formed by the decomposition of ONOOH that is responsible for the deleterious e¡ects of peroxynitrite is still questionable.

Consistent with the notion of peroxynitrite-induced oxidative stress, previous studies showed that peroxynitrite caused conformational and structural changes in brain cortical synaptosomal membrane proteins and led to increased protein carbonyl levels [3]. Peroxynitrite also led to inactivation of the highly oxidation-sensitive enzyme glutamine synthetase (GS), and caused cell death in hippocampal neuronal cultures [3]. In all these in vitro experiments when the cellular antioxidant glutathione (GSH) was added to the system prior to the addition of peroxynitrite, signi¢cant protection was observed against the peroxynitrite-induced damage [3]. Glutathione is a tripeptide (Glu-Cys-Gly) and a known scavenger of peroxynitrite [2]. Peroxynitrite was shown to nitrate glutathione and such S-nitrosothiol species have been found in ALS patients [4] and in rat cerebellar extracts [5].

The goal of the current study was to assess the susceptibility of synaptosomal membrane proteins to peroxynitrite-mediated oxidative stress by modulating the endogenous levels of glutathione in vivo and studying the protein damage to subsequently isolated cortical synaptosomes. Intracellular levels of glutathione were reduced employing i.p. injections of 2-cyclohexen-1-one (CHX), while the levels of intracellular glutathione were increased using N-acetylcysteine (NAC).

Peroxynitrite was synthesized using sodium azide and ozone as described earlier [6]. Before each experiment the solution containing peroxynitrite was thawed on ice and the absorbance measured at 302 nm to determine the concentration of peroxynitrite present. Male Mongolian gerbils, 3^5 months of age, were used. The animals were injected i.p. with 100 mg/kg body weight CHX for the glutathione depletion studies, and 200 mg/kg body weight NAC for glutathione enhancement studies. These doses were based on previous studies [7^9]. The animals given CHX were killed 1 h later, and those given NAC were killed 3 h after injection, by decapitation, and the brains quickly dissected on ice. The times chosen for the study were based on time response studies conducted (data not shown),

The isolation of synaptosomes by ultracentrifugation of the homogenized cortices was performed as described earlier [10]. Puri¢ed synaptosomes obtained at the 1.18 M/1.10 M sucrose gradient interface were washed three times with V 30 ml lysing bu¡er (10 mM HEPES, 2 mM EDTA and 2 mM EGTA in deionized water, pH 7.4) containing 100 WM diethylenenetriaminepentaacetic acid (DTPA), a chelator for iron. After the three washes the synaptosome membranes were resuspended in V 1 ml lysing bu¡er and the protein concentration of each homogenate was adjusted to 4 mg/ml. The protein aliquots were centrifuged at 14 000 rpm for 4 min. The protein pellets obtained were then treated with 250 WM peroxynitrite, in lysing bu¡er, for 10 min and the washed homogenates were spin-labeled with the protein-speci¢c spin label 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) and the electron paramagnetic resonance (EPR) spectra obtained by methods described previously [3,10]. The data were analyzed for statistical signi¢cance using Dunnett's test for one-way ANOVA followed by Student's t-test. A value of $P < 0.05$ was considered to be statistically signi¢cant for comparison between data sets.

Proteins are generally non-paramagnetic species and hence the thiol-speci¢c spin label MAL-6 is

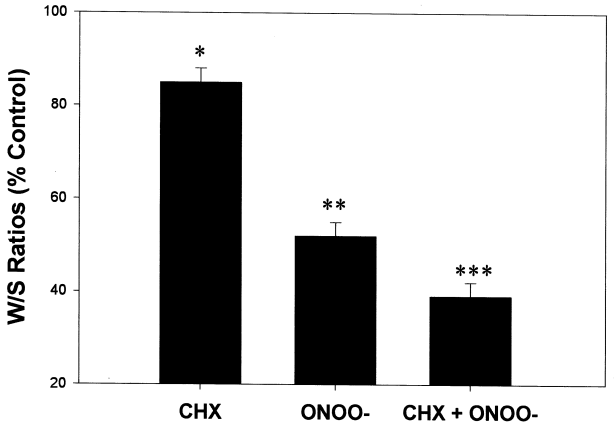

Fig. 2. Synaptosomal membranes were isolated from control animals and animals injected i.p. with CHX (100 mg/kg body weight) animals. The CHX-treated animals were decapitated 1 h after injection and synaptosomal membranes were isolated from brain cortices. W/S ratios of membrane proteins isolated from CHX-injected animals were statistically di¡erent from the W/S ratios of control animals (*P 6 0.01). 250 WM peroxynitrite added in vitro to membranes isolated from control animals showed lower W/S ratios, compared to the untreated membranes (**P 6 0.001). Membranes isolated from CHX-treated animals and treated with peroxynitrite for 10 min showed an even greater decrease in W/S ratios compared to both control untreated membrane proteins (***P 6 0.001) and control proteins treated with peroxynitrite (P 6 0.02). N = 6 was used for each group under study.

used to study the protein micro-environment by EPR. MAL-6 covalently labels sulfhydryl groups on proteins located on the protein surface or in deep pockets. The former group of -SH sites leads to spin label motion that is weakly immobilized, while reaction of MAL-6 with the latter group of -SH sites leads to spin label motion that is strongly hindered [11]. Accordingly, the EPR spectrum of MAL-6-labeled proteins in synaptosomal membranes has a weakly (W) and strongly (S) immobilized component (Fig. 1). The ratio of the signal amplitudes of these two components of the $M_I = +1$ low ¢eld region of the EPR spectrum, the W/S ratio, is extremely sensitive to alterations in the protein environment [11]. A decrease in the W/S ration results from increased protein-protein interactions, increased protein cross-linking, and changes in protein conformation [11]. Previous models of oxidative stress studied in our laboratory, such as hydroxyl radical generation [10], hyperoxia [12], ischemia/reperfusion [7], accelerated aging [13], and amyloid L–induced damage [14,15], have all shown decreased W/S ratios of spin-labeled synaptosomes with increasing oxidative stress.

Con¢rming the results of our previous study [3] and consistent with oxidative stress, synaptosomal membranes isolated from control animals and treated with 250 WM peroxynitrite for 10 min showed V 50% decrease in W/S ratios compared to the untreated control membranes (P 6 0.001, Fig. 2). The membranes isolated from the CHX-injected animals showed W/S ratios lowered to about 85% compared to those isolated from control animals (P 6 0.01, Fig. 2), con¢rming data previously obtained [7]. In contrast, there was essentially no di¡erence between the W/S ratios of the NAC-injected animals (102%) and control animals, P 6 0.2 (Fig. 3).

Synaptosomal membranes isolated from the CHX-injected animals, when treated with 250 WM peroxynitrite for 10 min, showed W/S ratios lowered to 39% compared to the untreated control samples (P 6 0.001, Fig. 2). This mean decrease in W/S ratio was statistically signi¢cant and V 11% lower than the mean decrease observed when the control samples were treated with peroxynitrite alone (P 6 0.02), suggesting that the membrane proteins isolated from

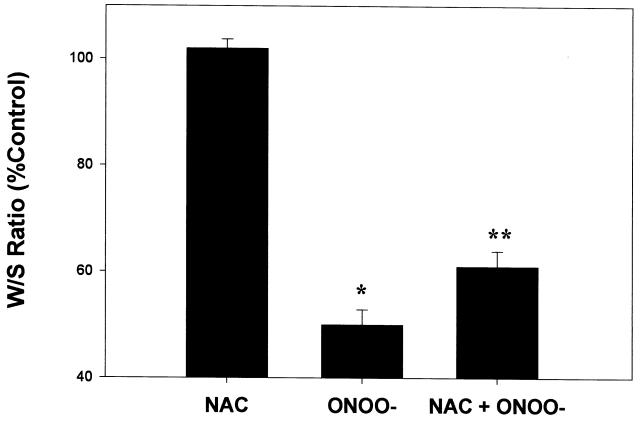

Fig. 3. Synaptosomal membranes were isolated from control animals and NAC-treated (200 mg/kg body weight) animals. Synaptosomal membranes were isolated from the animals 3 h after injection. W/S ratios of membrane proteins isolated from controls and NAC-injected animals were not statistically di¡erent (P s 0.2). 250 WM peroxynitrite added in vitro to membranes isolated from NAC-treated animals for 10 min showed a signi¢cant decrease in W/S ratios from untreated control values (*P 6 0.001) but was signi¢cantly higher than the values obtained with peroxynitrite-treated control membranes (P 6 0.01). N = 7 was used for each group in the study.

CHX-injected animals were more vulnerable to damage mediated by peroxynitrite. In contrast, the decrease observed in W/S ratios of membrane proteins isolated from NAC-injected animals and treated with peroxynitrite was about 61% (P 6 0.001), which is V 11% higher than the W/S ratios of membranes from control animals treated with peroxynitrite (P 6 0.01, Fig. 3), implying a lesser extent of protein damage. Thus, increased glutathione levels from NAC injections o¡ered partial protection against peroxynitrite-induced oxidative stress.

This study showed that injecting the animals with CHX caused a decrease in W/S ratios of MAL-6 covalently bound to cortical synaptosomal membrane proteins isolated from such animals. A previous study also showed signi¢cant changes in the physical state of membrane proteins after administration of CHX alone and CHX followed by ischemia/

ceivably could become a promising therapeutic strategy.