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EXPERIMENTAL ARTICLES

Antioxidant Defense in the Rat Brain Cortex, Cerebellum, Hippocampus, and Striatum and Its Alterations during Portacaval Shunting

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Abstract—Portacaval anastomoses spontaneously appear during liver insufficiency and lead to chronic hyperammonemia and hepatic encephalopathy. Ammonium induces oxidative stress in the brain. Here, we compared the state of the antioxidant system in different rat brain areas and studied the manner in which it is affected by portacaval shunting. At 4 weeks after the surgical operation, we measured the activities of catalase, Cu,Zn-superoxide dismutase, glutathione peroxidase, glutathione reductase, and glutathione transferase in cytoplasm and content of oxidized proteins in mitochondria of the neocortex, cerebellum, hippocampus, and striatum. All four brain regions differed according to the ammonium content and activity of each antioxidant enzyme. The activity of glutathione peroxidase decreased as compared to the control only in the cerebellum and striatum of animals with portacaval shunt. After portacaval shunting, the activities of catalase, Cu,Zn-superoxide dismutase, glutathione peroxidase, and glutathione reductase did not differ from the control in all the brain regions we studied. An inverse correlation between the content of oxidized proteins and activity of glutathione transferase suggests that the diminished activity of glutathione peroxidase in the cerebellum and striatum, but not other antioxidant enzymes and other brain regions, may play a role in the pathogenesis of hepatic encephalopathy.

Keywords: portacaval shunting, antioxidant enzymes, neocortex, cerebellum, hippocampus, striatum

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INTRODUCTION

Portacaval shunting (PCS) is a surgical method to create an artificial channel between the portal vein and the vena cava of the liver for drainage of venous blood into the systemic blood flow bypassing the liver. Many human diseases (cirrhosis, hepatitises, and alcoholism) induce hypertension in the portal vein and internal hemorrhage, which frequently results in the death of patients. In these cases, surgical PCS is necessary for correction of hemodynamics of portal hypertension, hemostasis of gastroesophageal bleeding, and to save a patient's life. On the other hand, portacaval anastomoses that spontaneously appear during liver insufficiency worsen ammonium detoxification and lead to chronic hyperammonemia and hepatic encephalopathy, the main cause of death during this disease.

The mechanisms of the disruption of brain function during hyperammonemia have not been well studied. Studies in animal models with acute liver insufficiency [1, 2] showed that ammonium causes oxidative stress in the brain. However, results of the analysis of oxidative stress during hepatic encephalopathy in patients or experiments with animals are not numerous and contradictory [3], whereas the data on antioxidant enzymes in brain regions and subcellular fraction are fragmentary or absent. Here, we performed a comparative study of the state of the antioxidant system in different brain regions of rats and its influence on the PCS. We determined the activities of Cu,Zn-superoxide dismutase (SOD), catalase, glutathione peroxidase (GP), glutathione reductase (GR), and glutathione transferase (GT) in the cytosol as well as the content of oxidized proteins in mitochondria of the neocortex, cerebellum, hippocampus, and striatum in the norm and 4 weeks after PCS.

MATERIALS AND METHODS

The experiments were performed in accordance with the European Rules on the use of laboratory animals 1986 (rules of laboratory practice in Russia. Order of Ministry of Health Care of Russia from June 19, 2003 no. 267).

We used male Wistar rats weighing 250–300 g. The animals were separated into two groups: sham-oper-

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Abbreviations: GP, glutathione peroxidase; GR, glutathione reductase; GT, glutathione transferase; PCS, portacaval shunting; SOD, superoxide dismutase.

ated ($n = 8$) and PCS operated ($n = 6$). The operation was performed under general anesthesia.[2] PCS was performed as described by Lee and Fischer [4]. Inasmuch as the body weight of animals gradually decreases after the operation (due to the consumption of smaller amounts of food), studies were performed in 4 weeks when the animals of both groups had insignificant differences in the body weight [5].

Reagents from the Sigma Chemical Co. (United States) were predominantly used in the study.

All preparative procedures were performed at 2–4°C.

The animals were decapitated, the brain was removed, and the neocortex, cerebellum, hippocampus, and striatum were dissected.

Mitochondria were isolated from each brain region by a previously described method [6]. The postmitochondrial supernatant was centrifuged at 100000 *g* and the supernatant we obtained was used as the cytoplasmic fraction. In the cytoplasm, we measured the activities of catalase, Cu,Zn-SOD, GP, GT, and GR.

One of the markers of oxidative stress is the carbonylation of proteins. In the rat brain, mitochondria are the major cell source of both hydrogen peroxide and protein carbonyl groups [7]. Therefore, we evaluated oxidative stress in the brain areas by an increase in the total amount of oxidized protein in mitochondria.

The activities of SOD (EC 1.15.1.1), catalase (EC 1.11.1.6), GP (EC 1.11.1.9), and GR (EC 1.8.1.7) were determined by methods that were described earlier [8, 9]. As a unit of SOD activity we took the amount of protein at which the rate of reduction of p-nitrotetrazolium blue in the xanthine-xanthine oxidase system decreases by a factor of 2.

The activity of GT (EC 2.5.1.18) was measured by a method described by Kozer et al. [10] with modifications. In 1 mL of 100 mM phosphate buffer at pH 7, which contained 1 mM EGTA, 1 mM glutathione, and 1 mM chlorodinitrobenzol we added 100 μg of cytosolic protein. The reaction was spectrophotometrically recorded at 340 nm and 37°C; for calculations, we used the absorption coefficient 9.6 mM^{-1} cm^{-1}.

The content of carbonylated proteins was determined by the method of Ahn et al. [11]. For this, we collected two equal parts of mitochondria with 400 μg protein in each. Both parts were incubated in a 1% solution of streptomycin sulfate for 15 min at 4°C to remove nucleic acids; the samples were then centrifuged at 14000 *g* for 10 min at 4°C. One supernatant was treated with 10 mM dinitrophenolhydrazine (DNPH) in 2 N HCl (at a ratio of 1 : 2) and the other with an equal volume of 2 N HCl. Both samples were incubated for 45 min at 4°C in the darkness with constant stirring on a shaker. The protein was then precipitated by an equal volume of 20% trichloroacetic acid, centrifuged, collected, and triply washed with a mixture ethanol–ethylacetate (1 : 1) at 14000 *g* for 10 min at 4°C. The final precipitate was dried, dissolved in 0.5 mL of 6 M guanidine hydrochloride with 20 mM phosphate buffer (pH 2.5) and incubated at 37°C for 15 min on a shaker with constant stirring. The content of oxidized carbonyl-containing proteins was determined by the difference in light absorption between the DNPG-treated sample and the HCl-treated control at 370 nm. The result was calculated with the use of the absorption coefficient of hydrazine of 22 mM^{-1} cm^{-1} and expressed in nmoles of DNPG per 1 mg protein. Since at different stages of washing, up to 15% of the protein was lost, the concentration of protein was measured in a precipitate dissolved in 6 M guanidine dihydrochloride by absorption at 260–280 nm. The amount of protein was calculated using a standard curve for bovine serum albumin dissolved in 6 M guanidine dihydrochloride.

The ammonium content in the blood plasma, neocortex, and striatum was measured by microfluorometry [12]. The data on ammonium in the plasma, cerebellum, and hippocampus were taken from a previous article [13].

The concentration of ammonium in the brain cytoplasm was calculated assuming that 1 g of tissue contains approximately 0.5 mL of cytoplasmic water [14].

Statistical analysis was performed using Prizm 5.0 software (GraphPad, United States). The results were expressed as the mean and the standard error of the mean. The normality of the distribution was confirmed using the Kolmogorov–Smirnov criterion. Paired comparisons were performed using the Student's statistical analysis; differences between the tissues were analyzed by ANOVA using the *t* criterion for multiple comparisons and the Bonferroni correction.

RESULTS

The ammonium concentration in blood plasma was 0.14 ± 0.02 mM in the control sham-operated rats. It significantly increased by a factor of 2 in the group of rats with portacaval anastomosis (to 0.27 ± 0.02 mM, $p = 0.0002$).

In four brain regions of control rats, ammonium content was different (Fig. 1). It was at its maximum in the cerebellum, much lower in other brain regions, and at a minimum in the neocortex. The differences between the cerebellum and other brain regions are significant.

In animals with PCS, the ammonium concentration in all brain regions was higher than in the control rats but this increase was not uniform: by 40, 99, 39, and 47% in the cerebellum, neocortex, hippocampus, and striatum, respectively (Table 1). In terms of cytoplasmic water [14], the ammonium concentration in the cytosol of the cerebellum reached 1 mM, in other regions it was 0.5–0.6 mM and it increased to

[2] Operation was performed in the Laboratory of Neurology, Valencia, Spain (Centre de Investigaciones Principe Felipe, Valencia, 46012 Spain). The authors are grateful to Ana Agusti and Vicente Felipo for technical help.

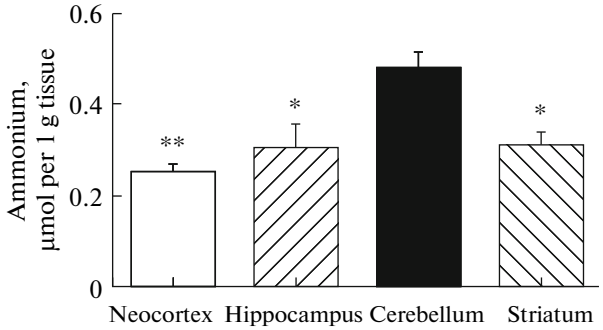

Fig. 1. The ammonium content in the cerebellum, hippocampus, neocortex, and striatum of control animals. The results are shown as M ± SE. *$p < 0.05$ and **$p < 0.01$ as compared to the cerebellum (the *t* test with the Bonferroni correction).

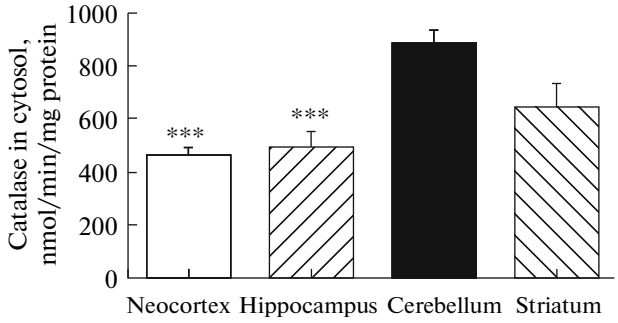

Fig. 2. The activity of catalase in the cerebellum, hippocampus, neocortex, and striatum of control animals. The results are shown as M ± SE. ***$p < 0.001$, as compared to the cerebellum (the *t* test with the Bonferroni correction).

1.32 mM and 0.85–1 mM after PCS, respectively (Table 1).

Under all conditions, the ammonium concentration in the cerebellum is significantly higher than in other brain regions and in all brain regions it is 3–7-fold higher than in the plasma. Thus, the four brain regions differ from each other in both the level of endogenous ammonium and the ability to accumulate exogenous ammonium (in systems of transport via the blood–brain barrier or in intracellular metabolic processes or other mechanisms) in vivo.

The activity of antioxidant enzymes in the brain regions is shown in Figs. 2–6 and Table 2.

In the control animals, catalase activity was substantially higher in the cerebellum than in the neocortex and hippocampus (by 78–94%) (Fig. 2). These differences were highly significant ($p < 0.001$). At 4 weeks after PCS, the catalase activity in all brain regions did not differ from the control.

The activity of Cu,Zn-SOD was at its maximum in the neocortex, its minimum in the striatum and hippocampus, and was at an intermediate level in the control animals (Table 2). The differences between the neocortex and hippocampus and the neocortex and striatum were highly significant. After PCS, the activity of Cu,Zn-SOD in all four brain regions did not change; however, the Cu,Zn-SOD activity in the cerebellum significantly exceeded the enzyme activity in the hippocampus, whereas the difference between the striatum and neocortex disappeared.

The specific activity of GP in the cerebellum (39.6 ± 2.8 nmol/min per 1 mg protein) and striatum (35.3 ± 2.3) in the control was higher than in the neocortex (27.6 ± 2.3) and hippocampus (31.8 ± 4.9) (Fig. 3). However, the differences between all four brain regions were insignificant. In animals with PCS, the GP activity in each of the four brain regions did not differ from the control.

The GR activity was at its maximum in the cerebellum (32.9 ± 2.1 nmol/min per 1 mg protein), while it was lower by 26–40% in the neocortex, hippocampus, and striatum (Fig. 4). The differences between the cerebellum and other brain regions were highly significant ($p < 0.01$). After PCS, the GR activity in each of the four brain areas was the same as in the control group; however, the differences between the cerebellum and striatum were insignificant.

Table 1. The concentration of ammonium in the cytoplasm of the cerebellum, hippocampus, neocortex, and striatum of rats in control group and 28 days after PCS operation

Brain area	Control		PCS	
	ammonium concentration, μM	ratio tissue/plasma	ammonium concentration, μM	ratio tissue/plasma
Cerebellum	946 ± 70	6.86	1320 ± 90**	4.89
Hippocampus	616 ± 92^{+}	4.46	856 ± 31^{+++}	3.17
Neocortex	500 ± 50^{++}	3.62	996 ± 66***, $^{+}$	3.69
Striatum	616 ± 58^{+}	4.46	906 ± 56**, $^{++}$	3.56

Concentration of ammonium in cytoplasm of brain areas was calculated using data of the previous study [13]. The results are shown as M ± SE and expressed in μmole per 1 L of cytoplasmic water. **, $p < 0.01$ and ***, $p < 0.001$ as compared to control (Student's *t* test); $^{+}$, $p < 0.05$, $^{++}$, $p < 0.01$, and $^{+++}$, $p < 0.001$ as compared to the cerebellum (*t* test with Bonferroni correction).

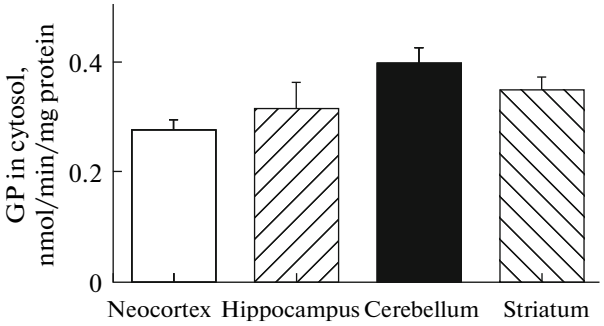

Fig. 3. Activity of GP in the cytosol in the cerebellum, hippocampus, neocortex, and striatum of control animals. The results are shown as M ± SE. All differences are insignificant.

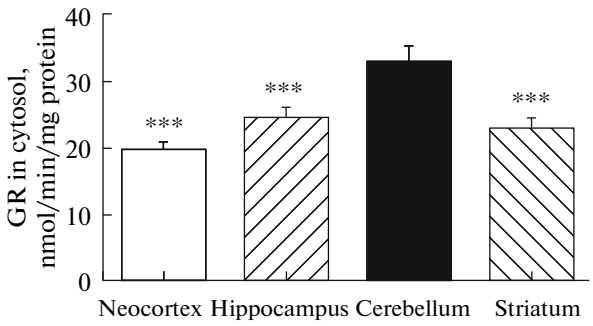

Fig. 4. The activity of GR in the cytosol in the cerebellum, hippocampus, neocortex, and striatum of the control animals. The results are shown as M ± SE. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to the cerebellum (*t* test with Bonferroni correction).

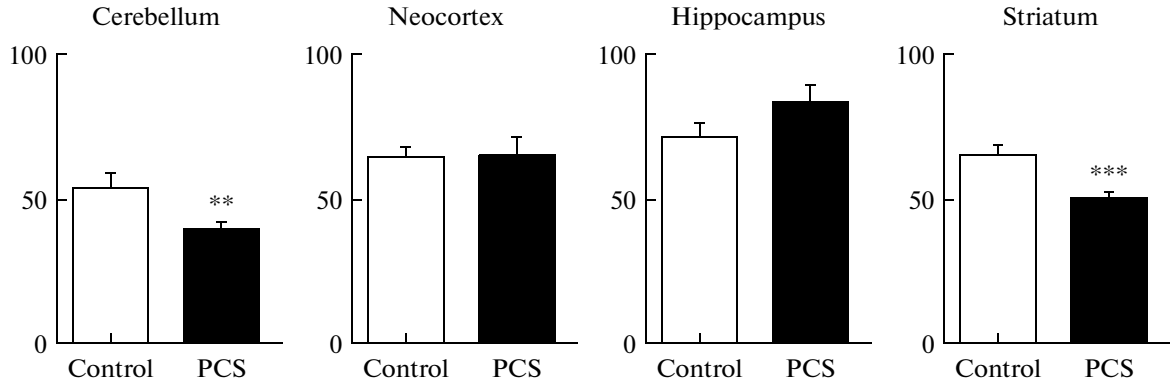

Fig. 5. The activity of GT in the cytoplasmic fractions of the cerebellum, hippocampus, neocortex, and striatum in the control and 4 weeks after PCS. The ordinate axes show the enzyme activity in nmol/min/mg protein. The results are shown as M ± SE. $^{**}p = 0.0036$ and $^{***}p$ 0.0003 as compared to the control (Student's *t* test).

The GT activities in the cerebellum, neocortex, hippocampus, and striatum were 53.1 ± 3.7, 62.4 ± 2.7, 68 ± 3.1, and 63.6 ± 1.8 nmol/min/mg protein, respectively (Fig. 5). Only the differences between the hippocampus and the cerebellum were significant ($p < 0.01$). In rats with PCS, the GT activity was lower than in sham-operated animals, viz., in the cerebellum by 31% ($p = 0.0036$) and in the striatum by 24% ($p = 0.0003$) and did not differ from the control in the neocortex and hippocampus (Fig. 5).

Table 2. The activity of Cu,Zn-SOD in the cytoplasm of the cerebellum, hippocampus, neocortex, and striatum of rats in the control group and 4 weeks after PCS operation

Brain area	Activity of Cu,Zn-SOD, un./min per 1 mg protein	
	control	PCS
Neocortex	15 ± 0.7	$12.3 \pm 1.1^{+}$
Hippocampus	$9.7 \pm 1^{+++}$	8.9 ± 0.8
Cerebellum	12.1 ± 0.4	$12.7 \pm 0.3^{+}$
Striatum	$9.6 \pm 0.8^{+++}$	11.6 ± 0.9

The results are shown as M ± SE. All differences between PCS group and control are insignificant. $^{+++}$, $p < 0.001$ as compared to the neocortex; $^{+}$, $p < 0.05$ as compared to the hippocampus(the *t* test with the Bonferroni correction).

The level of the carbonyl groups in mitochondrial proteins was at its maximum in the cerebellum (7.21 ± 0.34 nmol/mg protein), lower by 26% in the hippocampus (5.34 ± 0.31 nmol/mg protein, $p < 0.01$) and was insignificantly lower in the neocortex (by 13%, $p > 0.05$) and striatum (by 9%, $p > 0.05$) (Fig. 6). At 4 weeks after PCS, the content of carbonylated proteins increased in mitochondria of the cerebellum (by 26%, $p = 0.0008$) and striatum (by 27%, $p = 0.0104$), and was insignificantly altered in mitochondria of the neocortex and hippocampus.

There is a strong negative correlation between the content of oxidized proteins in the mitochondria and the GT activity in the cytosol: the coefficient of the Spearmen rank correlation is $r_s = -0.9998$ ($n = 32$, $p < 0.0001$) in rats of the control group and $r_s = -0.9996$ ($n = 24$, $p < 0.0001$) for PCS animals.

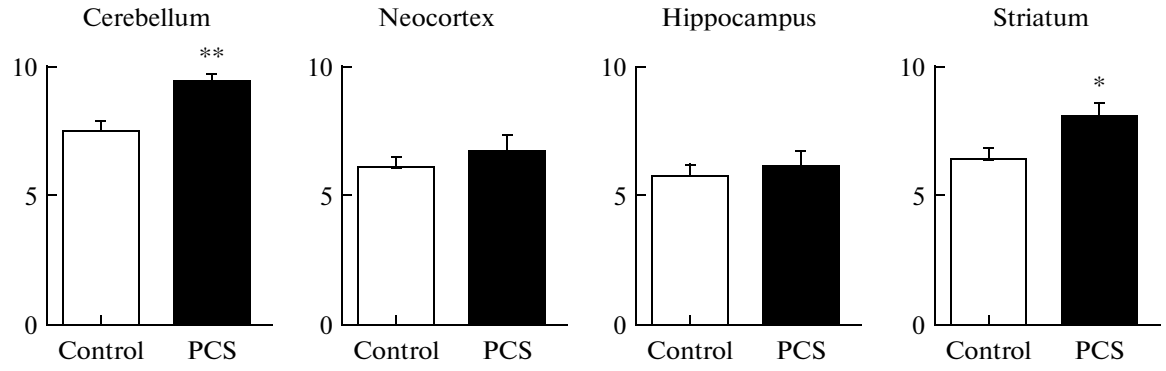

Fig. 6. The contents of carbonyl groups in proteins of mitochondria from the cerebellum, hippocampus, neocortex, and striatum in the control and at 4 weeks after PCS. The ordinate axes shows the content of carbonylated proteins in nmol/mg mitochondrial protein. The results are shown as M ± SE. $*p = 0.0104$, and $**p = 0.0008$ as compared to the control (Student's t test).

DISCUSSION

It is known that in a 4 week period portacaval anastomosis may result in liver atrophy and hyperammonemia [15]. High concentrations of ammonium induce oxidative stress in the brain [8] which appears to be one of the major factors in the pathogenesis of hepatic encephalopathy [2]. Here, the ammonium concentration in the blood plasma of rats 4 weeks after PCS increased to 0.27 mM, which is two times higher than the normal level and is in agreement with previous data [15, 16]. However, changes in the activity of this moderate hyperammonemia did not affect all antioxidant enzymes and all brain regions. Our results show that the neocortex, cerebellum, hippocampus, and striatum are characterized by different quantitative distribution of all analyzed antioxidant enzymes. The activities of these enzymes in different brain areas changes differently under conditions of chronic portacaval anastomosis. In the cytoplasm of the cerebellum and striatum, the GT activity was decreased by PCS and the activity of all the other enzymes did not differ from the control. In contrast, the content of oxidized protein increased after PCS only in the mitochondria of the cerebellum and striatum. The increase in the content of carbonylated proteins reflects the enhancement of oxidative stress.

The hippocampus had the smallest sensitivity to PCS: 4 weeks after the operation, the concentration of ammonium, the activities of the five analyzed enzymes in cytoplasm, and the total number of oxidized proteins in the mitochondria remained unaltered. In the neocortex, the activity of the enzyme in the cytoplasm and the content of carbonyl groups in mitochondrial proteins after PCS also did not differ from the control, although the ammonium concentration increased to a larger extent among other brain regions we studied. The absence of changes in the content of oxidized proteins indicates that a 4-week period after PCS is not enough time for the development of oxidative stress in the hippocampus and neocortex.

Our results are in general agreement with the data of Yan et al. [3], who showed that the contents of carbonylated protein in mitochondria of the rat forebrain do not change after 4 weeks of PCS. The authors concluded that PCS-induced hyperammonemia did not cause oxidative stress in the forebrain.

A very strong negative correlation between the content of carbonyl groups and GT activity in the brain regions ($r_s = -1.0$) indicates that protein oxidation is directly linked with weakening of GT capacity to detoxify 4-hydroxyalcenals and other cytotoxic products and lipid peroxidation [17, 18].

The literature contains only a single study by Bosoi et al. [19] on the activity of antioxidant enzymes in brain regions during PCS. In that study, the indices of oxidative stress in the forebrain, such as the concentrations of activated oxygen-containing metabolites; malonic dialdehyde; glutathione; the ratio between reduced/oxidized glutathione; and the activities of catalase, SOD, and GP remained unaltered. Our experiments showed that the activities of catalase, Cu,Zn-SOD, GP, and GR did not change after PCS in the cytoplasm of other brain areas as well, viz., the cerebellum, hippocampus, neocortex, and striatum.

Table 1 and Figs. 1–6 show the picture of the distribution of ammonium and the activities of all the enzymes. The cerebellum clearly stands apart among the four brain regions according to the biochemical indices in both the control and PCS animals. Recently, we described clear distinctions of the cerebellum from other brain regions in indices of homeostasis of mitochondrial calcium [20], which were found after feeding animals with ammonium acetate. However, it is hard to deeply discuss these unexpected observations in the absence of comparative studies in literature.

Our results suggest that moderate chronic hyperammonemia as a consequence of PCS results in an increase in the ammonium content in brain areas, as well as inducing oxidative stress in the cerebellum and striatum but not in the neocortex and hippocampus. The hippocampus is least sensitive to PCS and the cer-

ebellum is the most sensitive to hyperammonemia and oxidative stress [21]; hence, the observed biochemical changes in the cerebellum and striatum may reflect the onset of oxidative stress in the brain. This is associated with a decrease in GT activity and enhancement of protein oxidation only in these two brain areas. Thus, ammonium and oxidative stress in the cerebellum and striatum may play a collective role in the pathogenesis of hepatic encephalopathy.

CONCLUSIONS

Here we showed that the stationary concentration of ammonium and the activity of each of the analyzed antioxidant enzymes differ in the neocortex, cerebellum, hippocampus, striatum and undergo different changes during chronic PCS. After 4 weeks of portacaval anastomosis, oxidative stress was enhanced only in the cerebellum and striatum in parallel with a decrease in the GT activity in these brain areas. These results suggest that decreased GT activity in the cerebellum and striatum but not other antioxidant enzymes and other brain areas, may play a role in the pathogenesis of hepatic encephalopathy.

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