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Pharmacology, Biochemistry and Behavior 74 (2003) 891–900
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Effect of acute ozone exposure on locomotor behavior and striatal function

Selva Rivas-Arancibia[a,*], Claudia Dorado-Martínez[a], Laura Colin-Barenque[a], Keith M. Kendrick[b], Carlos de la Riva[b], Rosalinda Guevara-Guzmán[a]

[a]*Departamento de Fisiología, Facultad de Medicina, UNAM, AP 70-250, Mexico, DF 04510, Mexico*
[b]*Department of Neurobiology, The Babraham Institute, Cambridge, England CB24AT, UK*

Received 2 July 2002; received in revised form 19 October 2002; accepted 30 December 2002

Abstract

Ozone exposure, depending on the dose, is a noninvasive model of oxidative stress. The purpose of this work was to study striatal damage and cell death induced by oxidative stress. Sixty-three male Wistar rats were divided into two groups—Group 1: animals were exposed to an air stream free of ozone for 4 h; and Group 2: animals were exposed to 1 ppm of ozone for 4 h. Four subgroups in each treatment group were then tested 3 h after control or ozone exposure for: (1) exploratory and freezing behavior; (2) lipid peroxidation levels; (3) in vivo release of amino acid and monoamine transmitters, and metabolites and nitric oxide; and (4) striatal ultrastructural changes. Results showed that the ozone decreased exploratory and increased freezing behaviors. It also increased striatal lipoperoxidation levels and basal dopamine, glutamate, and nitric oxide (arginine, citrulline, and nitrate used as indices) concentrations and decreased those of 5-HT. Concentrations of GABA were initially decreased 3 h after ozone but then were increased 3 and 5 days afterwards. Increased lipofucsine, neuronal cytoplasm and dendrite vacuolation, and dilation of rough endoplasmic reticulum cisterns and dark cells were observed in striatal medium spiny neurons in ozone-exposed rats. These alterations suggest a neurodegenerative process caused by oxidative stress after acute ozone exposure.

Keywords: Striatum; Oxidative stress; Dopamine; Behavior; Ultrastructure; Microdialysis

1. Introduction

Ozone exposure, depending on the dose, causes an increase in free radicals (Dorado-Martínez et al., 2001; Pryor and Church, 1991), which leads to the formation of reactive oxygen species (ROS) in the organism. Whenever the ROS increase is not counterbalanced by the antioxidant systems, oxidative stress is produced (Sies, 1991). Oxidative stress plays a major role in aging (Barja and Herrero, 2000; Carney et al., 1995; Fukagawa et al., 2000; LaVoie and Hastings, 1999a,b) and age-related neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and Huntington's disease (Beckman and Ames, 1998; Luo and Roth, 2000; Olanow, 1993; Simonian and Coyle, 1996; Smith et al., 2000). Other pieces of evidence have shown that oxidative damage and free radical formation associated with the normal aging process produce altered hippocampal function and cognitive deficits (Burke and Taylor, 1992). Similarly, oxidative damage to the basal ganglia also produces deficits in locomotor function (Avila-Costa et al., 2001; Dorado-Martínez et al., 2001).

Ozone exposure provides a good noninvasive model for studying the participation of oxidative stress in the neurodegenerative process. Ozone increases ROS production in the lung. When the lung antioxidant defenses cannot counteract ROS, they reach the bloodstream and diffuse throughout the organism (including brain), producing a generalized oxidative stress state.

Free radicals increase lipoperoxidation and protein oxidation of cell membranes, alter enzymatic activity, and cause DNA destruction, which lead to cell apoptosis or necrosis (Floyd and Carney, 1992; Halliwell and Gutteridge, 1984). Additionally, free radicals react with fatty acids, producing epoxides and dialdehydes such as malondialdehyde, which extend cellular damage (Cross et al., 1992; Halliwell and Cross, 1994; Halliwell and Gutteridge, 1984; Van der Vliet et al., 1995; Wolff et al., 1986). The enzymatic antioxidant response (superoxide dismutase, catalase, and glutathione peroxidase) attempts to balance the production of free radicals.

* Corresponding author. Fax: +52-5-623-2241.
E-mail address: srivas@servidor.unam.mx (S. Rivas-Arancibia).

0091-3057/03/$ – see front matter
doi:10.1016/S0091-3057(03)00011-X

The nervous system is more vulnerable to the deteriorating effects of free radicals than other tissues (Colton and Gilbert, 1999; Olanow, 1993). This vulnerability is due to its high lipid content and oxygen consumption, low catalase and superoxide dismutase activity, and also to the moderate activity of glutathione peroxidase. Oxidative stress affects the entire brain; however, some areas are more vulnerable to free radical damage than others. In particular, oxidative stress produces an increase in the release and in the oxidation of dopamine. Reactive dopamine metabolites, such as dopamine quinones and ROS, directly alter protein functioning by oxidative modifications that induce a mitochondrial permeability transition pore (Berman and Hastings, 1999) and ATP depletion. Nitric oxide metabolites formed in the presence of oxidative stress contribute to the selective vulnerability of dopaminergic neurons (LaVoie and Hastings, 1999a,b), due to dopamine oxidation induced by peroxynitrite and nitrite.

Our previous experiments have shown that the brain regions associated with both cognitive and motor function are extremely vulnerable to ROS, with the striatum and hippocampus showing higher lipid peroxidation levels after ozone exposure than the frontal cortex (Dorado-Martínez et al., 2001; Rivas-Arancibia et al., 2000, 2001). Ozone exposure at low doses (0.7 ppm) causes memory alterations, and at higher doses (1.0 ppm), it produces motor activity alterations (Rivas-Arancibia et al., 1998, 2000). The aim of the present study was to elucidate the nature of the effects of oxidative stress caused by ozone exposure on locomotor function and on changes in the morphology and neurochemistry of the striatum.

2. Materials and methods

Sixty-three adult male Wistar rats (250–300 g) were individually housed in acrylic boxes with free access to food and water and randomly divided into two experimental groups—Group 1: control animals that were exposed to air stream free of ozone for 4 h; and Group 2: experimental animals that were exposed to 1 ppm of ozone for 4 h. Both groups were exposed on only a single occasion and then 3 h, 3 days, and 5 days afterwards, subgroups from each condition were given one of four different assessment procedures: (1) exploratory and freezing behavior ($n=20$); (2) lipid peroxidation levels ($n=24$); (3) in vivo neurotransmitter release, using microdialysis ($n=11$); and (4) ultrastructural changes in the striatum ($n=8$), which was only studied 3 h after ozone exposure. Animal care and handling were conducted in accordance with the National Institute of Health Guidelines for Animal Treatment.

2.1. Ozone exposure

Animals were exposed, as mentioned above, in a closed chamber with a diffuser connected to a variable-flux ozone generator (5 l/s). Ozone was generated from a tube through which a high-voltage current circulated. The tube contained aluminum chips, with two electrodes inside that allowed the conversion of oxygen that circulated around the tube into ozone. Ozone production levels were proportional to the current intensity and to the air flow. A PCI Ozone and Control System Monitor approved by the Environmental Protection Agency (EPA) was used to measure the ozone concentration inside the chamber throughout the experiment. The same chamber was used when treating the control group where flow of ozone-free air was used.

2.2. Exploratory and freezing behavior

Twenty rats were randomly assigned to two groups ($n=10$ for each treatment group). Both groups were measured at exactly the same conditions, and each animal was tested using its own housing box. The number and the duration of exploratory and freezing behaviors displayed by each animal were recorded during a 5-min test with measures being taken at 1-s intervals.

2.3. Lipid peroxidation

Twenty-four rats were randomly assigned to two groups ($n=6$ for each treatment group). The animals were sacrificed by decapitation 3 h, 3 days, and 5 days after ozone exposure concluded. Their brains were removed and the dorsal striatum of both hemispheres dissected out on an ice-cold plate and tissues weighed immediately. Each tissue sample was homogenated in PBS 1:20 and stored at -70 °C until the day of the assay in order to carry out lipid peroxidation measurements. Lipid peroxidation was measured using the K-assay test (Kamiya Biomedical), which uses ascorbic oxidase and lipoprotein lipase, chromogen reagent, buffer, and hemoglobin. Homogenates of each sample were centrifuged for 5 min, at 8 °C and 3000 rpm. The supernatant was separated and combined with the enzyme reagent (ascorbic oxidase and lipoprotein lipase). This mixture was incubated for 10 min at a temperature of 30 °C and the chromogen reagent [10-*N*-methylcarbamoyl-3,7-dimethylamino-10*H*-phenothiazine (MCDP)] was added. The solution obtained was incubated for 15 min at a temperature of 30 °C. Finally, absorbance was read (675 nm) in a spectrophotometer (Jenway 6405 UV/Vis Spectrophotometer). A curve with two calibration points was made using a saline blank (0 nmol/ml) and a 50-nmol/ml cumene hydroperoxide standard. Results were calculated using the following equation:

$$\text{LPO value [nmol/ml]} = (E_s - E_b) \times 50.0/(E_{std} - E_b).$$

where E_s = sample absorbance, E_{std} = absorbance of 50 nmol/ml standard, and E_b = blank absorbance.

2.4. Microdialysis and high-performance liquid chromatography (HPLC) analysis

Adult freely moving male Wistar rats (250–300 g) (control $n=5$, ozone $n=6$) were used. Animals were anesthesized with choral hydrate (400 mg/kg), and then placed in a stereotaxic frame with the incisor bar set at 5 mm above the interaural line. A guide tube (CMA) was implanted into the right striatum using the following coordinates: 1.0 mm rostral to bregma, 3 mm lateral to the midline, and 2 mm down from the surface of the brain (Paxinos and Watson, 1986). It was fixed with dental acrylic. Microdialysis sampling was then carried out starting 3 days after the animals had been allowed to recover.

For sampling, a microdialysis probe (CMA 12, 0.5 mm, OD 3 mm length) was introduced into the guide tube and a liquid swivel system was used to allow the animals to move about freely in the acrylic test box. The animals were sampled on four occasions for 3 h, once 24 h prior to being exposed to the air control or ozone exposures and again 3 h, 3 days, and 5 days after they had finished. On each occasion, the probe was placed in the striatum and sampling started 3 h later. A solution of Krebs ringer (in mM: NaCl 138, KCl 11, $CaCl_2$ 1.5, $MgCl_2$ 1, $NaHCO_3$ 11, NaH_2PO_4 1; pH = 7.4) with 10^{-5} M neostigmine methyl sulphate was passed through the probe at 1.5 μl/min using a syringe pump (CMA 100) and samples were collected from the outflow tube of the probe at 15-min intervals for 3 h.

Concentrations of glutamate, γ-aminobutyric acid (GABA), arginine, and citrulline were measured using an HPLC system with fluorescence detection following precolumn derivatisation with *o*-phthaldialdehyde (OPA) as described in a previous paper (Guevara-Guzmán et al., 2000). The method used reliably separates all classical

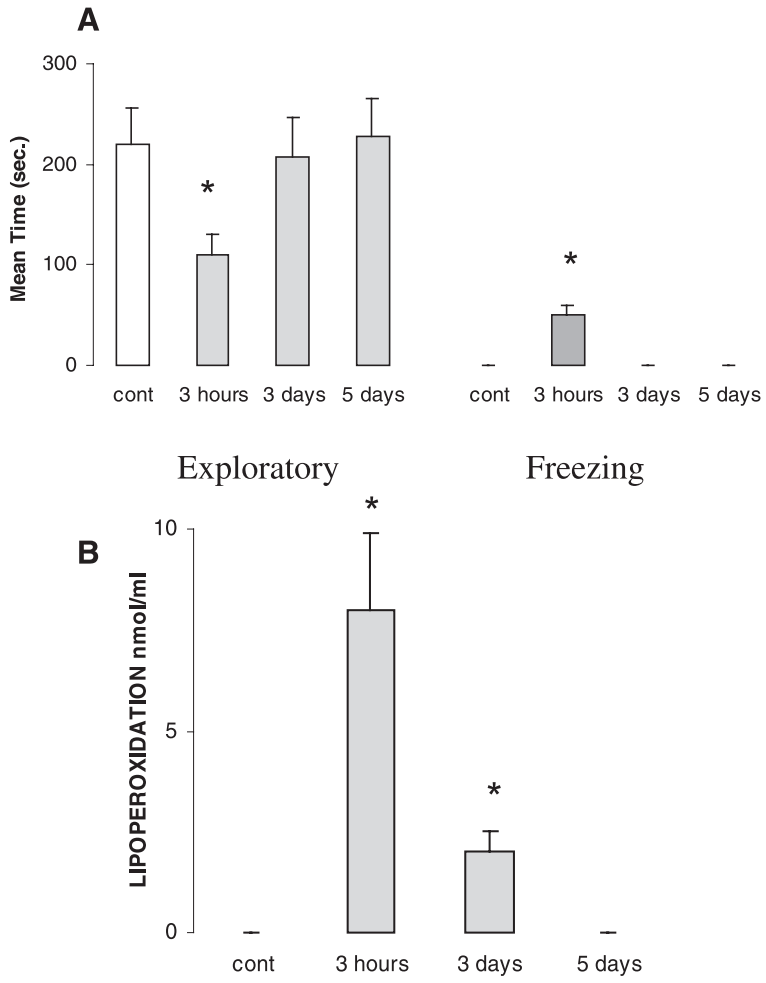

Fig. 1. (A) Effects of ozone on freezing and exploratory behavior. Freezing and exploratory behaviors in mean (n) ± S.E.M. (s) are depicted on the ordinate. The treatments, control, and 3 h, 3 days, and 5 days after ozone exposure had finished are depicted on the abscissa ($n=10$ for each group). Measures of the control (light bars) and the ozone-treated groups (dark bars) taken 3 h, 3 days, and 5 days after ozone exposure, mean ± S.E.M., respectively, were: exploratory: 220 ± 36, 110 ± 20, 207 ± 40, and 228 ± 38; and freezing: 0, 50 ± 9, 0, and 0. (B) * $P<.05$. Effects of ozone on lipid peroxidation levels in striatum. The lipid peroxidation levels (LPLs) in nanomoles per milliliter of homogenated striatal tissue are depicted on the ordinate. The treatments, control, and 3 h, 3 days, and 5 days after ozone exposure are depicted on the abscissa ($n=6$ for each group). Mean ± S.E.M. lipid peroxidation levels in the control and ozone-treated groups 3 h, 3 days, and 5 days were, respectively, 0, 8 ± 1.8, 2 ± 0.5, and 0 nmol/ml. * $P<.05$, two-tailed.

transmitter amino acids as well as citrulline and arginine with detection sensitivities of 1–5 nM. Previous studies have shown that citrulline is coproduced in stoichiometric amounts with nitric oxide and correlates well with altered concentrations of the nitric oxide metabolites nitrate and nitrite (Kendrick et al., 1996). Arginine was measured since it is the main nitric oxide precursor. Nitrate was measured by HPLC with UV detection using an anion exchange column as previously described (Kendrick et al., 1996) with a detection limit of 100 nM. Dopamine, dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hyroxyindoleacetic acid (5-HIAA) were measured using HPLC with electrochemical detection, as previously described (Guevara-Guzmán et al., 2000; Kendrick et al., 1996), with a detection sensitivity of 20–50 fg for a 10-μl injection. At the end of the final session, animals were given a lethal dose of anaesthetic (sodium phenobarbital) and the brain removed and frozen prior to being sectioned on a cryostat to determine probe localization.

2.5. Electron microscopy

The rats were sacrificed 3 h after air or ozone exposure concluded (control $n=3$, ozone-exposed $n=5$). Animals were given a lethal dose of sodium phenobarbital and perfused via the aorta with a saline solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (PB). The striatum was postfixed in 1% osmium tetroxide, buffered to pH 7.4 with PB, dehydrated in graded alcohols, and embedded in Araldite. Sections were obtained with a Reichert–Jung microtome, placed on a grid contrasted with uranyl acetate and lead citrate, and analyzed with a Zeiss EM 10 Electron Microscope. Ultrastructural analysis consisted of damage determination in 100 striatal medium spiny neurons from each rat. Evidence for neuronal damage was considered to be the presence of vacuoles, lipofucsine granules, and dark cells. Mean numbers of neurons with cytoplasm and dendrite vacuolation, lipofucsine granules, and necrosis were compared in ozone- and air-exposed groups.

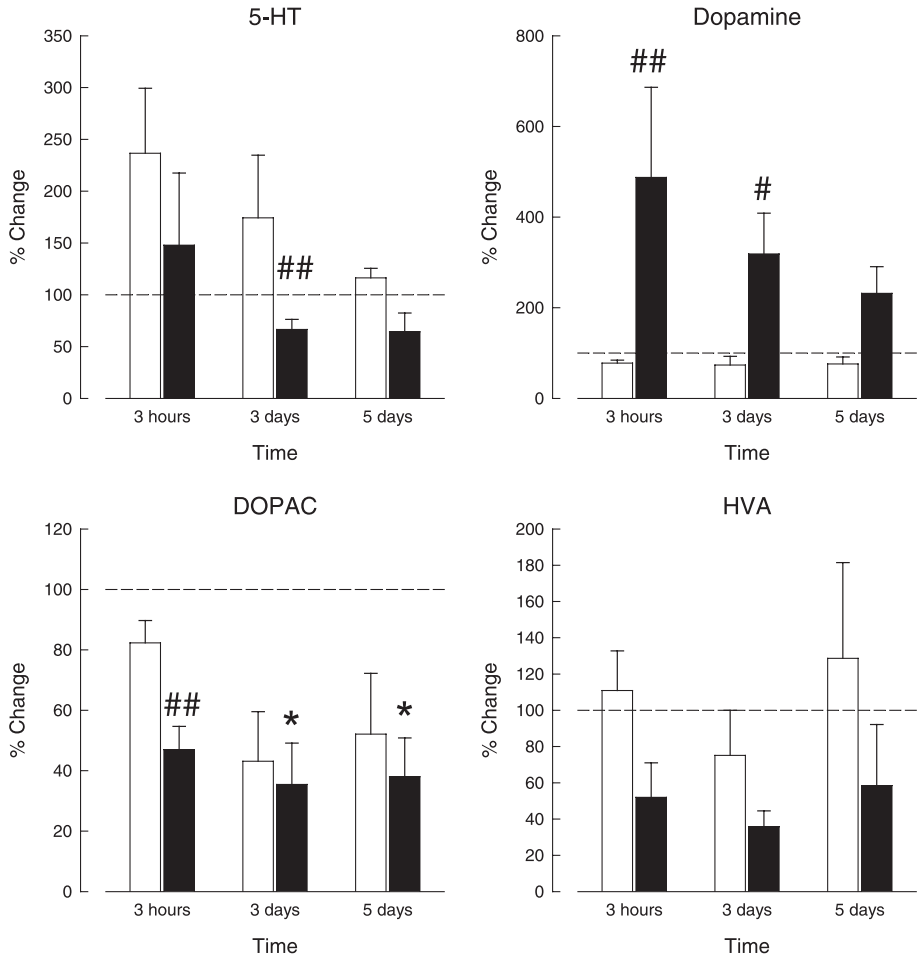

Fig. 2. Effects of ozone treatment on in vivo striatal monoamine concentrations. Histograms show mean (n) ± S.E.M. (%) concentration changes in 5-HT, DA, DOPAC, and HVA 3 h, 3 days, and 5 days after a 3-h ozone or control air exposure relative to basal levels measured 24 h before (expressed as 100%). Open bars represent controls ($n=5$) and black bars represent animals exposed to ozone ($n=6$). $^{*}P<.05$ (Dunnett's test) compared with concentrations 24 h prior to exposure; and $^{\#}P<.05$, $^{\#\#}P<.01$ (U test, two-tailed) compared with the same time point in the control group exposed to air. Mean ± S.E.M. basal concentrations 24 h before exposure in the control and ozone-treated groups, respectively, were: 5-HT, 29.5 ± 1.2 and 95.1 ± 24.7 pg/ml; DA, 90.4 ± 39.8 and 906 ± 473 pg/ml; DOPAC, 4.4 ± 1.6 and 19.2 ± 7.7 ng/ml; and HVA, 27.0 ± 12.3 and 14.1 ± 3.8 ng/ml.

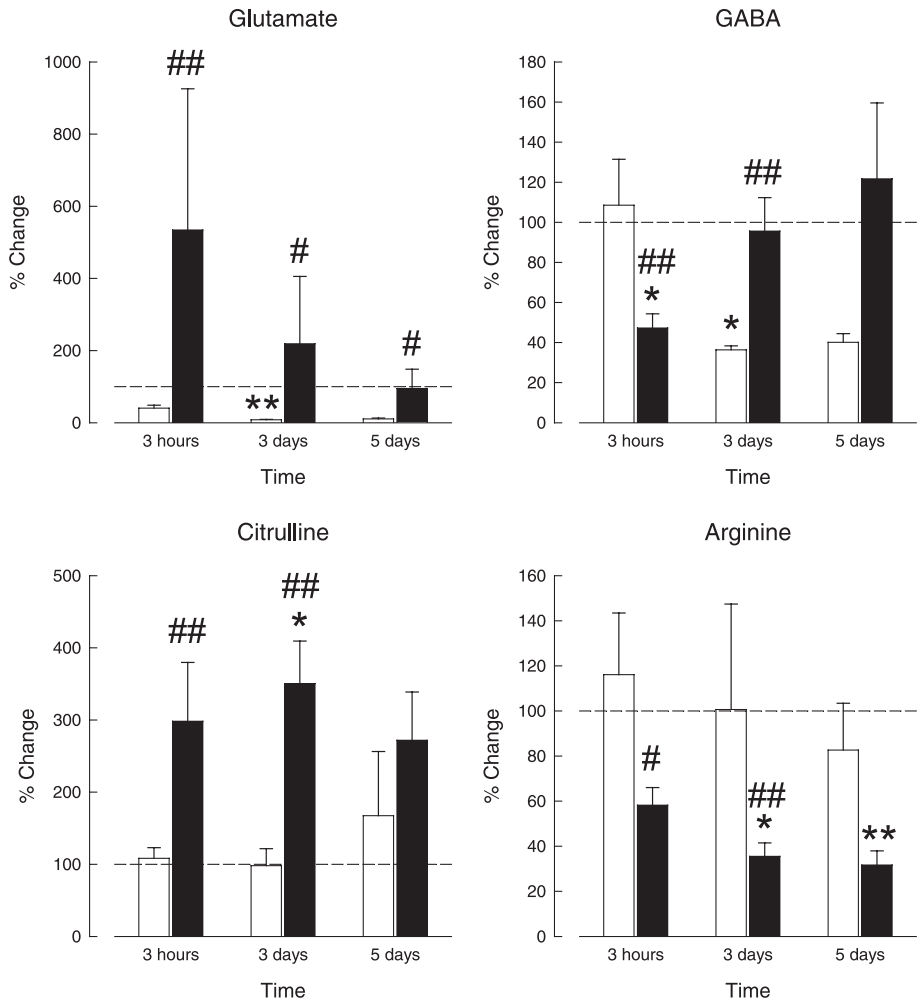

Fig. 3. Effects of ozone treatment on in vivo striatal amino acid concentrations. Histograms show mean (n) ± S.E.M. (%) concentration changes in glutamate, GABA, citrulline, and arginine 3 h, 3 days, and 5 days after a 3-h ozone or control air exposure relative to basal levels measured 24 h before (expressed as 100%). Open bars represent controls ($n = 5$) and black bars represent animals exposed to ozone ($n = 6$). $^{*}P < .05$, $^{**}P < .01$ (Dunnett's test) compared with concentrations 24 h prior to exposure; and $^{\#}P < .05$, $^{\#\#}P < .01$ (U test, two tailed) compared with the same time point in the control group exposed to air. Mean ± S.E.M. basal concentrations 24 h before exposure in the control and ozone-treated groups, respectively, were: glutamate, 11.6 ± 2.6 and 5.2 ± 1.5 μM; GABA, 97.7 ± 19.4 and 91.7 ± 19.8 nM; citrulline, 956 ± 204 and 404 ± 77 nM; and arginine, 1284 ± 665 and 769 ± 125 nM).

2.6. Statistics

Behavioral data from the two control and experimental groups were expressed as means and analyzed statistically using the nonparametric Mann–Whitney U test (Siegel, 1991). Lipid peroxidation levels were expressed in means. To compare changes in basal concentrations of neurochemical substances in the striatum within treatment groups, mean concentrations at different time points were analyzed by a nonparametric ANOVA followed by Dunnett's test (where $P < .05$). To compare differences between treatment groups, neurochemical concentrations from the microdialysis experiments were expressed as percentage changes from those measured in samples taken during the first control sampling session prior to either air (control) or ozone exposure. Differences between control and ozone groups at each time

Table 1
Striatal medium spiny neurons damage after ozone exposure

	Vacuoles	Lipofucsine	Necrosis	Proportion with damage
Air-exposed, mean ± S.E.M.	3 ± 3%	14.3 ± 3.5%	0	17.33 ± 1.5%
Ozone-exposed, mean ± S.E.M.	28.6 ± 14.5% *	24.8 ± 4.6% *	0.6 ± 0.2% *	54.0 ± 11.7% *

Mean proportions of damaged striatal medium spiny neurons in groups of air (control) and ozone-exposed rats. Data are from 100 neurons in each animal (three rats following air exposure and five rats following ozone exposure). Damage was indicated by the presence of neuronal cytoplasm vacuolation, lipofucsine granules, and necrotic cells.

* $P < .05$, two-tailed compared with control animals exposed to air.

point were then analyzed by Mann–Whitney *U* test. Changes in ultrastructural elements within the striatum following ozone exposure were analyzed by a *t* test.

3. Results

3.1. Exploratory and freezing behavior

Three hours after ozone exposure, we found that exploratory behavior had significantly decreased ($P < .05$, *U* test) and freezing behavior had increased compared with the control group. No changes were found when compared to controls 3 and 5 days later (Fig. 1A).

3.2. Lipid peroxidation

Striatal lipid peroxidation means increased significantly from undetectable (control group) to 8 nmol/ml (3 h after ozone exposure concluded), and decreased to 2 nmol/ml (3 days later) and undetectable (5 days after ozone exposure) (Fig. 1B). Striatal lipid peroxidation measures taken 3 h, 3

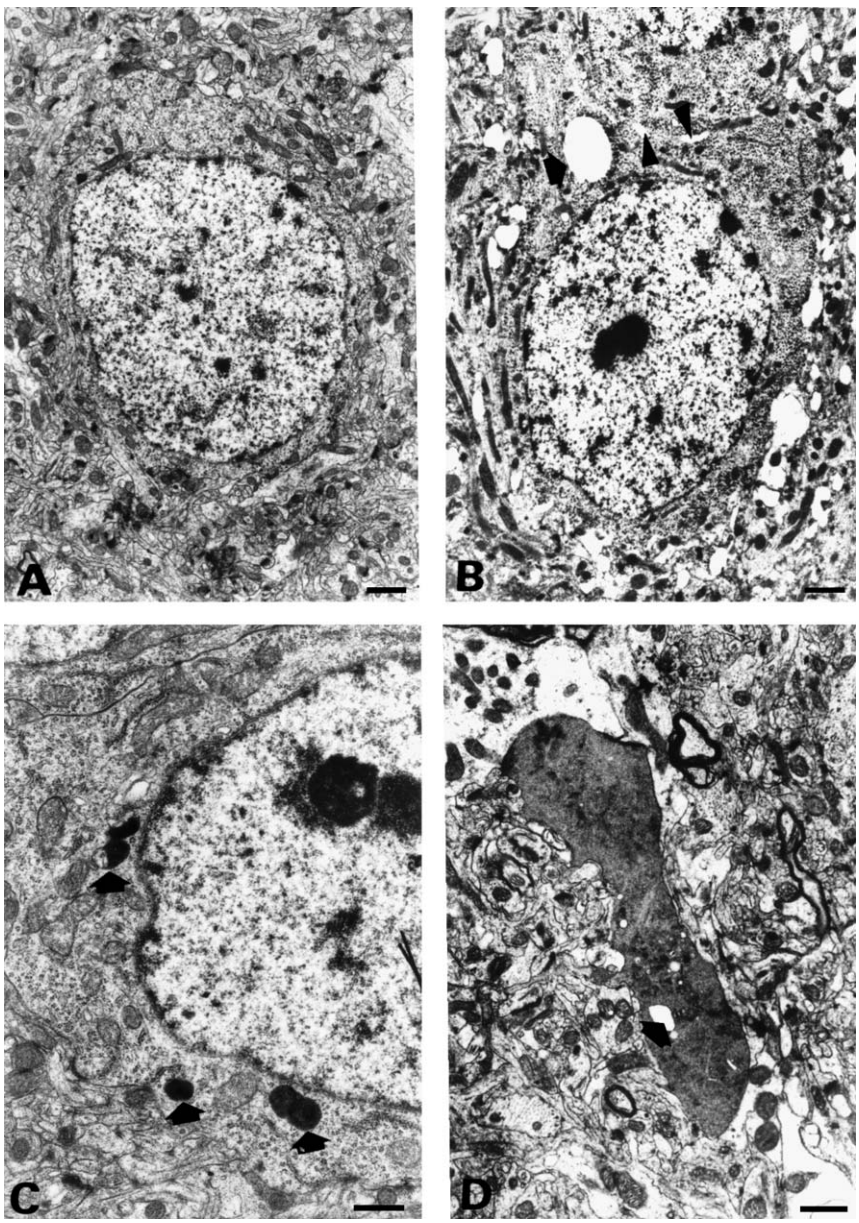

Fig. 4. Electron photomicrographs of medium spiny neurons of striatum. (A) Neurons of control rats 3 h after sham exposure (scale bar: 1 μm). (B) Neurons 3 h after ozone exposure, exhibiting a prominent vacuolation (arrow) and dilation of rough endoplasmic reticulum cisterns (arrowheads) (scale bar: 1 μm). (C) Lipofucsine granules in neurons exposed to ozone (arrows) (scale bar: 0.6 μm). (D) Medium spiny neuron necrotic body with vacuolation 3 h after ozone exposure (arrow) (scale bar: 1 μm).

days, and 5 days after control air exposure were undetectable (data not shown).

3.3. Microdialysis

Histology confirmed that in every experiment, microdialysis probe locations were within the medial portion of the striatum. Basal concentrations of substances during the first control sampling session did not differ significantly between treatment groups, although with repeated sampling in the control group, there was a significant decline in concentrations of glutamate and DOPAC but not in other substances. However, the proportionate change in basal concentrations of a number of substances was significantly different following ozone exposure compared with air-exposed controls (Figs. 2 and 3). For 5-HT, basal concentrations were significantly decreased 3 days after ozone exposure (P=.011, two-tailed) and showed a tendency to remain so at 5 days, but this did not quite achieve significance (P=.082). Dopamine levels were initially significantly elevated 3 h after ozone exposure (P=.0043) and remained so at 3 days (P=.017), while at 5 days, this just failed to achieve significance (P=.08). On the other hand, concentrations of DOPAC were significantly reduced by 3 h after ozone exposure (P=.009) but not at other time points. The pattern for HVA was similar but just failed to reach significance (P=.07) at the 3-h time point. Glutamate concentrations were significantly increased by 3 h after ozone exposure (P=.0043) and remained elevated at 3 days (P=.017) and 5 days (P=.03). By contrast, GABA levels were initially significantly decreased more 3 h after ozone exposure (P=.004) but then were elevated at the 3-day time point (P=.01) and showed a trend towards this at 5 days as well (P=.08). Concentrations of arginine were significantly decreased compared with controls at all three time points (3 h, P=.05; 3 days, P=.017; 5 days, P=.03), whereas those of citrulline were increased (3 h, P=.008; 3 days, P=.05; 5 days, P=.069). Nitrate levels showed a similar pattern as for citrulline, but insufficient samples were analyzed from the control group to allow statistical comparisons with the ozone group to be carried out. Taurine, glycine, and 5-HIAA concentrations were not differentially affected by the two treatment regimes.

3.4. Ultrastructural analysis

The group exposed to ozone showed a significant increase in the proportion of damaged medium spiny neurons increased. Neuronal cytoplasm and dendrite vacuolation increased significantly as did lipofucsine granules up and necrosis (Table 1). The total proportion of cells showing evidence for damage also increased significantly from 17.3% to 54% ($P<.05$ compared with the control group, t test). Dilation of rough endoplasmic reticulum cisterns also occurred in the ozone-exposed rats (Fig. 4).

4. Discussion

Ozone exposure at the doses used in this experiment is a good model to study both the short- and long-term effects of oxidative stress and ROS effects in brain. The loss of the oxide reduction balance leads to oxidative damage of lipids, proteins, DNA, and complex molecules. It also leads to the production of secondary reactive species, which increases redox imbalance and the expression of oxidative pathways. Neurotransmitters such as dopamine and nitric oxide play a protective role when an oxide reduction balance prevails. On the other hand, these neurotransmitters participate in metabolic changes that lead to the loss of the antioxidant efficacy, an increase in oxidative metabolites, and tissue damage, which is established when this oxide reduction balance is lost.

The increase in striatal lipid peroxidation levels found in this experiment indicates an oxidative stress state caused by ROS, secondary to ozone exposure (Fig. 1B). This oxidative stress state induces an increase in striatal dopamine release, as shown in Fig. 2. The initial high levels of dopamine are paralleled by a corresponding reduction in concentrations of the dopamine metabolites, DOPAC and HVA, suggesting altered transport or metabolism. However, elevated dopamine concentrations persisted 3 and 5 days after ozone exposure, when the metabolites had returned to normal, although by 5 days, this just failed to achieve significance. Dopamine plays a very important role in motor control and regulation. Ozone not only causes increased extracellular dopamine concentrations, but oxidative stress also promotes dysfunctional dopaminergic transmission, due to dopamine as well as dopamine receptor oxidation. This explains why, when oxide reduction balance is lost (although dopamine concentrations increase), the oxidation of this neurotransmitter and its receptor causes motor alterations that in this experiment are manifested by a decreased exploratory behavior and the elicitation of freezing (Fig. 1A).

Dopamine can oxidize to form superoxide and hydroxyl radicals in the presence of transition metals (Graham, 1978). Also, dopamine oxidation forms dopamine quinones that react with cysteine, leading to the formation of cystenyl residues that inhibit protein functions (LaVoie and Hastings, 1999). The sulfhydryl group of the cysteine reacts with glutathione and antioxidant levels consequently decrease. In various mitochondrial proteins, reactive dopamine metabolites also alter protein functions and disturb mitochondrial functions (Berman and Hastings, 1999). This leads to ATP depletion and further oxidative stress. It has been proposed that the peroxidase activity of prostaglandin H synthase is responsible for catalyzing the oxidation of dopamine to reactive quinones in the presence of arachidonic acid and hydrogen peroxide (Hastings, 1995; LaVoie and Hastings, 1999). Both metabolites increase during oxidative stress. These dopamine oxidative pathways are expressed mainly when oxide reduction balance is lost. They could also play a toxic role in striatal tissue damage by increasing neuro-

degenerative processes, contributing in this way to cell damage and death as evidenced by levels of necrosis found in the striatum after ozone exposure (Fig. 4, Table 1).

The effect of ozone in reducing basal 5-HT, but not 5-HIAA, concentrations in the striatum after 3 days suggests another mechanism whereby it promotes neurodegeneration. This amine is well established as a scavenger of ROS (Huether et al., 1997) and, therefore, one might expect that reduced basal concentrations would reflect a decreased capacity for the brain to counter the buildup of ROS.

Together with the alteration in dopamine and 5-HT release, we found an initial decrease in GABA levels 3 h after ozone exposure, although by 3 and 5 days afterwards, this had altered to become a significant increase. The cause of these biphasic GABA concentrations changes is unclear. Haughey et al. (1999) have reported that lipid peroxidation in synaptosomes resulted in the reduction of GABA release (Chan et al., 1983). However, like most neurotransmitters, GABA accumulates in the extracellular space during brain damage, such as in cerebral ischemia, and reaches normal levels within an hour (Globus et al., 1991; Phillis et al., 1994; Shuaib et al., 1994). It has also been proposed that during oxidative stress, GABA release increases whereas its uptake decreases (Rego et al., 1996; Saransaari and Oja, 1998). A large proportion of the medium spiny neurons within the striatum, which we have shown to exhibit ultrastructural changes indicative of neurodegeneration following ozone exposure, would be GABAergic. On the other hand, it has also been proposed that GABAergic neurons are more resistant to brain damage. For example, within the striatum, the enzyme glutamic acid decarboxylase (GAD) is relatively resistant to cerebral ischemia (González et al., 1992) despite the degeneration of GAD-containing neurons that project to the substantia nigra (Saji et al., 1994). Thus, altered GABA concentrations following ozone exposure may be both a reflection of neurodegenerative changes as well as altered function of undamaged cells. However, one might have expected levels of dopamine and glutamate to decrease and those of GABA to increase, but these changes may reflect compensatory changes brought on by oxidative stress that are still clearly insufficient to restore normal locomotor activity.

While our results indicated that repeated sampling within the striatum significantly depleted basal extracellular glutamate concentrations in the vicinity of the microdialysis probes, ozone exposure nonetheless stimulated a proportionate elevation in the levels of this transmitter compared with controls at all time points (Fig. 3). It has been reported that ROS formation stimulates increased glutamate release and this transmitter can cause neuronal damage either through a receptor-mediated pathway that produces excitotoxicity (Choi, 1988), or through a nonreceptor-mediated oxidative pathway (Murphy et al., 1989) that inhibits cysteine uptake. In addition, glutamate uptake inhibition occurs in astrocytes by blocking Na^+/K^+ ATPase activity during oxidative stress (Volterra et al., 1994). This leads to glutamate toxicity, depending on the amount of free radical production, contributing in this form to an increase in neuronal damage. The ultrastructural alterations found after ozone exposure support this conclusion (Fig. 4, Table 1).

Cells normally use glutathione peroxidase to remove H_2O_2. On the other hand, glutamate increase causes a decrease in glutathione levels (Murphy et al., 1989) and an increase in H_2O_2 levels before the overt manifestation of cell death. Monoamine metabolism is also involved in glutamate toxicity (Maher and Davis, 1996). Glutamate toxicity via the oxidative pathway requires monoamine metabolism as a source of free radicals and is inhibited by monoamine oxidase type A inhibitors.

Citrulline and nitric oxide are produced from arginine by nitric oxide synthase (Zhang and Snyder, 1995). Our results indicate that oxidative stress caused an alteration in nitric oxide such that the levels of its precursor, arginine, were reduced while those of nitric oxide itself (as indexed by citrulline) were increased (Fig. 3). These effects occurred immediately after ozone exposure and were maintained for the rest of the 5 days afterwards. This shows a pattern similar to that of increased glutamate concentrations, suggesting that it is glutamate that may be stimulating these nitric oxide changes through NMDA and AMPA receptors (Kendrick et al., 1996). Presumably, basal arginine levels may be depleted through its increased conversion into nitric oxide following ozone exposure. During oxidative stress, it is possible for nitric oxide to react rapidly with superoxide to form peroxynitrite, which induces dopamine oxidation and protein alteration (LaVoie and Hastings, 1999). Peroxynitrite also causes oxidative lesions to DNA (Wink et al., 1999), increasing oxidative damage. Nitric oxide reduces glutathione and transforms it into oxidized glutathione, causing cellular GSH depletion and making cells more vulnerable to the toxic effects of free radicals (Kroncke et al., 2000).

A state of oxidative stress induces metabolic changes that lead to the expression of oxidative pathways. Pro-oxidant metabolites are also produced and can interfere with enzymatic antioxidant defense systems. This increases oxide reduction imbalance and it is possible that the same messengers that carry out a specific physiological role during redox balance function differently during oxidative stress. When there is oxidative stress, cell signaling or metabolic pathways are modified, leading to damage or death of the cells. Ultrastructural alterations found in medium spiny neurons of striatum can be an example of this kind of damage (Fig. 4, Table 1) and could be associated with neuronal degeneration caused by the circulation of ROS in the blood as a secondary reaction produced by ozone exposure.

Studies carried out in our laboratory 24 h after ozone exposure showed that there is a significant reduction in the number of dendritic spines in primary and secondary dendrites in the olfactory bulb (Colín-Barenque et al.,1999). The Golgi apparatus and mitochondria in the olfactory bulb

(Colín-Barenque et al., 1999), hippocampus (Avila-Costa et al., 1999), striatum, and prefrontal cortex (Avila-Costa et al., 2001) are also swollen.

In summary, therefore, we hypothesise that the neurochemical and ultrastructural changes we have found following short-term exposure to ozone reflect a neurodegenerative process caused by oxidative stress. This model allows the study of the role of ROS in neural damage and cell death without any other complicating factors. Ozone disruption to redox homeostasis in vivo results in neural cell damage with rapid (by 3 h) concomitant alterations in dopamine, glutamate, GABA, and nitric oxide levels, which are still evident 5 days after exposure.

Acknowledgements

This work was partially supported by DGAPA IN-213501 to S.R.-A. We thank Ms. Karina Valencia-Juarez for her translation, and Mr. Gabino Borgonio-Pérez and Francisco Pasos for their technical assistance.

References

Avila-Costa MR, Colín-Barenque L, Fortoul TI, Machado-Salas JP, Espinosa Villanueva J, Rugeiro-Vargas C, et al. Memory deterioration in an oxidative stress model and its correlation with cytological changes. Neurosci Lett 1999;270:107–9.

Avila-Costa MR, Colín-Barenque L, Fortoul TI, Machado-Salas JP, Espinosa Villanueva J, Rugeiro-Vargas C, et al. Motor impairments in an oxidative stress model and its correlation with cytological changes on rat striatum and prefrontal cortex. Int J Neurocsi 2001;108: 193–200.

Barja G, Herrero A. Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. FASEB J 2000;14:312–8.

Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev 1998;78:547–81.

Berman SB, Hastings TG. Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria. J Neurochem 1999;73:1127–37.

Burke SP, Taylor CP. Glutamate, aspartate and GABA release from hippocampal CA1 slices during in vitro ischemia is calcium-independent. In: Dietrich WD, Globus MYT, editors. The role of neurotransmitters in brain injury. New York: Plenum; 1992. p. 45–52.

Carney JM, Smith CD, Carney AM, Butterfield DA. Aging and oxygen-induced modifications in brain biochemistry and behavior. In: Chiueh C, Gilbert D, Colton C, editors. The neurobiology of NO and OH. Ann NY Acad Sci., vol. 738. 1995. p. 44–53.

Chan PA, Kerlan R, Rishman RA. Reductions of γ-aminobutyric acid and glutamate uptake and (Na^+/K^+)-ATPase activity in brain slices in synaptosomes by arachidonic acid. J Neurochem 1983;40:309–16.

Choi DW. Glutamate neurotoxicity and diseases of the nervous system. Neuron 1988;1:623–34.

Colín-Barenque L, Avila-Costa MR, Fortoul TI, Rugeiro-Vargas C, Machado-Salas JP, Espinosa-Villanueva J, et al. Morphological alteration of the olfactory bulb after acute ozone exposure in rats. Neurosci Lett 1999;274:1–4.

Colton CA, Gilbert DL. Reactive oxygen species and neuronal function. In: Gilbert D, Colton C, editors. Reactive oxygen species in biological systems. New York: Plenum; 1999. p. 569–85.

Cross CE, Reznick AZ, Packer L, Davis PA, Susuki YJ, Halliwell B. Oxidative damage to human plasma proteins by ozone. Free Radic Res Commun 1992;15:347–52.

Dorado-Martínez C, Paredes-Carbajal C, Dieter M, Borgonio-Pérez G, Rivas-Arancibia S. Effects of different ozone doses on memory, motor activity and lipid peroxidation levels, in rats. Int J Neurosci 2001;108: 149–62.

Floyd RA, Carney JM. Free radical damage to protein and DNA: mechanisms involved and relevant observations on brain undergoing oxidative stress. Ann Neurol 1992;32:522–7.

Fukagawa NK, Timblin CR, Buder-Hoffman S, Mossman BT. Strategies for evaluation of signaling pathways and transcription factors altered in aging. Antioxid Redox Signal 2000;2:379–89.

Globus MY, Busto R, Martínez E, Valdes I, Dietrich WD, Ginsberg MD. Comparative effect of transient global ischemia on extracellular levels of glutamate, glycine and γ-aminobutyric acid in vulnerable and non-vulnerable brain regions in the rat. J Neurochem 1991;57:470–7.

González C, Lin RCS, Chesselet MF. Relative sparing of GABAergic interneurones in the striatum of gerbils with ischemia-induced lesions. Neurosci Lett 1992;135:53–8.

Graham DG. Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. Mol Pharmacol 1978;14:633–43.

Guevara-Guzmán R, Barrera-Mera B, de la Riva C, Kendrick KM. Release of classical transmitters and nitric oxide in the rat olfactory bulb evoked by vagina–cervical stimulation and potassium, varies with the oestrus cycle. Eur J Neurosci 2000;12:80–8.

Halliwell B, Cross CE. Oxygen-derived species: their relation to human disease and environmental stress. Cell 1994;10:5–12.

Halliwell B, Gutteridge JM. Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. Lancet 1984;23:1396–8.

Hastings TG. Enzymatic oxidation of dopamine: the role of prostaglandin H synthase. J Neurochem 1995;64:919–24.

Haughey HM, Brown JM, Fleckenstein AE, Hanson GR. Oxygen radicals differentially affect Na^+/Cl^- dependent transporters. Eur J Pharmacol 1999;379:107–10.

Huether G, Fettkotter I, Keilhoff G, Wolf G. Serotonin acts as a radical scavenger and is oxidized to a dimer during the respiratory burst of activated microglia. J Neurochem 1997;69:2096–101.

Kendrick KM, Guevara-Guzmán R, de la Riva C, Christensen J, Ostergaard K, Emson PC. NMDA- and kainate-evoked release of nitric oxide and classical transmitter the striatum: in vivo evidence that nitric oxide may play a neuroprotective role. Eur J Neurosci 1996;8:2619–34.

Kroncke KD, Suschek CV, Kolb-Bachofen V. Implications of inducible nitric oxide synthase expression and enzyme activity. Antioxid Redox Signal 2000;2:585–605.

LaVoie MJ, Hastings TG. Dopamine quinone formation and protein modification associated with the striatal neurotoxicity of methamphetamine: evidence against a role for extracellular dopamine. J Neurosci 1999a; 19:1484–91.

LaVoie MJ, Hastings TG. Peroxynitrite and nitrite-induced oxidation of dopamine: implications for nitric oxide in dopaminergic cell loss. J Neurochem 1999b;73:2546–54.

Luo Y, Roth GS. The roles of dopamine oxidative stress and dopamine receptor signaling in aging and age-related neurodegeneration. Antioxid Redox Signal 2000;2:449–60.

Maher P, Davis JB. The role of monoamine metabolism in oxidative glutamate toxicity. J Neurosci 1996;16:6394–401.

Murphy TH, Miyamoto M, Sastre A, Coyle JT. Glutamate toxicity in a neural cell line involves inhibition of cysteine transport leading to oxidative stress. Neuron 1989;2:1547–58.

Olanow CA. A radical hypothesis for neurodegeneration. TINS 1993;16: 439–44.

Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 2nd ed. London: Academic Press; 1986.

Phillis JW, Smith-Barbour M, Perkins LM, O'Regan MH. Characterization of glutamate, aspartate, and GABA release from ischemic rat cerebral cortex. Brain Res Bull 1994;34:457–66.

Pryor WA, Church DF. Aldehydes hydrogen peroxide and organic radicals as mediators of oxygen toxicity. Free Radic Biol Med 1991;11:41–6.

Rego AC, Santos MS, Oliveira CA. Oxidative stress, hypoxia and ischemia-like conditions increase the release of endogenous amino acids by distinct mechanisms in cultured retinal cells. J Neurochem 1996;66: 2506–16.

Rivas-Arancibia S, Dorado-Martínez C, Borgonio-Pérez G, Hiriart-Urdanivia M, Verdugo-Diaz L, Durán-Vázquez A, et al. Effects of taurine on ozone-induced memory deficits and lipid peroxidation levels in brains of young, mature and old rats. Environ Res 2000;82:7–17.

Rivas-Arancibia S, Vázquez-Sandoval R, González-Kladiano D, Schneider-Rivas S, Lechuga-Guerrero A. Effects of ozone exposure in rats on memory and levels of brain and pulmonary superoxide dismutase. Environ Res 1998;76:33–9.

Rivas-Arancibia S, Colín Barenque L, Dorado-Martínez C, Fortoul T. Estrés oxidativo y neurodegeneración. In: Velázquez MR, editor. Temas selectos de neurociencias. México: UAM; 2001. p. 351–74.

Saji M, Cohen M, Blau AD, Wessel TC, Volpe BT. Transient forebrain ischemia induces delayed injury in the substantia nigra reticulata: degeneration of GABA neurons, compensatory expression of GAD mRNA. Brain Res 1994;643:234–44.

Saransaari P, Oja SS. Release of endogenous glutamate, aspartate, GABA and taurine from hippocampal slices from adult and developing mice under cell damaging conditions. Neurochem Res 1998;23:563–70.

Shuaib A, Ijaz S, Miyashita H, Mainprize T, Kanthan R. Progressive decrease in extracellular GABA concentration in the postischemic period in the striatum: a microdialysis study. Brain Res 1994;666:99–103.

Siegel S. Non-parametric statistics for the behavioral sciences. New York: McGraw-Boob; 1991.

Sies H. Oxidative stress: from basic research to clinical application. Am J Med 1991;91:31S–8S.

Simonian NA, Coyle JT. Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol 1996;36:83–106.

Smith MA, Nunomura A, Zhu X, Takeda A, Perry G. Metabolic, metallic, and mitotic sources of oxidative stress in Alzheimer disease. Antioxid Redox Signal 2000;2:413–20.

Van der Vliet A, O'Neil CA, Eiserich JP, Cross CE. Oxidative damage to extracellular fluids by ozone and possible protection effects of thiols. Arch Biochem Biophys 1995;321:43–50.

Volterra A, Trotti D, Tromba C, Floridi S. Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. J Neurosci 1994;14: 2924–32.

Wink DA, Feelisch M, Vodovotz Y, Fukuto J, Grisham B. The chemical biology of nitric oxide. In: Gilbert D, Colton C, editors. Reactive oxygen species in biological systems. New York, USA: Kluwer Academic Publishing/Plenum; 1999. p. 245–91.

Wolff SP, Garner A, Dean RT. Free radicals, lipids and protein degradation. TIPS 1986;11:27–31.

Zhang J, Snyder SH. Nitric oxide in the nervous system. Annu Rev Pharmacol 1995;35:213–33.