Abstract
CuZn superoxide dismutase (CuZnSOD) activity in hippocampus and brain cortex of Wistar male rats exposed to acute stress (immobilization or cold for 2 h), chronic stress (isolation or social crowding for 21 days), or to their various combinations was examined. The highest CuZnSOD activity in both brain structures was observed in chronic isolation and it decreased after additional exposure to either of the acute stressors. Contrary to that, chronic crowding had no effect on CuZnSOD activity in hippocampus, while in brain cortex it even caused the suppression of enzyme activity. Additional exposure to acute stresses differently affected the enzyme activity depending on the brain region and type of stress: immobilization increased the activity of CuZnSOD in hippocampus, while cold decreased it in cortex. Acute stress by immobilization caused the elevation of CuZnSOD activity in cortex, and acute cold exposure decreased enzyme activity in hippocampus. The observed region specific alterations of SOD activity indicate that neuroendocrine stress most probably generate cellular imbalance between production and elimination of ROS, which may provide some insights into a variety of neuropsychological processes as well as their treatment.

Introduction
Exposure to stress alters the normal body homeostasis and leads to the development of various pathologies, which might involve alterations in the antioxidant defense system. CuZn superoxide dismutase is the antioxidant enzyme that catalyses the dismutation of the highly reactive superoxide anion to O2 and to the less reactive species H2O2 (1), and it is believed to play a major role in the antioxidant defense system of nervous tissue (2). The importance of CuZnSOD in the CNS is emphasized by the finding that this enzyme exerts a protective action against brain injury and neuronal death both in vitro and in vivo (3).

The relation of CuZnSOD activities and stress has been reported in several studies. Long term emotional stress activated glutathione peroxidase, and activation was accompanied by a decrease in superoxide dismutase and glutathione transferase activities (4). Immobilization stress is followed by an increase of lipid peroxidation, measured in plasma and brain, and in the inhibition of antioxidant enzymes (5-7). Studies of cytosolic fractions of cerebral cortex also demonstrated that there was a significantly greater increase in lipid peroxidation and protein oxidation after immobilization stress (8).

In the present study, our interest was focused on the cytosolic CuZnSOD which is responsible for 80% of SOD activity in the brain (9). Tissues chosen for analysis were the hippocampus and the brain cortex of Wistar male rats. We investigated which kind of stress (acute, chronic or combined)
led to pronounced changes in CuZnSOD activity.

Materials and Methods

Animal treatments: Adult Wistar rat males, aged three months, weighing 330-400 g, were housed in groups of four individuals per cage and offered water and food ad libitum. The light was kept on between 7:00 a.m. and 7:00 p.m. The 'Guiding Principles for the Care and Use of Animals' based upon Helsinki Declaration (1964) and 'Protocol of the "Vinča" Institute on care and treatment of laboratory animals' were strictly followed. The experiment had two parts. In the part I rats were exposed to either of the two types of acute stress: cold (Cold) or immobilization (Immob) for 2 h; or to the chronic stresses: isolation (Isol) or social crowding (Crowd) which lasted for 21 days. In case of isolation one animal was housed per cage, while in case of crowding eight animals were housed per cage; the untreated animals served as controls. In the part II rats previously exposed to either of the chronic stresses were subjected to immobilization or cold for 2 h. Immobilization stress was induced as described by Kvetnansky and Mikulaj (10). The animals exposed to cold were initially kept at ambient temperature and than carefully transferred into a cold chamber at 4°C. Unstressed controls or stressed animals were sacrificed 2 hrs following the end of the stress procedure by decapitation with a guillotine (Harvard-Apparatus, USA) under ether anaesthesia. Brain cortex and hippocampus were immediately excised and placed on ice.

Preparation of neuronal cell extracts and CuZnSOD enzyme activity assay: The extracts of brain cortex and hippocampus of 6 animals per experimental sample were prepared by the following procedure: tissues were weighed and homogenized (1:6 w/v, Potter-Elvehjem teflon-glass homogenizer) in 0.25 M sucrose buffer containing 0.05 M Tris-HCl and 1 mM EDTA, pH 7.4. The homogenates were vortexed for 15 s three times, with intermittent cooling on ice, and left frozen at -70°C for 20 h in order to disrupt the membranes. They were defrosted at room temperature, vortexed 1 min and centrifuged (Eppendorf centrifuge 5417R, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) at 9000 rpm for 15 min at 4°C, and the supernatants were collected. SOD activity was measured by the method of Misra and Fridovich (11), which is based on the inhibition of auto-oxidation of adrenaline to adrenochrome by SOD contained in the examined samples. The reaction was performed in an incubation mixture containing 0.05 M Na₂CO₃, 0.1 M EDTA, pH 10.2, and monitored spectrophotometrically at 480 nm and 26°C (Cecil CE 2040 spectrophotometer, Cecil Instruments Ltd., Cambridge, UK). After assaying the total SOD activity, the samples were treated with 4 mM KCN in order to inhibit CuZnSOD (12) and subjected again to the enzyme assay as described above. The values thus obtained and the differences between the two measurements were considered as mitochondrial SOD and CuZnSOD activities, respectively. The results were expressed as specific activity of the enzyme in units per mg protein (U/mg protein). One unit of SOD was defined as the amount of protein which causes 50% inhibition of the conversion rate of adrenaline to adrenochrome between the 3rd and 4th minute under specified conditions. Total protein concentration (U/mg) was measured according to the method of Lowry et al (13).

Data analysis: Results are reported as mean±SEM. Departures from normal distribution were determined by Shapiro-Wilks test. Since the observed variables did not show significant departures from normal distribution, no data transformation was employed.

Part I. Differences of CuZnSOD activity were analyzed by a One-way ANOVA. To
Fig. 1. CuZnSOD activity in hippocampus of rats exposed to acute stress: Immobilization (Immob) or Cold (Cold); chronic stress: Isolation (Isol) or Crowding (Crowd), or combined stress. The values are means±SEM of 6 animals. Symbols: *p<0.05, **p<0.01, when compared to Control (t-test); ## p<0.01, when compared to Crowding (t-test).

analyze the effects of acute stress (Immob, Cold) and chronic stress (Isol, Crowd) in comparison to untreated controls (C), as well as the effects of Immob and Cold in comparison to Isol and Crowd pre-treated animals, t-test was used.

Part II. The effects of combined stress treatment were analyzed by a Two-way ANOVA to test for the two main effects (chronic and acute stress) and for the interaction between them. When a significant p-value was obtained, Tukey HSD test was employed to determine the differences between groups. Correlation between CuZnSOD activity between brain regions was estimated by the Pearson's correlation coefficient. The level of statistical significance was set to 5%.

Results and Discussion
CuZnSOD in Hippocampus of Animals Subjected to Acute, Chronic and Combined Stress
The activities of CuZnSOD in hippocampus are shown in the Fig. 1. The One way ANOVA analysis revealed significant variations of CuZnSOD activity in hippocampus (F_{8,45}=11.79, p<0.001) under the examined stress conditions. For example, CuZnSOD activity was decreased after acute cold exposure (p<0.05, t-test) (Fig. 1, left intersection), while its profound increase (p<0.01, t-test) was recorded after chronic isolation (Fig. 1, middle intersection). Additional acute stress of either immobilization or cold had no significant additional effect (p>0.05, t-test) on already increased enzyme activity after chronic isolation. In the group chronically exposed to crowding, only additional immobilization led to a significant increase (p<0.01, t-test) in CuZnSOD activity (Fig. 1, right intersection). The Two-way ANOVA analysis of combined stress treatment (Table) showed a significant main effect of chronic stress (F_{1,20}=41.78, p<0.001) on CuZnSOD activity. Post-hoc comparison revealed significant differences in stress induced CuZnSOD activity (p<0.001, p<0.01, Tukey test) when comparing chronically
Fig. 2. CuZnSOD activity in brain cortex of rats exposed to acute stress: Immobilization (Immob) or Cold (Cold); chronic stress: Isolation (Isol) or Crowding (Crowd), or combined stress. The values are means±SEM of 6 animals. Symbols: *p<0.05, **p<0.01, ***p<0.001, when compared to Control (t-test); +++p<0.001, when compared to Isolation, # p<0.05, when compared to Crowding (t-test).

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<th>Hippocampus</th>
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<td>Acute stress</td>
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<td>Immobilization</td>
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<tr>
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<td>13.85±0.43***</td>
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<td>32.93±6.55</td>
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<tr>
<td>Crowd</td>
<td>11.51±1.07**</td>
<td>9.64±0.47***</td>
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Values are means ± SEM, (n=6); Two-way ANOVA: a significant (p<0.001) main effect of chronic stress was observed in both hippocampus and cortex and a significant (p<0.001) main effect of acute stress was observed in cortex only (p<0.001); a significant (p=0.001) interaction effect was observed in cortex; Symbols: *p<0.05, **p<0.01, *** p<0.001, significantly different from the isolation group, ****p<0.001 significantly different from the immobilization group.

isolated animals with animals exposed to chronic crowding.
cant interaction effect ($F_{1,20}=13.66$, $p=0.001$) of both kind of stresses, as well as significant effect of both chronic ($F_{1,20}=74.54$, $p<0.001$) and acute stress ($F_{1,20}=24.71$, $p<0.001$) exposures. Similarly to hippocampus, significant differences ($p<0.001$, $p<0.05$, Tukey test) of CuZnSOD activity were recorded when chronic isolation and chronic crowding were compared. Also, a significant difference ($p<0.001$, Tukey test) was found in the Isolation + Cold vs. Isolation + Immobilization comparison.

Also, no significant correlation ($p>0.05$) was found between CuZnSOD activity in hippocampus and enzyme activity in brain cortex in all examined stress groups.

Cerebral formation of ROS is a physiological process originating from antioxidant enzymes catalyzed redox reactions triggered by the turnover of endogenous and exogenous substrates. Brain generally exhibits high rate of oxygen consumption, high content of polyunsaturated fatty acids which are primer targets for free radical attack, and low levels of natural antioxidants (14). Thus, stress conditions with high production of superoxide make brain particularly vulnerable to oxidative damage.

Within the last decade ROS were shown to play an important role in signal transduction by modifying activity of transcription factors and altering expression of numerous genes including antioxidant enzymes (AOE) such as CuZnSOD. Comparative studies of the level of CuZnSOD and specific activity of this enzyme showed that complex interactions of various sites of signal transduction with ROS via modifications of transcription factors may lead to alterations in signal transduction to the nucleus and consequently in altered gene expression. On the other side, different effects of the same stress conditions on the level and activity of CuZnSOD pointed out to alterations of structural and functional characteristics of enzyme molecules (15,16).

In addition to other factors, neuroendocrine stress was also shown to increase the basal level of ROS production in cell. Such conditions are shown to increase vulnerability of brain neurons due to altered neuronal AOE defense capacity against oxidative damage (17). To evaluate effects of acute, chronic, and combined neuroendocrine stress conditions, such as those described in this study, changes of SOD activity should be considered. The results of this study indicated that elevation of CuZnSOD activity could be observed after acute immobilization in brain cortex only, and also after chronic stress by isolation in both brain regions. The elevated CuZnSOD activity could indicate that those stress conditions, either directly or indirectly, acted on the brain cell-redox equilibrium by shifting it toward prooxidant state. Namely, it was shown that immobilization activated sympathoneural and adrenomedular system as evidenced by increased plasma catecholamines noradrenaline (NOR) and adrenaline (A) (18,19). Catecholamines, containing two free hydroxyl residues, could exert neurotoxic effect due to highly reactive quinones and superoxide radicals (20), thus elevation of CuZnSOD after the applied stressors might reflect the preventive action against stress neurotoxicity.

We also observed the suppression of CuZnSOD activity after acute stress by cold in hippocampus and also in the brain cortex after chronic crowding. Cold stress is known to alter the AOE activity, which is explained by a mechanism resisting the negative effects of ROS (21) and it is also known as the activator of sympathoneural system (22). Among the changes that occur as a result of acute stress are decrease in brain NOR content (23) and the increase in NOR turnover (24). Since hippocampus possesses the highest density of adrenergic neurons (25), and consequently the highest activity of NOR and its byproduct $H_2O_2$,
we might expect that observed decrease of CuZnSOD activity could be partially the consequence of its inactivation by H$_2$O$_2$ (26) generated in that brain region. Also, the chronic crowding as a psychosocial stress could lead to the impairment of CuZnSOD activity since it affects emotionality and various neuroendocrine and other brain functions in rats (27-29). Interestingly, it was also recorded that crowding stresses male rats but not the females (30). However, judging by the effect that psychosocial stressors exert on CuZnSOD activity, isolation seems to be a stress of higher intensity than crowding.

In hippocampus of rats chronically pre-treated with isolation, although no significant changes were observed after additional immobilization or cold, activity of CuZnSOD remained elevated indicating a sort of adaptive response to the altered ROS production triggered by neuroendocrine stress. Meanwhile, this combined stress suppressed the activity of CuZnSOD in cortex. Chronic crowding followed by immobilization resulted in slight, but significant elevation of CuZnSOD activity in hippocampus, while the slight suppression of activity was recorded in cortex after additional cold stress. The available literature shows that chronic exposure to several kinds of stressors results in a potentiation of the adrenocorticotropic (ACTH) and/or corticosterone (CORT) response to novel acute stressful stimuli (31-33). Moreover, a crowding condition produces the amplification of the adrenocortical response to various acute stressors (27,34,35). Also, following chronic exposure to stress, the reduction in brain NOR levels that occur after acute stress (23) no longer are observed (36,37) but may actually be increased in response to chronic stress exposure (24,38-41). Based on such results, it has been hypothesized that chronic stress leads to a compensatory increase in the biosynthesis of NOR which then permits a sustained increase in NOR release such that NOR content does not decline and may even increase, thus contributing to the changes of brain cell-redox equilibrium.

The observed region specific alterations of SOD activity indicate that both acute and chronic neuroendocrine stress most probably generate cellular imbalance between production and elimination of ROS. Further examination of such prooxidant cellular conditions and their functional implications may provide some insights into a variety of neuropsychological processes as well as their treatment.

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REFERENCES