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EXPERIMENTAL ARTICLES

The Effect of Prenatal Stress on Glutathione-Associated Antioxidant Enzyme Activity in Subcellular Fractions of Neocortical Neurons and Neuroglia of Rats during the Period of Intensive Myelination

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Abstract—We studied the effect of prenatal stress on the activity of glutathione-associated enzymes in subcellular fractions of neurons and neuroglia from the neocortex of 20-day-old male rats (during the period of intensive myelination). The following changes were observed in prenatally stressed animals compared to the control. The activity of glutathione peroxidase (EC 1.11.1.9) in the cytosol and the nuclear fraction of neurons and neuroglia increased, but decreased in the mitochondrial fraction. The activity of glutathione reductase (EC 1.8.1.7) increased in the cytosol and in the nuclear fraction of the neuroglia. The activity of glutathione transferase (EC 2.5.1.18) increased in the mitochondrial fraction of neurons and in the cytosol of neuroglia but decreased in the nuclear fraction of neurons and in the mitochondrial fraction of neuroglia. We believe that the changes in the activity of the studied enzymes in the subcellular fractions of neocortical neurons and neuroglia in prenatally stressed rats observed at the age of 20 days negatively affect the processes of myelination in the neocortex and may contribute to accelerated aging and the development of neurodegenerative diseases in later life.

Keywords: prenatal stress, glutathione peroxidase, glutathione-S-transferase, glutathione reductase, neurons, glia, subcellular fractions, myelination

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INTRODUCTION

Numerous studies of stress effects on neuroglial relationships have revealed critical periods in the processes of neurogenesis and gliogenesis. Disruption of the formation of neuroglial complexes during these periods undoubtedly affects the functioning of the adult organism. The consequence of this disorder is changes in the differentiation of the cortex in the process of prenatal ontogeny [1]. The rat is a convenient model object for studying these processes, since the critical periods of both prenatal and postnatal ontogeny are well studied. One such period of neocortex formation in rats is the period of the second and third weeks of postnatal development. It was shown that by 20 days rat pups have maximal exploratory activity [2]. At this age, the weight of the neocortex in rats, along with other brain structures, reaches its maximum values [2]. It has been shown that the growth of neuron bodies in the cortex is completed by the 17th–20th day. At the same time, intense myelination occurs in the deep layers of the cortex, in addition, during the first 3 weeks of postnatal development, intense synaptogenesis occurs, and by the end of the 3rd week of postnatal development, the maximum oxygen consumption in the brain is established [3].

As is known, one of the consequences of exposure to prenatal stress (PS) is the generation of an excessive amount of reactive oxygen species (ROS) by various mechanisms. The organ-specific response depends on the relative balance between ROS generation and antioxidant resources of the cell [4, 5]. Flerov et al. [6] found no differences in the level of diene conjugates and Schiff bases in the neocortex of prenatally stressed rats at the age of 20 days compared with the control group of the same age. However, in the study of neurons and neuroglia isolated from the neocortex of prenatally stressed rats at the age of 20 days, the levels of diene and triene conjugates and Schiff bases were reduced in neuroglia compared with the control group. In the same study, an increase in the level of oxidative modification of proteins, both in neurons and in neuroglia, was found in prenatally stressed 20-day-old male rats compared with controls. At the same time, the activity of the antioxidant enzyme Cu-Zn-superoxide dismutase was lower in neurons, while it did not change in neuroglia [7].

In our previous study [8], we detected changes in the activity of glutathione-dependent antioxidant enzymes in neurons and neuroglia in prenatally

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stressed adult rats. However, these changes are undoubtedly the result of processes occurring at earlier stages of pre- and postnatal ontogeny, especially during critical periods.

Therefore, the objective this work was to study the activity of antioxidant glutathione-dependent enzymes in subcellular fractions of neurons and neuroglia in prenatally stressed rat pups at the age of 20 days, which is critical for the processes of neurogenesis and gliogenesis and the formation of the neocortex.

MATERIALS AND METHODS

The work was carried out on animals from the nursery of the Pavlov Institute of Physiology of the Russian Academy of Sciences, in compliance with the recommendations on the ethics of working with animals proposed by Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Experiments were carried out on Wistar rats. The animals were kept under standard vivarium conditions with a 12-h light regimen and free access to food and water. All manipulations with animals were performed from 9 to 11 am.

To produce prenatally stressed offspring primiparous pregnant females (age 5 months, weight 250–270 g) were subjected to one-hour immobilization stress under conditions of high illumination from the 15th to the 19th day of gestation [9]. In the experiment, 12 females were used (6 females in the control group and 6 females in the stressed group), to which males were placed (1 male to 3 females). A vaginal smear was taken daily from the femails to determine the phase of the estrous cycle. Day zero of pregnancy was considered the day when spermatozoa were found in the vaginal smear of the female in the estrus stage. On the 18th day of pregnancy, the females were placed in separate cages, where they remained until giving birth and during the process of feeding offspring. Further, 4 males at the age of 20 days were randomly selected from each brood (without using a blind method).

Male rats were decapitated, the brain was removed from the skull and the neocortex of both hemispheres were dissected. One sample included cortices from four animals; in total there were six samples ($n = 6$). Thus, 48 male rats were used in the experiment (24 rats in the control group, 24 rats in the prenatally stressed (PS) group).

Cell fractions enriched in neurons and neuroglia were isolated using the Sellinger method modified by Flerov [10]. The principle of the method is to obtain a cell suspension with subsequent isolation and purification of neuronal and neuroglial fractions by ultracentrifugation (VAC-60 ultracentrifuge, SWOUT 50 × 3 rotor, Germany) in a density gradient of sucrose and ficoll (sucrose, Vecton, Russia; Ficoll 400, Merck, Germany). In this case, the cell suspension was obtained by disintegrating the tissue by passing it through nylon and metal meshes with successively decreasing pore size. To facilitate disintegration, the tissue was preliminarily treated with a solution of polyvinylpyrrolidone (PVP-K30, Merck, Germany). After isolation, the fractions of neurons and neuroglia were washed from sucrose with physiological saline and then centrifuged for 10 minutes in a centrifuge at 3500 *g* (Eppendorf 5430R centrifuge, Germany). The resulting precipitate was homogenized (Potter's homogenizer, Sartorius, Germany) in 1 mL of 0.25 M sucrose solution containing 1 mM EDTA (Vekton, Russia), pH 7.4 (for neurons), or in 0.25 M sucrose containing 1 mM EDTA, pH 7.4 (for neuroglia), in a volume equivalent to the sediment volume. Further, the isolation of subcellular fractions was performed by the standard method of differential centrifugation as previously described [11].

Enzyme activity of glutathione peroxidase (GPO), glutathione reductase (GR), and glutathione transferase (GT) were determined using kits (Glutathione Peroxidase Assay Kit CatNo:EGPX-100, Glutathione Reductase Kit CatNo:ECGR-100, Glutathione S-transferase Assay Kit CatNo:DGST-100, BioAssay Systems, United States) on a Thermo Scientific Multiscan fs photometer (United States). The unit of activity of the studied enzymes was taken as the number of nmol of the reaction product formed in 1 min per 1 mg of protein (nmol/min/mg of protein). The amount of total protein was determined by the Lowry method.

Statistical processing The results were analyzed using the Mann–Whitney *U* criterion in the "IBM SPSS Statistics 21" software. The normality of the distribution of the values of the groups under consideration was performed using the Shapiro–Wilk test. Statistical hypotheses were tested at a significance level $p < 0.05$. When describing quantitative data, the following indices were used: Me is the median; IQR is the interquartile range between the values of 25–75 percentiles.

RESULTS

Table 1 summarizes the activity of enzymes of the glutathione pool in the cytosol, nuclear and mitochondrial fractions of neurons and neuroglia in the control group of rats at the age of 20 days.

Of the three enzymes studied in the cytosol of neurons, GR activity was absent. In the nuclear fraction, GR and GT activity was detected, while GPO activity was absent. In the mitochondrial fraction of neurons, GR and GT activity was not detected.

In the cytosol of neuroglia, the activity of GPO and GT was lower compared to neurons; in addition, GR activity was detected, in contrast to neurons ($p < 0.05$). In the nuclear fraction of neuroglia, the activity of all three enzymes was higher compared to the activity of enzymes in the nuclear fraction of neurons ($p < 0.05$). In the mitochondrial fraction of neuroglia, GPO

Table 1. Activity of glutathione peroxidase (GPO), glutathione reductase (GR), and glutathione transferase (GT) (nmol/min/mg protein) in subcellular fractions of neurons and neocortex neuroglia of control rats at the age of 20 days, Me(IQR), ($n = 6$ in all groups)

	Neurons			Neuroglia		
	C	N	M	C	N	M
GPO	27.7 (18.5–38.9)	ND	401.5 (229–574)	6.17 (1.8–9.7)#	21.3 (11.1–40.8)#	137.0 (114–229)#
GR	ND	13.1 (8.8–17.6)	ND	14.3 (0–17.9)#	28.1 (25–31.2)#	ND
GT	7.9 (7.8–11.5)	2.1 (0–2.7)	ND	1.1 (1.06–1.19)#	9.2 (9.2–9.6)#	17.3 (13.8–34.6)#

Me(IQR), where Me is the median, IQR is the interquartile range between the values of 25–75 percentiles; C, cytosol; N, fraction of nuclei; M is the fraction of mitochondria; ND is the activity was not determined; # difference between neuroglia and neurons at $p < 0.05$.

Table 2. Activity of glutathione peroxidase (GPO), glutathione reductase (GR), and glutathione transferase (GT) (nmol/min/mg protein) in subcellular fractions of neurons and neocortex neuroglia of prenatally stressed rats at the age of 20 days, Me(IQR), ($n = 6$ in all groups)

	Neurons			Neuroglia		
	C	N	M	C	N	M
GPO	305.5 (305.5–496)*	9.2 (5.1–10.2)*	ND*	99.4 (66.9–121.6)*	85.1 (76.1–107.7) *	ND*
GR	ND	11.6 (10.0–19.6)	ND	22.4 (16.1–29.5)*	35.4 (19.6–41.2)	ND
GT	8.6 (6.9–9.5)	ND*	27.7 (15.6–44.9)*	5.2 (4.8–5.9)*	9.9 (6.4–12.7)	5.2 (0–6.4)*

Symbols same as in Table 1. *, difference between the group of prenatally stressed rats and the control group at $p < 0.05$

activity was lower than in neurons in the mitochondrial fraction ($p < 0.05$). GR activity in the mitochondrial fraction of neuroglia was not detected, nor was it detected in the mitochondrial fraction of neurons. However, GT activity was detected in the mitochondrial fraction of neuroglia. It should be noted that GPO activity is an order of magnitude higher in the mitochondrial fraction of both neurons and neuroglia compared to the activity of this enzyme in all other fractions. Also noteworthy is the absence of GR activity in the mitochondrial fraction of both neurons and neuroglia.

Changes in the activity of the studied enzymes in the subcellular fractions of neurons and neuroglia in prenatally stressed rats at the age of 20 days are shown in Table 2.

In prenatally stressed animals, GPO activity in the cytosol of neurons increased 10 times compared to the control, GR activity, like in the control, was absent, and GT activity did not change compared to the control. In the fraction of neuron nuclei, in contrast to the control, GPO activity was detected, and GR activity did not have significant differences from the control, while GT activity was not detected. In the mitochondrial fraction of neurons from PS animals, GPO and GR activity was not detected, while GT activity, in contrast to the control, was detectable.

In neuroglia of prenatally stressed rats, the activity of all studied enzymes in the cytosol increased compared to control rats ($p < 0.05$). GPO activity significantly increased in the nuclear fraction ($p < 0.05$), while the activities of GT and GR did not differ from the control group. In the mitochondrial fraction, GPO activity was absent, in contrast to the control, GT activity decreased compared to the control, and GR activity, as in control animals, was not detectable.

DISCUSSION

In our previous study [8], we revealed the effects of prenatal stress on the activity of antioxidant enzymes of the glutathione pool in subcellular fractions of neurons and neuroglia in the neocortex of mature male rats. We concluded that in adult rats, changes in the activity of the studied enzymes are both compensatory and pathological. However, the formation of neuroglial relationships in ontogenesis is also important for behavioral and cognitive properties, and the probability of developing neurodegenerative diseases in adult individuals. Therefore, here we studied juvenile rats at

Table 3. Changes in the activity of the studied glutathione enzymes in subcellular fractions of neurons and neuroglia in prenatally stressed rats compared with the control group

	Neurons			Neuroglia		
	C	N	M	C	N	M
GPO	↑	↑	↓	↑	↑	↓
GR	–	–	–	↑	–	–
GT	–	↓	↑	↑	–	↓

Symbols as in Table 1. "↓", decrease in activity; "↑", increase in activity; "–", no change.

the age of 20 days, when the myelination processes have maximum intensity [3]. The literature notes the fact that the brain during early postnatal development has a significant reserve of low molecular weight antioxidants, while the concentrations of the antioxidant enzymes superoxide dismutase and GPO are relatively low [12]. However, it should be noted that it is the activity of antioxidant enzymes that is more important than their absolute amount. The results of this study showed that in 20-day-old rat pups in the control group, the activity of enzymes in the fraction of neuroglial nuclei is higher compared to neurons (Table 1). It can be assumed that it is most important for neuroglia to maintain the required level of antioxidant activity of glutathione-dependent enzymes in the nuclear fraction. At the same time, in neurons, the activity of GPO in the cytosol and in the mitochondrial fraction is almost 4 times higher than in neuroglia. These data suggest that the ability to maintain a lower level of ROS in neurons than in neuroglia [13] at the early stages of ontogeny is due not only to the specific organization of complex 1 in mitochondria [14] but also due to increased GPO activity. At the same time, in 20-day-old rat pups, a high activity of GPO in comparison with other studied enzymes is observed in the mitochondrial fraction both in neurons and neuroglia. Apparently, this is important for the viability and survival of developing neurons [15]. The activity of GT in the nuclear and mitochondrial fractions is significantly higher in neuroglia than in neurons, while the activity of GT in the cytosol of neurons is higher than in neuroglia. GT is an important regulator of Nrf-2 (nuclear erythroid-associated factor 2) activity [16], which, in turn, through ARE (antioxidant responsive element) induces the expression of a wide range of antioxidant enzymes, including those associated with the synthesis and metabolism of glutathione [17]. Such a picture during the period of enhanced myelination can be explained by the need to regulate the antioxidant status through Nrf-2 not only in neuroglia, but also in neurons, since neurons themselves are directly involved in the regulation of myelination processes, as shown in the study by Simons et al. [18].

Changes in the activity of the studied enzymes in subcellular fractions of neurons and neuroglia in 20-day-old rat pups as a result of PS exposure are presented in the form of a scheme in Table 3.

As can be seen from Table 3, GPO activity in the mitochondrial fraction of neurons and neuroglia in prenatally stressed rats is lower compared to the control group. While in neurons this is partly compensated by the appearance of GT activity, in neuroglia the GT activity decreases by 3 times. It is likely that PS disrupts energy processes, which affects the processes of myelination in juvenile rat pups and may contribute to the development of neurodegenerative diseases [19]. At the same time, in the cytosol of neuroglia after PS, the activity of all three studied enzymes increases, and in the cytosol of neurons, only the activity of GPO increases. It can be assumed that antioxidant mechanisms are activated in neuroglia, which make it possible to compensate for the negative consequences of PS in the neocortex. This is confirmed by the previously obtained results on the unchanged level of lipid peroxidation and protein oxidative modifications in neurons and neuroglia [7], as well as on the less effective glutathione system for neuronal peroxide detoxification [20], and data on the greater resistance to ischemia of juvenile oligodendrocytes [21].

In the fraction of neuron nuclei after PS, GT activity is not detected, while GPO activity is detected, which may be of a compensatory nature. It should be noted that both in prenatally stressed rat pups and in control rat pups, in the fraction of nuclei of neuroglia, the activity of the studied enzymes is higher compared to neurons (Tables 1, 2). The distribution of the activity of the studied enzymes in the fraction of neuroglial nuclei in prenatally stressed rats, similar to control animals, may indicate that the mechanisms of antioxidant protection of the genetic material of this cell population are preserved.

In order to trace the effect of changes in the activity of the studied enzymes at the critical age for postnatal formation of neuroglial relationships (20 days) on the activity of these enzymes in adult animals, we compared age-related changes separately in control and prenatally stressed rats using our previously obtained data [8] that is shown in Table 4 (control) and Table 5 (PS).

As can be seen from Table 4, control animals are characterized by a significant decrease with age in the activity of the studied enzymes in neurons, with the exception of GPO in the cytosol. Whereas in neuroglia, on the contrary, the activity of all studied antioxidant enzymes increases with age. This increase is especially noticeable for GPO in the cytosol (by 5 times) and in the mitochondrial fraction (by 6 times). Studies by Galkina et al. [22, 23] showed that the activity of antioxidant enzymes of the glutathione pool in 20-day-old rat pups in the brain is lower than in adult animals. The authors attribute this to the effect of these enzymes on the GSH/GSSG ratio, believing that this may be a factor in "switching" the proliferation phase

Table 4. Comparison of the activity of the studied glutathione enzymes in subcellular fractions of neurons and neuroglia in control rats at the age of 90 days compared with control rats at the age of 20 days

	Neurons			Neuroglia		
	C	N	M	C	N	M
GPO	↑ (27–274)	– (ND–7)	↓ (401–ND)	↑ (6–32)	– (21–29)	↑ (137–898)
GR	– (ND–ND)	↓ (13–ND)	– (ND–ND)	↑ (14–41)	– (28–36)	– (ND–ND)
GT	↓ (7–ND)	↑ (2–6)	– (ND–ND)	↑ (1–13)	↑ (9–25)	↑ (17–79)

Symbols same as in Table 1. "↓", decrease in activity; "↑", increase in activity; "—", no changes; (A–B) is shown in parentheses, where A is the enzyme activity in rats at the age of 20 days and B is the enzyme activity in rats at the age of 90 days. Enzyme activity values are presented as medians (Me).

Table 5. Comparison of the activity of the studied glutathione enzymes in subcellular fractions of neurons and neuroglia in prenatally stressed rats at the age of 90 days compared with groups of prenatally stressed rats at the age of 20 days.

	Neurons			Neuroglia		
	C	N	M	C	N	M
GPO	↓ (305–23)	↑ (9–28)	↑ (ND–164)	↑ (99–197)	↓ (85–62)	↑ (ND–244)
GR	– (ND–ND)	↑ (11–29)	– (ND–ND)	↑ (22–77)	– (35–43)	– (ND–ND)
GT	↓ (8–ND)	↑ (ND–6)	↑ (28–63)	↑ (5–18)	↑ (9–20)	↑ (5–74)

Symbols same as in Table 1. "↓", decrease in activity; "↑", increase in activity; "–", no changes; (A–B) is shown in parentheses, where A is the enzyme activity in a rat at the age of 20 days; B is the enzyme activity in a rat at the age of 90 days, the values of the enzyme activity are presented as medians (Me).

and the differentiation phase of nerve cells. At the same time, this may be due to the formation of specific supercomplexes from complex 1 in neuron mitochondria and the production of more ROS in glial cells [14].

When considering the age dynamics in prenatally stressed rats (Table 5) is noteworthy the decrease in the activity of GPO in the cytosol of neurons in 90-day-old animals (by 13 times) and the appearance of the activity of this enzyme in the mitochondrial fraction of neurons (164 nmol/min/mg of protein), whereas in prenatally stressed rats aged 20 days, the activity of this enzyme was not detected. In addition, in adult (90 days) prenatally stressed animals, the activity of GT increased in the mitochondrial fraction of neurons, while in the control, the activity of this enzyme was not detected at both stages of development (Table 4). Thus, in the neurons of prenatally stressed rats during maturation, multidirectional changes in the activity of GPO in the cytosol and mitochondrial fraction are observed in comparison with control animals.

In the cytosol of neuroglia in prenatally stressed rats, the activity of all studied enzymes increases with age, as well as in control rats. However, it should be noted that both at the age of 20 and 90 days in prenatally stressed rats in the cytosol of neuroglia, the level of activity of all the studied enzymes is higher than in control animals. When considering changes from 20 to 90 days in neuroglia in control animals, it can be seen that GPO activity in the mitochondrial fraction increases sixfold to 898 nmol/min/mg of protein (Table 4), while the activity of this enzyme in prenatally stressed animals varies from undetectable values to 244 nmol/min/mg of protein (Table 5). At the same time, GT activity in the mitochondrial fraction of neuroglia in prenatally stressed animals reaches values comparable to control animals at the age of 90 days (Table 5), increasing by 14.8 times, while in control animals the activity of this enzyme, having a higher level on the 20th day, increases only by 4.6 times by the 90th day (Table 4). Apparently, PS does not change the program for the formation of neuroglial complexes but causes changes in the rate of morphogenetic processes, which is possibly associated with simultaneous processes of differentiation, pathological changes, and compensatory processes. As indicated in our previously published works [6, 7], PS has the greatest effect on free radical protein oxidation and lipid peroxidation in neurons and neuroglia in 20-day-old animals compared to adult animals. However, this restructuring of the age-related dynamics of the oxidative modification of biomolecules may indicate processes of tissue adaptation, which are forced to develop under conditions of numerous desynchronoses. At the same time, abrupt metabolic rearrangements during ontogenesis in rats subjected to prenatal stress, as a result of the interaction of subcellular populations, lead to compensation of pathological changes if we consider the indicators of lipid peroxidation and oxidative modification of proteins in the neocortex in adult rats. The above comparative data may indicate that, in prenatally stressed animals, the compensatory mechanisms of antioxidant activity are switched on to a sig-

nificant extent both in neurons and in neuroglia. However, when considering the extent of this compensation, one can assume a disruption in the functioning of mitochondria in prenatally stressed animals, which does not allow the studied antioxidant enzymes to reach the level of activity determined in control animals. The constant activation of compensatory mechanisms of this level can probably lead to faster "wear and tear" of the antioxidant system and, as a result, accelerated aging.

CONCLUSIONS

The study showed that prenatal stress leads to a change in the level of activity of glutathione-dependent antioxidant enzymes in subcellular fractions of neurons and neocortical neuroglia in rats aged 20 days. An analysis of these changes allows us to conclude that PS has a negative effect on the processes of postnatal ontogeny in the neocortex, and suggests that one of the causes for the increased predisposition of prenatally stressed animals to neurodegenerative diseases and accelerated aging are changes in the activity of glutathione-dependent enzymes both in neurons and in the neuroglia of the neocortex. To understand the pathological mechanisms of the negative effect of PS on the CNS, it is necessary to study the functions of mitochondria in prenatally stressed animals. Further research in this direction may be important for clinical practice in the prevention of neurodegenerative diseases and their treatment with mitochondria-targeted therapy.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Ethical approval. All applicable international, national and/or institutional principles for the care and use of animals have been observed.

AUTHORS' CONTRIBUTIONS

Each of the authors equally participated in the planning of the experiment, literature analysis, work with animals, biochemical studies, statistical processing and discussion of the results.

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