Enzyme Immunoassay of NSE and GFAP as the Criterion of Dynamic Evaluation of the Rat Blood-Brain Barrier in Perinatal Hypoxic Ischemic Injury of the CNS

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Enzyme immunoassay of the serum neurospecific antigens (gliofibrillar acid protein and neurospecific enolase) was used for evaluation of the resistance of the blood-brain barrier in Wistar rats with perinatal hypoxia and ischemia of the CNS. Perinatal hypoxia and ischemia of the CNS was modeled by two methods: ligation of the common carotid artery in 7-day-old rats followed by 3.5-h hypoxic hypoxia or 15-min anoxic exposure of fetuses isolated via hysterectomy on day 21 of gestation. Enzyme immunoassay of serum gliofibrillar acid protein and neurospecific enolase in control an experimental rat pups was carried out once a week during 3 months. In controls serum levels of gliofibrillar acid protein and neurospecific enolase virtually did not change during postnatal development, while in animals with cerebral hypoxia and ischemia induced in fetuses by both methods serum concentration of neurospecific enolase sharply increased 1 week after the injury and increased on weeks 6 and 10. The content of gliofibrillar acid protein was maximum on week 1 and later considerably varied, the peaks of its concentrations observed on weeks 3 and 8 preceded the increase in neurospecific enolase activity in peripheral blood.

Key Words: *experimental perinatal hypoxia and ischemia of CNS; enzyme immunoassay of serum gliofibrillar acid protein and neurospecific enolase*

Neurospecific proteins (NSP) as serum markers of brain diseases and injuries resulting from impairment of the blood-brain barrier (BBB), attracted much attention during two last decades of the twentieth century [1,6,8,11,12]. Enzyme immunoassay of neurospecific antigens is widely used for quantitative assessment of BBB permeability in the brain-blood direction [1]. The best studied antigens adequately characterizing BBB functions are gliofibrillar acid protein (GFAP), a structural protein of astrocyte intermediate filaments [6], and neurospecific enolase (NSE), a neuronal glycolytic cytoplasmatic enzyme catalyzing transformation of 2-phosphoglycerate into 2-phosphoenolpyruvate [12].

Unique potentialities of GFAP and NSE as natural markers of BBB functions explain special interest to the studies demonstrating the prospects of using assays of neurospecific antigens for the analysis of the severity of hypoxic ischemic injuries to the CNS during the perinatal period and for monitoring the disease course and predicting its outcome [3,4,7,8,10]. However, despite similar results of dynamic clinical and experimental studies of the phenomenon of neurospecific proteins elimination into the blood and cerebrospinal fluid, these reports described only the acute period of conditions associated with disorders of

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cerebral circulation and the resultant hypoxia and ischemia of the nervous tissue [8,11,14]. On the other hand, BBB functions in delayed periods after perinatal hypoxic ischemic damage of fetal brain (HIDFB) are little studied; the causes of chronic transformation of the neurodegenerative process, the key point for the course and outcome of HIDFB in children, remain unclear [1,3].

This problem is important for better understanding of some aspects in the pathogenesis of HIDFB, the role of impairment of the BBB permeability for NSP in chronic transformation of the neurodegenerative process after perinatal CNS injury.

The aim of our study was enzyme immunoassay of BBB permeability for GFAP and NSE over 3 months after induction of HIDFB in rats.

MATERIALS AND METHODS

The known models of HIDFB do not completely reflect numerous pathophysiological and pathomorphological processes associated with perinatal ischemia and hypoxia of human fetal brain [2]. Therefore we used two experimental models of HIDFB: the model developed by J. Rice *et al.* [13] and reproducing predominantly unilateral HIDFB and the model proposed by B. Lubec *et al.* [5,10] and reproducing intrauterine fetal hypoxia and diffuse involvement of the nerve tissue in both hemispheres of rat brain.

Experiments were carried out on 270 rat pups of both sexes from 20 litters of Wistar females. The animals were kept under standard conditions with free access to food and water.

Two experimental series were carried out. HIDFB was induced by Rice's method (61 experimental animals and 67 controls, series I) and by Lubec's method (66 experimental and 76 control animals, series II). The numbers of males and females in the groups were approximately the same.

In the first series experiments carried out on 7-day-old rat pups the common carotid artery (CCA) was mobilized through the median incision on the neck and ligated with a silk thread under inhalation narcosis (3-min exposure in a 3-liter excicator with a cotton tampon wetted in 5.0 ml ether pro narcosis). Bloodflow arrest in CCA was controlled visually. The rats were left in an incubator at 37°C for 1 h, after which 3.5-h hypoxic hypoxia was modeled in a 2-liter chamber through which wet gaseous nitrogen-oxygen (8% O_2) mixture was let at a rate of 5-6 liters/min at 37°C. After the exposure the rats were put back into cages to their mothers. Control animals were treated similarly except CCA ligation and hypoxic hypoxia. At the age of 25-28 days the rat pups were separated from mothers and kept in cages (5 per cage).

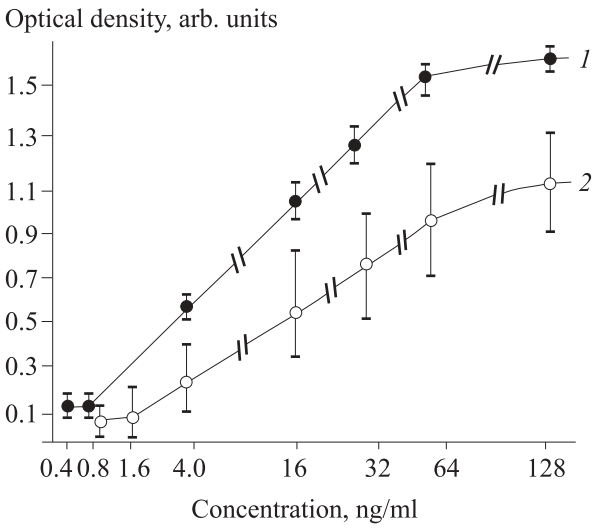

Fig. 1. Calibration curves of enzyme immunoassay of gliofibrillar acid protein (GFAP, *1*) and neurospecific enolase (NSE, *2*).

In the second series pregnant females were sacrificed on day 21 of gestation by cervical dislocation, and hysterectomy was immediately carried out. Uterine horns with fetuses were put into water bath (37°C, 15 min) for anoxic exposure, after which the fetuses were removed from the uterine horns, umbilical cords were ligated, and the fetuses were placed in an incubator (37°C). Each fetus was isolated within 1-2 min. After 1 h these pups were weighed, pups weighing more than 4.5 g were selected and put into cages with feeding females. Control group consisted of animals isolated directly after hysterectomy without anoxic exposure.

In both series of experiments the blood was collected from the vena cava inferior under ketamine anesthesia (100 mg/kg) 1, 2, 3, 4, 6, 8, 10, and 12 weeks after HIDFB modeling (5-8 experimental and control rats per term). After blood collection the brain was perfused with 4% paraformaldehyde. Blood samples were stored at -80°C.

Serum GFAP and NSE were measured by enzyme immunoassay using special test systems developed on the basis of the corresponding monoclonal antibodies at Laboratory of Immunochemistry, V. P. Serbskii Center of Social and Forensic Psychiatry [1]. The results were recorded on a single-channel photometer (Bio-Teck Instruments) at λ=450 nm.

Statistical analysis was carried out using Student's *t* test and Pierson's coefficient of correlations.

RESULTS

The developed test systems for enzyme immunoassay of GFAP and NSE allowed analysis of these antigens in concentrations from 0.5 to 64 ng/ml (Fig. 1).

In the first experimental series serum concentrations of NSP in controls (Fig. 2, *a-b*, and 3, *a-b*)

1 week after the intervention were 2.84±2.40 ng/ml for GFAP and 1.7±1.3 ng/ml for NSE; in the second series these values were 0.48±0.30 ng/ml and 1.9±0.9 ng/ml, respectively. After 2 weeks these concentrations decreased and then virtually did not change throughout observation.

In experimental groups in series I and II the maximum concentrations of GFAP 1 week after injury were 24.9±11.0 ng/ml (p=0.01) and 18.6±8.4 (p=0.02), those of NSE were 19.6±5.8 ng/ml (p=0.02) and 24.0±7.4 ng/ml (p=0.005), respectively. After 2 weeks serum levels of NSP decreased 2-5-fold, but remained above the control (p<0.05) in all cases. Wave-like changes in NSP concentrations were observed during weeks 2-12 (Fig. 2, *c*, and 3, *c*). At the peak of the curve the mean serum concentrations of NSP in animals with HIDFB differed significantly (p<0.05) from the respective values of controls (Fig. 2, *c*). In series I a maximum drop of NSE concentration corresponded to the peak of GFAP concentration (weeks 3, 8, and 12), and vice versa, the increase of NSE release into peripheral blood (weeks 2, 6, and 10) was paralleled by a decrease in serum GFAP concentration. In series II (Fig. 3, *c*) pronounced wave-like changes were observed only for GFAP (peaks on weeks 3 and 10), while regression of NSE concentrations was more even, though the profiles of GFAP and NSE differed considerably on weeks 3 and 8. Analysis of correlations of the results in series I revealed a negative relationship between changes in serum levels of NSE and GFAP in the same animals during weeks 2, 6, and 12 after injury (correlation coefficients -0.517, -0.467, and -0.452, respectively); the respective correlations in series II were observed during weeks 3 and 8 (-0.40 and -0.33, respectively). In the latter case blood concentrations of GFAP and NSE on weeks 4 and 10 of the experiment positively correlated (0.38 and 0.96, respectively).

The results of enzyme immunoassay of NSP in the peripheral blood of control animals coincide with the data obtained by other scientists [15]. Our findings suggest that serum levels of GFAP and NSE in Wistar rat pups in early ontogeny (<3 months) did not exceed

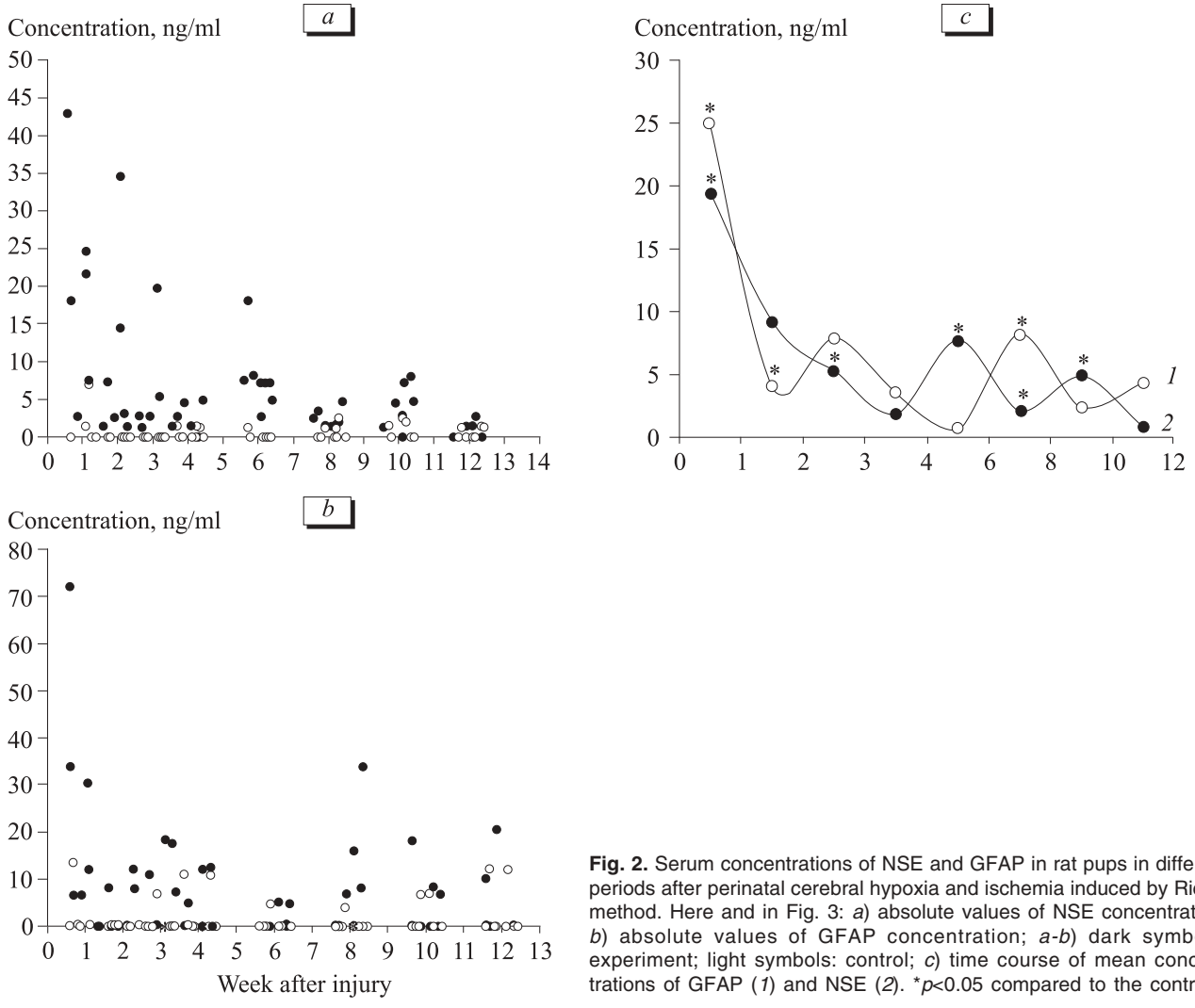

Fig. 2. Serum concentrations of NSE and GFAP in rat pups in different periods after perinatal cerebral hypoxia and ischemia induced by Rice's method. Here and in Fig. 3: *a*) absolute values of NSE concentration; *b*) absolute values of GFAP concentration; *a*-*b*) dark symbols: experiment; light symbols: control; *c*) time course of mean concentrations of GFAP (*1*) and NSE (*2*). *p<0.05 compared to the control.

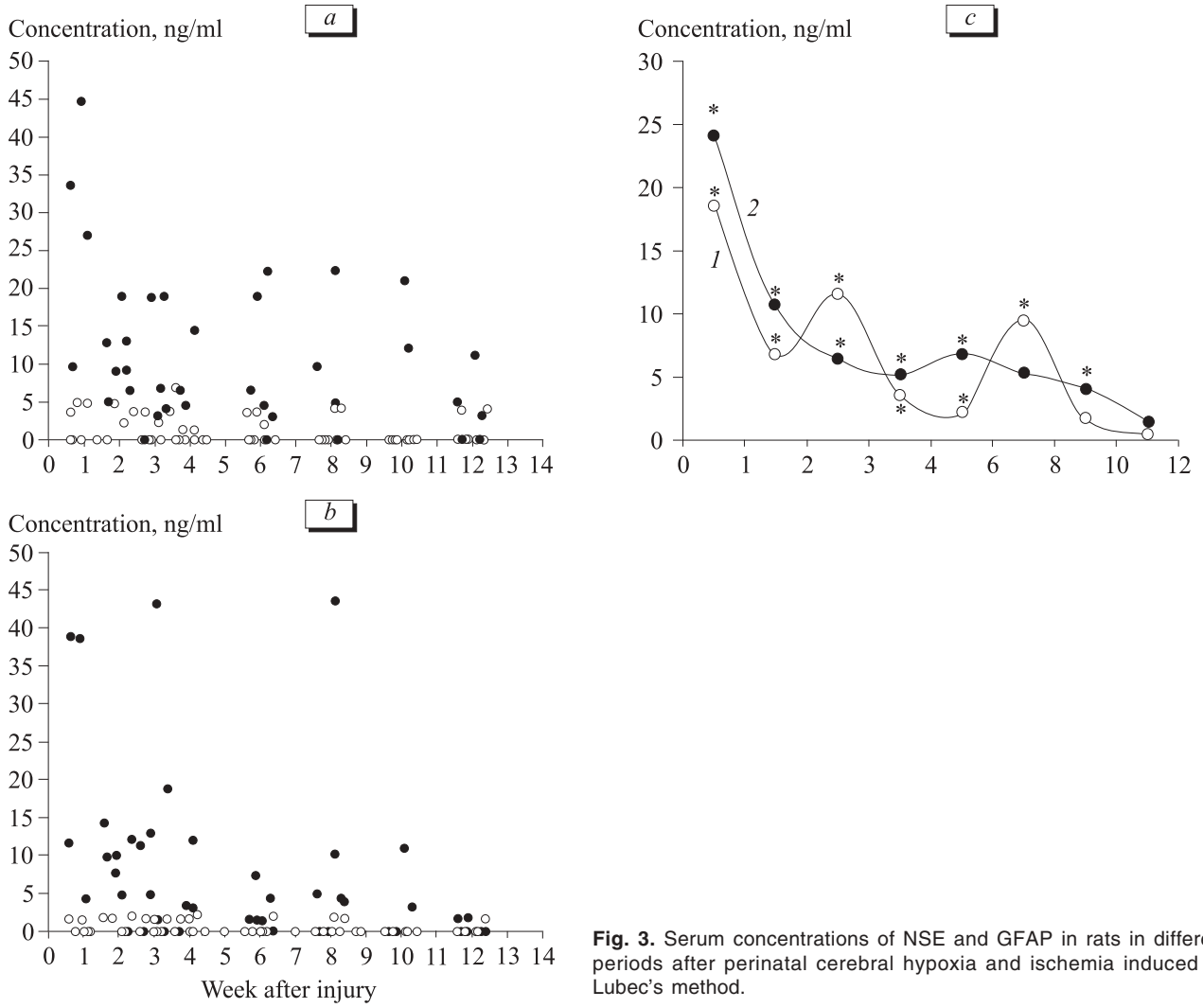

Fig. 3. Serum concentrations of NSE and GFAP in rats in different periods after perinatal cerebral hypoxia and ischemia induced by Lubec's method.

2.8 and 1.7 ng/ml, respectively. Experiments showed that the 1st week after HIDFB induction was characterized by massive release of NSE and GFAP into peripheral circulation (primary impairment of BBB structures), which was followed by long lasting (10-12 weeks) periodical increases in BBB permeability for these antigens. The increase in GFAP content preceded the increase of NSE content during weeks 2-3. It is known that elimination of neurospecific antigens into peripheral blood is associated with a complex of specific immune reactions, for example, production of the corresponding antibodies [1]. The peak of their blood concentrations was observed also after 12-20 days, i.e. coincided with the dynamics of impairment of BBB permeability for NSE and GFAP in rats with HIDFB observed in our experiments (Fig. 2, *c*, and 3, *c*). Presumably, the repeated increases in BBB permeability for NSP in HIDFB are caused by autosensitization of the rat pups' lymphocytes with NSE and GFAP, production of the corresponding autoantibodies, and subsequent autoimmune (complement-dependent) damage to BBB cell structures. The studied HIDFB models involve structural organic injuries to cerebral tissue, and therefore we cannot exclude the possibility of penetration of anti-NSP autoantibodies into damaged areas of the brain and subsequent propagation of the neurodegenerative process initiated by a complex of processes associated with primary damage to the nerve tissue during HIDFB induction. It seems that this hypothesis explains the phenomenon of the second and third waves of increased NSP concentrations in the peripheral blood after HIDFB induction by the method of Rice (Fig. 2, *c*), attesting to chronic transformation of the neurodegenerative process. The absence of pronounced fluctuations in serum NSP levels of rat pups with HIDFB induced by Lubec's method (Fig. 3, *c*) can be explained by the type of cerebral tissue damage (in our case predominating diffuse cytoneuropathological process without apparent ischemic foci) [9].

Hence, the detected rhythmic elimination of NSP into peripheral blood during early postnatal period in

animals with induced HIDFB is one more argument in favor of the autoimmune mechanisms of chronic transformation of the neurodegenerative process.

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