 kDa and the known physiological pattern of structural expression of protein were observed in immunoblots with anti GLAST antibody. The protein expression was of the highest magnitude in cerebellum and hippocampus and of the lowest levels in forebrain. Under Pb toxicity conditions, the

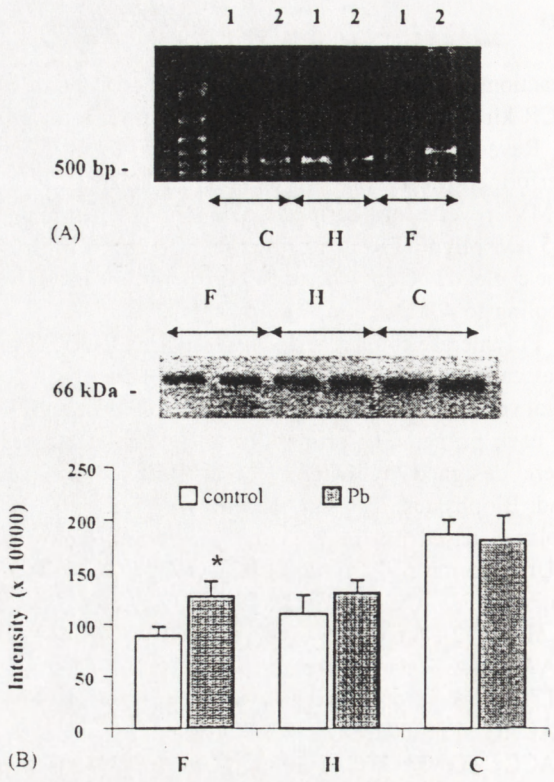

Fig. 1. (A) Expression of EAAC1 mRNA in three regions (C, cerebellum; H, hippocampus; F, forebrain) of control (1) and Pb-exposed (2) rat brains. Single band at 500 bp is visible. Mean results of relative density of bands measured against GAPDH and statistical analysis are shown in Table 2. (B) Expression of EAAC1 protein in brain homogenates (F, forebrain; H, hippocampus; C, cerebellum) obtained from control and Pb-treated rats. Results of densitometric analysis of Western blots ($n = 4$) done using homogenates from distinct animal are presented in the graph. $^{*}P < 0.05$ (one-way ANOVA with post hoc Tuckey test).

increased expression of GLAST protein was found in forebrain, whereas no changes were apparent in hippocampus and cerebellum (Fig. 2B), an observation which does not correlate with that of intense mRNA expression in cerebellum.

Western blots with anti GLT-1 antibody revealed an immunoreactive band of ~70 kDa consistent with the known molecular weight of GLT-1. A loss of immunoreactivity in hippocampus and especially in forebrain of Pb-exposed rats was observed. Densitometric analysis performed to quantify the changes in immunoreactivity showed that GLT-1 protein expression decreased relative to controls by about 40 and 25%, respectively (Fig. 3B). These results

Table 2
Relative expression of glutamate transporters mRNA in different regions of control and Pb-exposed rat brains

	Cerebellum		Hippocampus		Forebrain	
	Control	Pb-treated	Control	Pb-treated	Control	Pb-treated
EAAC1	1.58 ± 0.08	1.82 ± 0.55	2.32 ± 0.01	2.06 ± 0.31	1.13 ± 0.07	2.57 ± 0.02*
GLAST	5.07 ± 0.38	6.90 ± 0.25*	5.05 ± 0.42	4.10 ± 0.87	1.45 ± 0.26	4.72 ± 0.53**
GLT-1	4.48 ± 0.11	6.11 ± 0.69*	4.68 ± 0.52	2.70 ± 0.32*	6.56 ± 0.89	1.88 ± 0.30***

Data are the means ± S.D. of densitometric analysis done on independent gels; $n = 3$ (EAAC1) and $n = 4$ (GLAST and GLT-1). Results are presented as a ratio of respective transporter's and GAPDH signal. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (one-way ANOVA with post hoc Tuckey test).

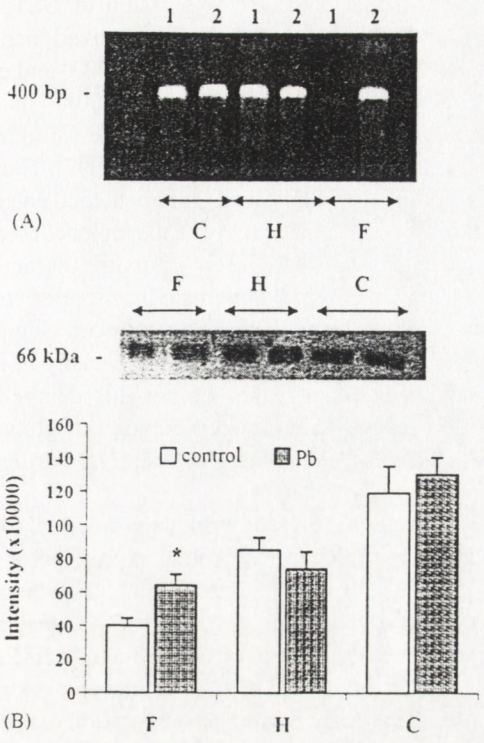

Fig. 2. (A) Expression of GLAST mRNA in three regions of control (1) and Pb-treated (2) rat brain (C, cerebellum; H, hippocampus; F, forebrain). Mean results of relative density of bands measured against GAPDH and statistical analysis are shown in Table 2. (B) Representative immunoblot demonstrating the expression of GLAST protein in three regions of control and Pb-treated rat brains (F, forebrain; H, hippocampus; C, cerebellum). The graph presents the summary of results from four independent immunoblots, each done from distinct brain. Data are the means ± S.D. compared with one-way ANOVA followed by post hoc Tuckey test. $^{*}P < 0.05$ compared with respective control.

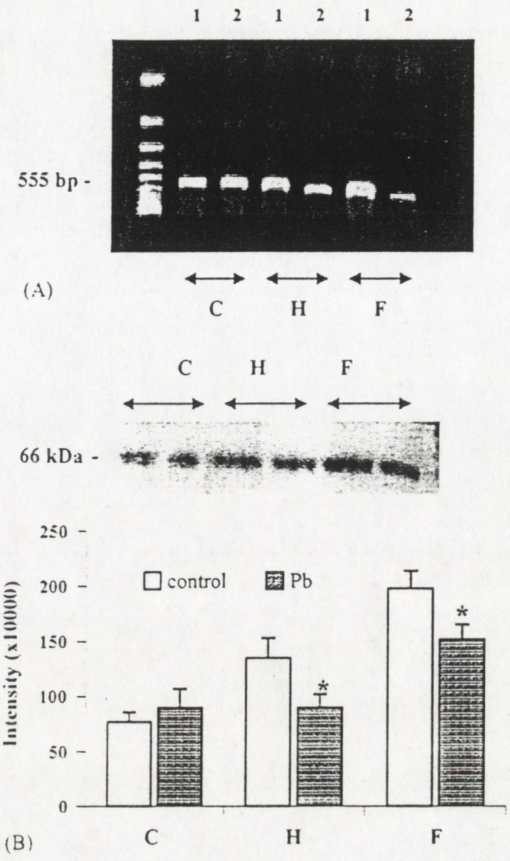

Fig. 3. (A) Expression of GLT-1 mRNA in three regions of control (1) and Pb-treated (2) rat brain (C, cerebellum; H, hippocampus; F, forebrain). Mean results of relative density of bands measured against GAPDH and statistical analysis are shown in Table 2. (B) Representative immunoblot showing the expression of GLT-1 protein in three regions of control and Pb-treated rat brains (F, forebrain; H, hippocampus; C, cerebellum). The graph presents the results of densitometric measurements of four independent immunoblots, each done from distinct brain. $^{*}P < 0.05$ compared with respective control (one-way ANOVA with post hoc Tuckey test).

were consistent with the simultaneously observed changes in mRNA expression of the transporter. Inconsistently, there was no enhancement in protein expression of GLT-1 in cerebellum, although mRNA expression tends to increase in this structure.

The expression of protein did not mirror the expression of mRNA in the case of both GLAST and GLT-1 transporters, an observation which may reflect the existence of post-translational modifications or the influence of regulative mechanisms that mask epitopes distinguished by applied antibodies (Susarla et al., 2004).

4. Discussion

Glial cells play a key role in the in normal brain function both during development and in the adult. These cells are also essential for neuronal response to different negative stimuli (including Pb neurotoxicity) and this response can be modified through action on glial cells (Perez-Capote et al., 2004). Reactive astroglia may play a dual role inducing either neuroprotective or neurodegenerative mechanisms with the actual effect observed being a result of these two functions. Neuron–glia interactions are essential for synaptic function and glial glutamate transporters play a key role in limiting the activity of metabotropic glutamate receptors at glutamatergic synapses. Glutamate transporters regulate glutamate uptake and are modulated on many levels including DNA-transcription, mRNA splicing and protein synthesis (Gegelashvili et al., 2001). Rapid regulation may occur in response to protein kinase C activation (Susarla et al., 2004) and to neuronal glutamate release (Munir et al., 2000; Poitry-Yamate et al., 2002).

4.1. Expression of neuronal glutamate transporter subtype EAAC1

In our previous study using the same model of Pb toxicity, we suggested that the glutamate transporter EAAC1, overexpressed in synaptosomal fraction obtained from whole brain of Pb-treated rat, may contribute to the excessive extracellular glutamate level via operation in the reverse direction (Strużyńska and Sulkowski, 2004).

The present study using homogenates from several parts of brain revealed enhanced expression of EAAC1 mRNA and protein particularly in forebrain (Fig. 1A and B). This may be the result of the increased extracellular glutamate concentration. However, unlike the astrocytic glutamate transporters, EAAC1 does not appear to play a major role in clearance of glutamate from the extracellular space (Rothstein et al., 1996; Tanaka et al., 1997). It has been suggested that the EAAC1 system is the primary route of neuronal cysteine uptake (Chen and Swanson, 2003; Himi et al., 2003). Therefore, overexpression of EAAC1 may reflect enhanced transport of cysteine—the rate-limiting substrate for glutathione synthesis in neurons (Bains and Shaw, 1997). Because glutathione is an important antioxidant, EAAC1 may regulate cellular glutathione content and affect the antioxidant capacity of neurons.

This is in agreement with previously published data, which reported increased GSH content after Pb delivery (McGowan and Donaldson, 1987; Strużyńska et al., 2002) and stimulation of the GSH biosynthetic pathway by Pb (Hsu, 1981).

4.2. The effect of Pb exposure on the expression of astrocytic glutamate transporters—GLAST and GLT-1 in different regions of rat brain

We have also provided evidence that the two astrocytic glutamate transporters, GLAST and GLT-1, are affected under conditions of Pb toxicity. The RT-PCR assay revealed that GLT-1 mRNA and protein expression tend to decrease in forebrain and hippocampus after Pb exposure. This finding is of particular interest in light of the crucial role of this transporter subtype in controlling glutamate level in these sections of the brain (Rothstein et al., 1996). Simultaneously, GLAST was highly overexpressed in forebrain (where its normal expression is typically lowest) but not in hippocampus. This may reflect the protective activity of the astroglial transporter in this structure of brain since a high level of GLAST expression would be required to regulate the enhanced glutamate level during Pb toxicity and to prevent cellular damage.

It is of particular interest that the expression of GLT-1 and GLAST is different under conditions of Pb toxicity. Both astrocytic transporters are Na^+-dependent and specifically up-regulated by substrate (unlike neuronal EAAC1) in a dose- and time-dependent manner (Munir et al., 2000). Astroglial GLAST expression was found to be correlated with neuronal glutamatergic activity and glutamate has been shown to increase GLAST gene expression (Gegelashvili et al., 1996) and translocation of GLAST protein to the plasmalemma (Duan et al., 1999).

The nature of the factors affecting and regulating astrocytic glutamate transporters are not fully understood and are currently the subject of intense investigation. It seems, however, that both transporters are regulated via different signalling mechanisms. Neuronal soluble factors may determine the induction of GLT-1, while having little effect on the expression of GLAST (Campiani et al., 2003). It was shown that enhanced expression of GLT-1 but not GLAST may be induced by brain-derived neurotrophic factor (BDNF) (Rodriguez-Kern et al., 2003) and epidermal growth factor (EGF) (Zelenaia et al., 2000).

The astrocytic GLT-1 is the quantitatively dominant form of Glu transporter in brain (Tanaka et al., 1997). Thus, a lower rate of clearance of the released glutamate (and enhanced risk for excitotoxic cell damage) may be the major consequence of decreased expression of GLT-1. It is possible that activation of GLAST, the other glial glutamate transporter, modulates glutamate neurotoxicity during Pb treatment. Compensatory upregulation of GLAST is observed, especially in forebrain. The question arises: whether or not this compensation is enough to prevent the prolonged duration of glutamate and overstimulation of glutamatergic receptors. Further investigation may clarify this question.

In conclusion, the present work demonstrates, for the first time, changes in mRNA and protein expression of neuronal (EAAC1) and astroglial (GLAST and GLT-1) glutamate transporters in adult rat brain as a result of short lead exposure.

The enhanced expression of GLAST and GLT-1 suggest a protective role of astroglia against elevated extracellular glutamate in the early period of lead toxicity in rodents al least in cerebellum and possibly in forebrain where, however, lowered expression of main astroglial transporter, GLT-1 coexists. Undoubtedly, the hippocampus, which is a critical structure for learning and memory, appears to be the most vulnerable structure, in light of the observation of downregulation of GLT-1 without compensation by GLAST. The simultaneously observed tendency towards decreased levels of EAAC1 mRNA, although statistically not significant, may potentially influence the susceptibility of neurons to the injury due to the disturbed GSH synthesis. Since the hippocampus contributes to cognitive processes, the possibility arises that cognitive impairment can be a result of Pb neurotoxicity in adult brain.

The model of Pb toxicity used in the present study fulfils the conditions of acute type of poisoning characteristic for occupational exposure or occasional incidents of Pb administration. Blood Pb levels in the present experimental study are comparable with those reported for occupationally exposed workers (Hirata et al., 2004; Kasperczyk et al., 2004) (or even lower). However, rats are more resistant to Pb than humans and the similar clinical signs of toxicity are observable in rats with higher blood lead levels. Thus, it may be expected that changes in the expression of glutamate transporters could exist in humans even at lower blood lead concentration.

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K O M E N T A R Z

do rozprawy habilitacyjnej:

„REAKCJA ASTROGLEJU WE WCZESNYM OKRESIE TOKSYCZNOŚCI OŁOWIOWEJ U SZCZURA W ŚWIETLE ZALEŻNOŚCI ASTROCYT-NEURON"

WSTĘP

Problem toksyczności ołowiu w świetle aktualnych badań.

Ołów (Pb) jest pierwiastkiem powszechnie występującym w środowisku, lecz nie mającym znaczenia fizjologicznego. Jego obecność w organizmie uważana jest za przejaw zanieczyszczenia środowiska. Ze względu na rosnącą świadomość szkodliwości Pb dla środowiska i zdrowia człowieka, ogranicza się jego zastosowanie. Dotyczy to jednak głównie państw rozwiniętych gospodarczo, a globalna antropogenna emisja Pb do atmosfery stale kształtuje się na wysokim poziomie. Dodatkowe znaczenie ma fakt, że związki ołowiu nie ulegają biologicznej degradacji, tak więc, jeśli raz dostaną się do środowiska, nie są z niego usuwane.

Toksyczność ołowiu wciąż stanowi problem zdrowotny, wynikający zarówno z narażenia środowiskowego, jak i zawodowego. W centrum szczególnego zainteresowania jest neurotoksyczne działanie tego metalu. Na świecie w badaniach nad neurotoksycznością Pb obserwuje się tendencję do obniżania progu "bezpiecznego stężenia" tego pierwiastka. Niektórzy badacze uznają wręcz, że nie ma bezpiecznego poziomu. Oznacza to, że przy każdym stężeniu Pb w organizmie, możemy spodziewać się nieprawidłowości w przebiegu procesów biochemicznych w tkance nerwowej. Dodatkowo stwierdzono, że metal ten ma zdolność kumulowania się w niektórych tkankach/narządach, skąd może być uwalniany w określonych warunkach (n.p. ciąża, osteoporoza, zaburzenia hormonalne). Może prowadzić to do wzrostu jego stężenia we krwi i w następstwie tego, również w mózgu. Nawet tzw. pula kostna ołowiu, która uważana była wcześniej za miejsce depozytowe tego pierwiastka, będące jednocześnie formą jego detoksykacji, może być wg. najnowszych badań pulą mobilną (O'Flaherty, 2000; Latorre i in., 2003).

Najbardziej drastyczne objawy kliniczne ze strony o.u.n., pod postacią encefalopatii ołowiowej (obrzęk mózgu, drgawki, śpiączka), występują w przypadku ostrego zatrucia wysokimi dawkami metalu, co obecnie ma miejsce rzadko. Stwierdzono jednak, że przy narażeniu na niższe dawki, mogą występować subtelne, niespecyficzne zaburzenia czynności mózgu pod postacią obniżonej percepcji, zaburzeń zdolności uczenia się, słuchu i widzenia, obniżonego IQ, a nawet zaburzeń rozwoju osobowości takich jak agresja (Mendola i in., 2002; Canfield i in., 2003). Tego typu odstępstwa od prawidłowego funkcjonowania obserwuje się głównie u organizmów młodych, jednakże coraz częściej zwraca się uwagę na Pb jako czynnik etiologiczny podobnych zaburzeń występujących u dorosłych.

Niedojrzałość organizmu ma ogromne znaczenie w toksyczności Pb (jak w toksyczności w ogóle). Z jednej strony niedojrzałość bariery jelitowej oraz bariery krew-mózg ma wpływ na

zwiększoną absorpcją ołowiu z przewodu pokarmowego oraz zwiększona penetrację z łożyska naczyniowego do naczyń mózgowych i do parenchymy mózgowej, z drugiej zaś niedostateczne rozwinięcie komórkowych mechanizmów obronnych skutkuje zwiększoną podatnością na czynnik toksyczny. Dlatego ołów został uznany za tzw. „toksynę rozwojową" i z oczywistych względów, uwaga badaczy skupiała się na organizmach młodych. Jeszcze do niedawna pokutował pogląd, że organizmy dorosłe są „odporne" na neurotoksyczność Pb z racji dojrzałej bariery krew-mózg i wykształconych mechanizmów obronnych. Dlatego też epidemiologiczne badania przesiewowe pod kątem zawartości Pb i/lub metabolitów świadczących o jego obecności w organizmie, wykonywane są nieczęsto na populacjach osób dorosłych (z wyjątkiem grup ryzyka zawodowego). Również w badaniach eksperymentalnych nad mechanizmami neurotoksyczności Pb, najczęściej wykorzystywano modele zwierzęce (osobniki młode lub wręcz oseski), oraz hodowle komórkowe.

Jednakże w piśmiennictwie z ostatnich lat coraz częściej zwraca się uwagę na wpływ środowiska na występowanie deficytów neurologicznych (Costa i in., 2004). Sugeruje się, że upośledzenie funkcji poznawczych u dorosłych może być skutkiem długotrwałego działania czynników środowiskowych, wśród których ołów wymienia się na pierwszym miejscu (Maruff i in., 1998: Mendola i in., 2002). Nie wyklucza się również, że neurotoksyczność Pb może mieć związek z występowaniem w mózgu zmian o charakterze neurodegeneracyjnym. Dyskutowana jest hipoteza, że Pb może stanowić jeden z czynników przyspieszających i/lub wzmagających rozwój chorób o podłożu neurodegeneracyjnym (Prince, 1998; Haraguchi i in., 2001). Badania epidemiologiczne wskazują na dodatnią korelację pomiędzy zwiększonym poziomem Pb w mózgu a występowaniem choroby Parkinsona (Gorell i in., 1999; Kuhn i in., 1998). Sugeruje się również udział ekspozycji na Pb w etiologii stwardnienia zanikowego bocznego (ALS). Dane Narodowego Instytutu Zdrowia Stanów Zjednoczonych wykazały wzrost ryzyka wystąpienia tej choroby u ludzi deklarujących narażenie na Pb związane z wykonywanym zawodem. Ryzyko wzrastało prawie dwukrotnie wraz z podniesieniem się poziomu Pb we krwi o 1 mg/dl i 3,5-krotnie przy wzroście Pb w kościach o 1 jednostkę (Kamel, 2003). Badania wykonane w USA wykazały silną korelację pomiędzy zawartością Pb i podwyższonym poziomem homocysteiny we krwi sugerując, że działanie poprzez homocysteinę może być jednym z mechanizmów toksyczności Pb w ośrodkowym układzie nerwowym (Schafer i in., 2005). Tak więc, obserwuje się coraz większe zainteresowanie wpływem Pb na układ nerwowy w ciągu całego życia, nie tylko w okresie zwiększonej wrażliwości t.j. w okresie rozwojowym.

Włączenie astrogleju w patologię neurotoksyczności Pb

Wiadomo, że Pb powoduje zarówno funkcjonalne jak i metaboliczne zmiany w mózgu, jednakże mechanizmy leżące u podstaw neurotoksycznego działania Pb nie są do końca poznane. O ile wpływ Pb na funkcję komórek nerwowych był badany dokładniej, o tyle niewiele wiadomo na temat oddziaływań tego metalu z komórkami astrocytarnymi, szczególnie *in vivo*. Na poziomie biochemicznym i molekularnym Pb wywołuje szereg zaburzeń zarówno w przekaźnictwie nerwowym, jak też własnościach receptorów dla neuroprzekaźników, w tym również dla glutaminianu. Blokowanie aktywności jonotropowego receptora glutamatergicznego NMDA i hamowanie procesu długotrwałego wzmocnienia (LTP) uważa się za mechanizm leżący u podłoża nerotoksyczności Pb w okresie rozwojowym, objawiającej się m.in. zaburzeniem procesów poznawczych (Mendola i in., 2002; Marchetti, 2003). Ostatnie doniesienia sugerują, że ekspozycja na Pb może również u dorosłych zwierząt wpływać na procesy uczenia się i pamięci, w których to zjawiskach przekaźnictwo glutaminianergiczne odgrywa podstawową rolę (Garcia-Arenaz i in., 2004; Vazquez i Pena de Ortiz, 2004).

Liczne badania nad neurotoksycznością Pb wykazały wielość „punktów uchwytu" tego metalu w procesach komórkowych, jak również niejednakową wrażliwość poszczególnych rodzajów komórek na jego toksyczne działanie. Wpływ Pb na komórki astrogleju zasługuje na szczególną uwagę, ze względu na szereg istotnych ról, jakie pełnią one w utrzymaniu homeostazy środowiska okołoneuronalnego (m.in. kontrola zewnątrzkomórkowego stężenia glutaminianu, wydzielanie czynników neurotroficznych i in.). Wcześniejsze badania nad toksycznością metali ciężkich, w tym również ołowiu, wykazały że komórki astroglejowe mają zdolność ich kumulowania (Holtzman i in., 1984; Tiffany-Castiglioni i Qian, 2001). Stanowi to z jednej strony mechanizm ochronny w stosunku do bardziej wrażliwych neuronów, z drugiej zaś tworzy specyficzny rezerwuar tego metalu, potencjalnie toksyczny dla innych rodzajów komórek. Same astrocyty należą do komórek stosunkowo opornych na toksyczne działanie Pb, co związane jest z ich szczególnym metabolizmem oraz generalnie, z pełnieniem funkcji ochronnych w mózgu. Jednocześnie eksperymenty z użyciem hodowli astrocytarnych dowiodły, że mogą one być również celem toksycznego działania Pb.

W różnego typu patologiach ośrodkowego układu nerwowego astroglej zwykle ulega pobudzeniu. Pobudzenie to może mieć charakter protekcyjny w stosunku do neuronów, poprzez uaktywnienie mechanizmów ochronnych (synteza glutationu, enzymów oksydacyjnych, czynników wzrostu). Może też przybierać formę patologiczną, przyczyniając

się do propagacji procesów uszkadzających, poprzez zaburzenie mechanizmów biorących udział w utrzymaniu homeostazy środowiska zewnątrzkomórkowego.

Biorąc pod uwagę szczególną rolę astrogleju w kontroli zewnątrzkomórkowego stężenia glutaminianu, jego metabolizmie i energetyce mózgu, jest pewne, że komórki te mają podstawowe znaczenie dla prawidłowego funkcjonowania OUN, a wiele procesów patologicznych w tkance nerwowej należy postrzegać jako konsekwencję zaburzeń funkcji astrocytów i zależności astrocyt-neuron.

Dlatego **celem** badań objętych rozprawą było przede wszystkim zdobycie wiedzy o roli astrocytarnej puli komórkowej w warunkach toksycznego działania Pb w mózgu dorosłego szczura oraz określenie mechanizmów komórkowych, wiążących metabolizm astrocytów i neuronów, jakie zachodzą we wczesnej fazie tej toksyczności.

Prace będące tematem niniejszej rozprawy włączają się poza tym w ogólny nurt badań nad rolą astrogleju oraz współzależności pomiędzy astrocytem a neuronem w patologii ośrodkowego układu nerwowego.

Model badawczy

Dane literaturowe oraz doświadczenia własne wskazują, iż w przypadku badań nad toksycznością Pb istotne znaczenie ma wybór modelu eksperymentalnego. Uzyskane wyniki mogą różnić się znacząco w zależności od użytej dawki metalu, czasu ekspozycji oraz wieku zwierzęcia w momencie ekspozycji. Podstawowe znaczenie ma jednak fakt, czy działanie Pb zachodzi *in vivo* - w wybranym modelu zwierzęcym, czy też *in vitro* - z wykorzystaniem materiału zwierzęcego, bądź też hodowli komórkowych. Wyniki uzyskane w obydwu tych modelach często są przeciwstawne. Hodowle komórkowe (szczególnie monokultury) nie są dobrym modelem odzwierciedlającym rzeczywiste warunki patologii zatruć Pb, z uwagi na wysoką reaktywność chemiczną kationu Pb^{2+} , który tworzy wiązania z wieloma anionami i wytrąca się w mediach hodowlanych, co stwarza trudności w ocenie rzeczywistego stężenia wolnych jonów Pb odpowiedzialnych za obserwowane efekty. Co więcej, monokultury komórkowe nie dają możliwości oceny efektów Pb wynikających z oddziaływań międzykomórkowych (jak to ma miejsce *in situ,* w mózgu). Dlatego, temat rozprawy realizowano z użyciem modelu zwierzęcego, uznając go za bliższy patologii ludzkiej i lepiej oddający zależności pomiędzy pulami komórek astrocyt-neuron w warunkach ekspozycji.

Wszystkie eksperymenty wykonano na dorosłych szczurach rasy Wistar o masie 200-220 g, którym w iniekcji dootrzewnowej podawano octan ołowiu w dawce 25 mg/kg m.c. przez 3 dni. Grupie kontrolnej podawano wodę destylowaną. Taki sposób podawania Pb jest

powszechnie używany w modelowaniu krótkotrwałej ekspozycji i odzwierciedla narażenie o charakterze ostrym (Zhang i in., 2004) imitującym zatrucie typu incydentalnego, bądź spotykane u osób narażonych zawodowo. Po 3-dniowym okresie podawania Pb, szczury dekapitowano i przeprowadzano preparatykę mózgów. W zależności od potrzeb, tkankę mrożono i przetrzymywano w temp. -70°C do dalszych doświadczeń, bądź też przygotowywano homogenaty i odpowiednie frakcje komórkowe.

Zastosowany w modelu reżim podawania Pb, skutkuje wzrostem Pb we krwi i w mózgu do poziomów charakterystycznych dla krótkotrwałych ekspozycji (Goyer i Chisolm, 1972). Pomiar zawartości metalu we krwi jest testem z wyboru oceniającym stopień narażenia. Choć jego przydatność jest krytykowana, stanowi jedyny parametr umożliwiający oszacowanie zależności dawka-efekt oraz porównanie otrzymanych wyników z danymi literaturowymi. Średnie zakresy zawartości Pb we krwi były zróżnicowane, co wynika z zastosowania dwóch metod pomiaru zawartości Pb (w zależności od dostępności). Początkowo korzystano z metody spektrofotometrii absorpcji atomowej, a następnie z metody spektrofotometrii absorpcji atomowej połączonej z kuwetą grafitową. Poziomy Pb we krwi oznaczone przy użyciu pierwszej z metod były wyższe i obarczone większym rozrzutem. Z uwagi na większą czułość drugiej z metod oraz większą powtarzalność wyników, należy przyjąć, iż stężenie Pb we krwi w zastosowanym modelu wynosiło odpowiednio 2,8 ±0,8 μg/dl dla zwierząt kontrolnych oraz 59±11,8 μg/dl dla eksponowanych. Poziomy te są porównywalne (a nawet niższe) do tych jakie obserwuje się obecnie u osób narażonych zawodowo (Hirata i in., 2004; Kasperczyk i in., 2004). Należy jednak pamiętać, iż szczury są bardziej odporne na toksyczne działanie Pb, a podobne kliniczne objawy zatrucia obserwuje się u nich przy znacznie wyższych poziomach tego metalu we krwi. Można więc oczekiwać, że podobne mechanizmy zatrucia będą występować u ludzi przy niższej zawartości Pb w organizmie.

I. Aktywacja astrogleju jako reakcja na czynnik toksyczny.

Wiadomo, że jedną z właściwości astrogleju jest zdolność reakcji na różnorodne czynniki patologiczne tzw. reaktywną gliozą – związaną ze zmianami morfologicznymi komórek, jak i z biochemicznymi, wyrażającymi się m.in. zwiększoną syntezą wielu substancji (Norenberg, 1996). Reaktywne astrocyty odgrywają szczególna rolę w odpowiedzi na uszkodzenie, gdyż generują czynniki odpowiedzialne za przywrócenie równowagi zaburzonego środowiska zewnątrzkomórkowego i pobudzenie procesów naprawczych. W pewnych warunkach jednak