Acta Veterinaria (Beograd), Vol. 55, No. 5-6, 375-383, 2005.

UDK 619:617-089

TOTAL ANAESTHESIA, RATS BRAIN SURGERY, NITRIC OXIDE (NO) AND FREE RADICALS

JELENKOVIĆ ANKICA*, JOVANOVIĆ MARINA**, NINKOVIĆ MILICA**, MAKSIMOVIĆ M** and BOŠKOVIĆ B**

*Institute for Biological Research "Siniša Stanković, Belgrade
**Military Medical Academy, Belgrade

(Received 14. February 2005)

It is expected that clinical recovery after surgically induced brain trauma is followed by molecular and biochemical restitution. Seven days after surgery, we investigated whether the plastic cannula implanted in the left brain ventricle of adult Wistar rats (n=6-7), performed in pentobarbital anesthesia, could influence oxidative stress elements (superoxide anion and lipid peroxidation), as well as the antioxidative system (superoxide dismuthase-SOD). Also, we investigated whether nitric oxide (NO) is involved in these processes. Biochemical analyses was performed in the forebrain cortex, striatum and hippocampus.

Clinical recovery was complete seven days after surgery. Thereafter, thirty minutes before decapitation, through the cannula, one group of rats received saline intracerebroventricularly (control group), and the treated group receaved Nω-nitro-L-arginine methyl ester (L-NAME). The third group was left unoperated and untreated. Before and after the treatments, rectal body temperature was measured.

Compared to the untreated group the index of lipid peroxidation was increased in all three brain structures in the group that received saline ($p<0.05$ to 0.01). Application of L-NAME deteriorated it in the striatum and hippocampus ($p<0.01$ compared to the both other groups), but the value in the forebrain cortex was similar to the untreated group. Supeoxide anion level was decreased in the L-NAME treated group only in the striatum ($p<0.01$ compared to control and untreated groups), but SOD was increased in the hipppocampus, compared to the saline treated group ($p<0.05$).

Seven days after brain surgery in pentobarbital anesthesia, recovery of biochemical disturbances was not parallel to clinical recovery. Long lasting biochemical changes are rather the consequence of brain injury than to pentobarbital anesthesia. In this experimental model, NO had protective effects, acting against lipid peroxidation in the striatum and hippocampus, but not in the forebrain cortex i. e. NO involvement in the free radical processes strongly depends on the observed brain region.

Key words: brain, lipid peroxidation, nitric oxide, oxidative stress, pentobarbital, surgery

INTRODUCTION

After brain trauma (mechanical or chemical) consequent surgical treatment induces further tissue damage with macro, micro and biochemical changes. Injury initiates a cascade of biochemical changes. The production of free radical species is the most important among them. This is the result of excitatory amino acids (EAA) toxicity, involving glutamate, quinolonic and others excitatory acids. Such reactions are found not only in a number of degenerative disorders, but also in the brain previosly affected by ischemia-reperfusion, trauma (Lipton and Rosenberg, 1994), etc. Activity of EAA is realized through ionotropic (N-methyl-D-aspartate-NMDA, non-NMDA), as well as methabotropic receptors. Among other EAA effects, Ca2+ inward initiation is the leading one. In excessive gluthamatergic activity evokes enormous Ca2+ cell level, which is followed by a number of pathological processes, with cell energy depletion as the last stage, and destruction or conformational changes in all kinds of molecules (Lewen *et al.,* 2000). Lipid membranes are especially prone to that processes, as they are rich in unsaturated free fatty acids (Dawson, *et al.*, 1991). In the case of imbalance between prooxidants (free radicals) and anti oxidants (enzymes, vitamins and chemical substannces), the above mentioned disturbances can be develop.

Production of nitric oxide (NO) is a part of EAA activity. Glutamate binding to NMDA receptors activates nitric oxide synthase enzymes (NOS). Only uncontrolled synthesis of NO is harmful. Otherwise, i. e. in physiological conditions, it mediates intracellular and intercellular communication (Stamler *et al.,* 1997), and regulates homeostasis of, for example blood preassure, learning and memory, neuroendocrine processes, sleep, pain, appetite, etc.

Glutamate and NO are involved in reactive oxygen and nitrogen species metabolism, such as superoxide anion, hydroxyl and NO radical, and also in peroxynitrite (ONOO-) formation. The last compound is known as a stable and long acting toxic metabolite of NO. The superoxide anion can produce a number of free radicals (Goss *et al.*, 1999). Thus, we investigated the consequences of brain surgery performed in pentobarbital anesthesia on oxide-reductive processes in the brain, as well as NO production and body temperature.

MATERIAL AND METHODS

Animals and surgery

Experiments were performed on 13 weeks old male Wistar rats (*Rattus norvegicus*). They were kept in an aired-conditioned room: temperature (23±2^{o}C), relative humidity (60-70%), and dark/light cycles (11/13 hours). Food (commercial rat diet) and water were not restricted. Experiments were conducted within two weeks, every day between 10 am and 3 pm.

The animals were anaesthetized by pentobarbital sodium (0.045 g/kg body weight-bw, applied intraperitoneally, Vetanarcol® , Werfft-chemie, Wien). On the sterotaxic frame, polyethylene plastic cannula was implanted into the left lateral ventricle of the brain (coordinates: 1.3 mm behind the bregma, 1.8 mm left from the midline suture, 3.7 mm ventral from the dura) (Paxinos and Watson, 1982).

The investigated substances were applied intracerebroventricularly (icv) throught the inserted cannula, which was fixed to the skull with dental cement and two jeweler screws.

Treatment

Seven days after surgery (recovery period), two groups of rats (n=7 in each) were assigned randomly to receive two different treatments. The third group was unoperated and untreated (n=7). During the recovery period, rats were examined clinically (motor activity, changes in nasal and eye secretion and hair inspection).

After clinical recovery (seven days after surgery), one of the operated groups received 0.9% saline (control group). A nonselective NOS antagonist, Nω-nitro-L-arginine methyl ester (L-NAME, Sigma), dissolved in 0.9% saline, was applied to the second group. Both solutions were given in a volume of 10 microliters. Thirty minutes later, rats were sacrificed by decapitation. Heads were immediately frozen in liquid nitrogen. Time of decapitation was selected according to pharmacokinetic and pharmacodynamic characteristics of L-NAME. Heads were stored at -70^{o}C until preparation of brain structures for biochemical analysis. Before decapitation, rectal temperature was registered twice: once before the solution was applied, and the second time 30 minutes later, just before decapitation. The same measuresments were performed in unoperated rats, too.

Biochemical analysis

Three brain regions: forebrain cortex, striatum and hippocampus were dissected on ice and prepared for spectrophotometric biochemical analyses.

Superoxide anion content was determined thought the reduction of nitroblue-tetrazolium (Merck) in an alkaline, nitrogen-saturated medium. Analysis was performed at 515 nm (Sun and Zigman, 1985).

Superoxide dismutase (SOD) activity was determined as inhibition of epinephrine autooxidation at 480 nm. After adding 10 mM of epinephrine (Sigma), the kinetics was monitored in sodium carbonate buffer (50 mM, pH 10.2; Serva) containing 0.1 mM EDTA (Sigma) (Auclair and Voisin, 1985).

Lipid peroxidation index was measured as malondialdehyde produced after stimulated peroxidation with 0.01 mM ferrosulphate (Merck) and 0.5 mM ascorbic acid (Serva). Thiobarbituric acid reagent (TBAR), consisting of trichloracetic acid (Merck), thiobarbituric acid and HCl, reacts with malondialdehyde, the final product of polyunsaturated fatty acid peroxidation, measured at 533 nm (Villacara, *et al.*, 1989).

STATISTICS

Data were expressed as the mean ± standard deviation (SD). Results were analyzed by Students t-test. Differences among means were considered statistically significant at $p<0.05$. For the comparison of body temperature within and between groups, two-way analysis of variance was used with a level of significance at $p<0.05$.

RESULTS

All operated rats recovered without any motor activity changes, unusual movements, changes in nasal and eye secretion, or in the hair color or body coverage pattern. There were no clinical signs of any disorder.

In all three groups, the rectal body temperature was similar before and after treatment, within and between groups (Table 1).

Table 1. Rectal body temperature in saline-treated, untreated and L-NAME-treated rats (n=7, intracerebroventricular application), before and 30 minutes after the treatment

Groups	Temperature (°C±SD)		Significance
	Before treatment	30 minutes after treatment	
Saline treated	37.7±0.34	37.8±0.31	N.S.
Untreated rats	38.1±0.27	37.9±0.29	N.S.
L-NAME treated	38.0±0.51	38.1±0.59	N.S.

Values are expressed as mean±SD. *(**) $p<0.05$-significance to corresponding values within groups (saline treated group), (Two-way analysis of variance)

Biochemical changes were not parallel to clinical recovery. Some parameters of oxidative stress were increased seven days post operation. They have been developed in the structures directly damaged by cannula insertion (hippocampus), as well as in the striatum and forebrain cortex. All examined brain regions were very vulnerable in the saline treated group, compared to unoperated

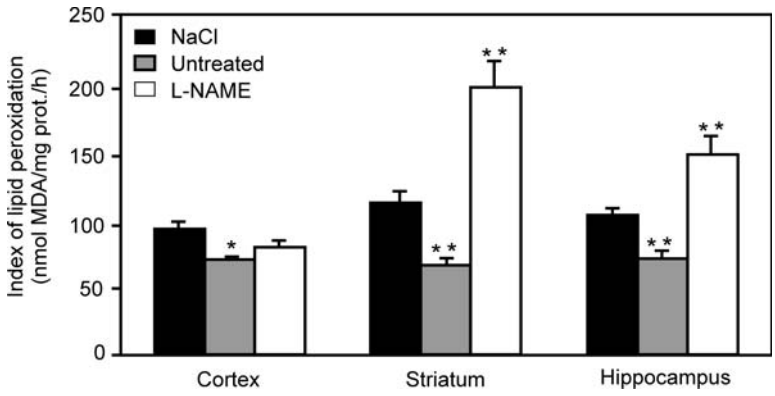

Figure 1. Index of lipid peroxidation in saline-treated, untreated and L-NAME-treated rats (n=7, intracerebroventricular application)
Values are expressed as mean±SD. *$p<0.05$-significance to corresponding values of saline treated group, ** $p<0.01$-significance to corresponding values of saline treated and untreated group (Student t-test)

rats, with a more pronounced increase of lipid peroxidation in the striatum and hippocampus ($p<0.01$ for both structures), than in the cortex ($p<0.05$) (Figure 1). Further increment of lipid peroxidation index was obtained by L-NAME treatment ($p<0.01$ in striatum and hippocampus, compared to saline treated rats). In the forebrain cortex it was only slightly above the values for untreated rats.

Superoxide anion decreased significantly only in the striatum of the L-NAME treated group, compared to both other groups ($p<0.05$) (Figure 2).

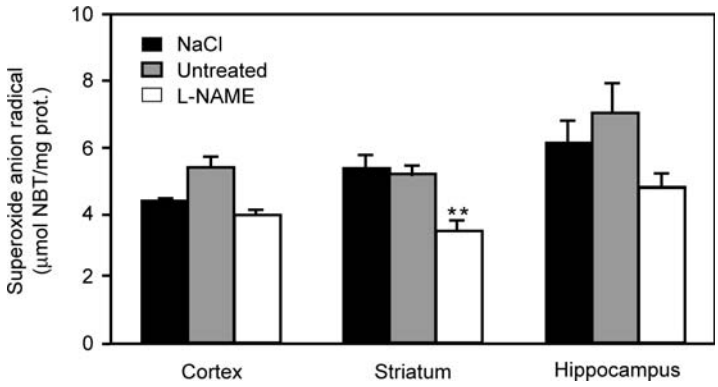

Figure 2. Superoxide anion radical content in saline-treated, untreated and L-NAME-treated rats (n=7, intracerebroventricular application)
Values are expressed as mean ±SD. **$p<0.01$-significance to corresponding values of saline treated and untreated group (Student t-test)

Activity of SOD was increased only in the hippocampus of rats treated with L-NAME, compared to the saline treated group ($p<0.05$) (Figure 3).

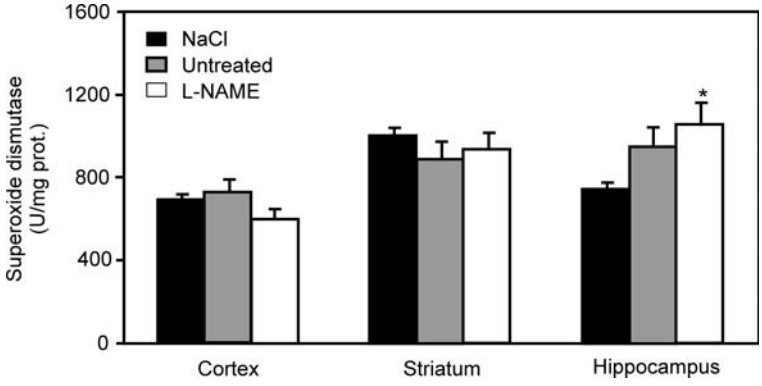

Figure 3. Activity of superoxide dismutase in saline-treated, untreated and L-NAME-treated rats (n=7, intracerebroventricular application)
Values are expressed as mean ±SD. *$p<0.05$-significance to corresponding values of saline treated group (Student t-test)

DISCUSSION

Seven days after surgery under pentobarbital sodium anesthesia rats were completely clinically recovered. Contrary to expectations, biochemical changes were still present, although seven days seemed to be long enough for the termination of such processes. The presence of biochemical disturbances was documented by the increased index of lipid peroxidation in the three brain regions examined, especially in the striatum and hippocampus. This could be due to anesthesia, surgery, or both.

There is not enough evidence about anesthesia-evoked lipid peroxidation. We used pentobarbital sodium, a barbiturate with extremely long plasma half-live i.e. from 80-120 hours in humans. Such pharmacokinetics may be of great importance in the case of substances which can induce oxidative damage, but data on prooxidative effects of pentobarbital are not available. In some circumstances pentobarbital sodium protects cells against oxidative damage, like in red blood cells, where halothane-increased lipid peroxidation is prevented by pentobarbital pretreatment (Yesilkaya *et al.*, 1998). Also, short-term (60 minutes) after pentobarbital anesthesia, NO and cyclic guanosine monophosphate (cGMP) production was not affected. The obtained results are of importance since cGMP is in part under NO control (Galley *et al.*, 2001).

In the performed experiment, nitric oxide was powerfully involved in the observed biochemical processes. Strong antioxidative effects of NO registered in the striatum and hippocampus were confirmed by L-NAME application. Unselective NOS antagonism achieved by L-NAME inhibited NO production, which was followed by a more profound lipid peroxidation in two structures (striatum and hippocampus), and almost returned to normal values in one part of the brain (forebrain cortex). At the same time, we registered protective and toxic effects of NO.

Data about neuroprotective and neurotoxic effects of NO, apart from its physiological role, are not equivocal, with a predominant evidence of toxic effects of NO. These differences most probably are determined by the experimental model and protocol, but also by the neurochemical complexity of the brain as a whole, as well as in different structures of the brain.

To elucidate the dual role of NO (neuroprotective and neurotoxic), Chiueh has been investigating for a long time the interaction of NO with a number of molecules in different experimental models. Nitric oxide interacts with oxygen and superoxide anion, followed by reactive nitrogen species generation, like peroxynitrite and S-nitrosothiols. Chiueh together with his coworkers found more evidence of NO protective role against oxidative stress. He recognized the so called atypical antioxidants that integrate NO in their molecules. One of them is a very strong antioxidant S-nitrosoglutathione, which serves as an endogenous reservoir of NO. In suppressing iron-induced oxidative stress, S-nitrosoglutathione is about 100-fold more potent antioxidant than its precursor, reduced glutathione (GSH) (Rauhala *et al.*, 1998). The same author also discovered a new antioxidative protein, named thioredoxin, with NO involved in its synthesis (Andoh *et al*., 2002).

There are new data about antioxidative effects of NO. In one of them, such effects are the consequence of hidroperoxide decomposition (d'Ischia *et al.*, 2000). In the other one, L-NAME decreases glutathione peroxidase activity in the brain and increases markers of lipid peroxidation (Yargicoglu, *et al.*, 2004). Also, in suboptimal arginine and tetrahydrobiopterine concentration (a cofactor for NOS activity), NOS syntheses hydrogen peroxide and reactive oxygen species (Rengasamy, *et al.,* 1996).

Bidmon and coworkers (Bidmon, *et al.*, 2002) in focal ischemia documented higher survival rate of perilesional neurons containing constitutive NOS than neurons without it. This could be due to co-expression of antioxidative enzymes (Mn, Zn-SOD, glutathione peroxidase) in these neurons. These enzymes are a part of defense against free radical cascade reactions. It can be supposed that such neuronal organization was involved in the brain healing after performed surgery and it was suppressed by L-NAME application.

At the some time, superoxide anion production in vulnerable structures was decreased after L-NAME treatment. Having in mind a very strong affinity of NO for the superoxide anion, scavenging of superoxide anion by NO could be eliminated after the inhibition of NO synthesis, with superoxide anion transformation to more toxic radicals that could initiate membrane phospholipid peroxidation. This can result in cell membrane damage.

So, in the circumstances where NO has neuroprotective effects, every reduction of its synthesis could be harmful. In this research, it was expressed in augmentation of lipid peroxidation index, obtained by L-NAME application in the left cerebral ventricle through the inserted plastic cannula. The answer to the question about missing protective effects of NO in the forebrain cortex is still a matter of investigation. It is suggested that NO could exert protective effects (striatum and hippocampus) and to be harmful (forebrain cortex) at the some time, due to complex neuronal and neurochemical interplay in the brain, i. e. NO involvement in the free radical processes strongly depends on the brain region.

Finally, recovery of biochemical disturbances in the brain evoked by brain surgery in pentobarbital anesthesia, are not in parallel to clinical healing. Increment of lipid peroxidation in the forebrain cortex, striatum and hippocampus was more likely to be due to mechanical trauma, rather than to be the result of the pentobarbital used for anaesthesia. In the applied protocols, NO had antioxidative properties (striatum and hippocampus), which were deteriorated by inhibition of NO synthesis (gained by L-NAME application). In the forebrain cortex, protective effects of NO against lipid peroxidation were not registered.

Address for correspondence:
Ankica Jelenković
Institute for Medical Research
Military Medical Academy
Crnotravska, 17
11000 Beograd
Serbia&Montenegro
E-mail: aka950@yahoo.com

REFERENCES

1. *Andoh T, Chock PB, Chiueh CC,* 2002, Preconditioning-mediated neuroprotection: role of nitric oxide, cGMP, and new protein expression, *Ann N Y Acad Sci,* 962, 1-7.
2. *Auclair C, Voisin E*, 1985, Nitroblue tetrazolium reduction, In: Greenwald RA, editor. *Handbook of methods for oxygen radical research*, Boca Raton: CRC Press Inc, 123-32.
3. *Bidmon HJ, Embe B, Kowalski T, Schmidtt M, Mayer B, Kato A, et al,* 2002, Nitric oxide synthase-I containing cortical interneurons co-express antioxidative enzymes and anti-apoptotic Bcl-2 following focal ischemia: evidence for direct and indirect mechanisms towards their resistance to neuropathology. *J Chem Neuroanatom,* 22, 167-84.
4. *D'Ischia M, Palumbo A, Buzzo F*, 2000, Interaction of nitric oxide with lipid peroxidation products under aerobic conditions: inhibitory effects on the formation of malondialdehyde and related thiobarbituric acid-reactive substances, *Nitric oxide,* 4, 4-14.
5. *Dawson VL, Dawson T, London E, Bredt D, Snyder S*, 1991, Nitric oxide mediates glutamate toxicity in primary cortical cultures, *Proc Natl Acad Sci USA,* 88, 6368-71.
6. *Galley HF, Le Cras AE, Logan SD, Webster NR*, 2001, Differential nitric oxide synthase activity, cofactor availability and cGMP accumulation in the central nervous system during anaesthesia, *Br J Anaesth,* 86, 388-94.
7. *Goss SPA, Singh RJ, Hogg N, Kalyanaraman B*, 1999, Reactions of .NO, .NO2 and peroxynitrite in membranes: physiological implications, *Free Rad Res*, 31, 597-606.
8. *Lewen A, Matz P, Chan PH,* 2000, Free radical pathway in CNS injury, *J Neurotrauma,* 17, 871-90.
9. *Lipton S, Rosenberg P*, 1994, Excitatory amino acids a final common pathway for neurologic disorders, *N Engl J Med,* 330, 613-22.
10. *Paxinos G, Watson C,* 1982, The rat brain stereotaxic coordinates, *Acad Pres.*
11. *Rauhala P, Lin AMY, Chiueh C,* 1998, Neuroprotection by S-nitrosoglutathione of brain dopamine neurons from oxidative stress, *FASEB J,* 12, 165-73.
12. *Rengasamy A, Johns R*, 1996, Determination of Km for oxygen of nitric oxide synthase isoforms, *J Pharmacol Exp Ther,* 276, 30-3.
13. *Stamler J, Toone E, Lipton S, Sucher N*, 1997, NO signals: translolcation, regulation, and a consensus motif, *Neuron,* 18, 691-6.
14. *Sun M , Zigman S,* 1978, An improved spectrophotometric assay for superoxide dismutase based on epinephrine autooxidation, *Anal Biochem*, 90, 81-9.
15. *Villacara A, Kumami K, Yamamoto T, Mršulja BB, Spatz M,* 1989, Ischemic modification of cerebrocortical membranes: 5-hydroxytryptamine receptors, fluidity, and inducible *in vitro* lipid peroxidation, *J Neurochem*, 53, 595-601.
16. *Yargicoglu P, Yaras N, Agar A, Gumuslu S, Abidin I, Bilmen S,* 2004, Effects of nitro-l-arginine methyl ester (l-NAME), a potent nitric oxide synthase inhibitor, on visual evoked potentials of rats exposed to different experimental stress models, *Acta Physiol Scand,* 180, 307-16.
17. *Yesilkaya A, Ertug Z, Yegin A, Melikoglu M, Baskurt OK,* 1998, Deformability and oxidant stress in red blood cells under the influence of halothane and isoflurane anesthesia, *Gen Pharmacol,* 31, 33-6.

OPŠTA ANESTEZIJA, OPERACIJA NA MOZGU PACOVA, AZOT OKSID (NO) I SLOBODNI RADIKALI

JELENKOVIĆ ANKICA, JOVANOVIĆ MARINA, NINKOVIĆ MILICA, MAKSIMOVIĆ M i BOŠKOVIĆ B

SADRŽAJ

Posle hirurške intervencije na mozgu, očekuje se paralelizam između kliničkog, sa jedne strane, i molekulskog i biohemijskog oporavka, sa druge strane. Da bi to utvrdili, u tri moždane strukture (kora prednjeg mozga, strijatum, hipokampus) odraslih Vistar pacova muškog pola, ispitivali smo promene pojedinih prooksidativnih i antioksdativnih parametara, nastalih posle usađivanja plastične kanile u bočnu komoru mozga, kroz koju su ubrizgavane ispitivane supstance (10 μl). Kao opšti anestetik korišćen je pentobarbiton natrijum (0,045 g/kg).

Eksperiment je nastavljen sedam dana posle operacije, kada su pacovi bili klinički potpuno oporavljeni. Pre ubrizgavanja 0,9% NaCl jednoj grupi (kontrola) i Nω-nitro-L-arginin metil estra (L-NAME, 10 mikrograma, rastvoren u 0,9% NaCl) drugoj grupi, kao i 30 minuta posle toga, merena je rektalna temperatura kod sve tri grupe pacova (treću su činili intaktni pacovi, 6-7 pacova u svakoj grupi). Porast indeksa lipidne peroksidacije u sve tri moždane strukture operisanih pacova koji su dobili NaCl bio je statistički značajan u odnosu na intaktnu grupu. Ubrizgavanjem L-NAME, ove promene su u strijatumu i hipokampusu postale statistički još izraženije u odnosu na grupu koja je dobila NaCl, dok je u kori prednjeg mozga registrovan sasvim slab porast u odnosu na intaktnu grupu. Istovremeno, ometanje sinteze NO bilo je praćeno statistički značajnim padom superoksidnog radikala u strijatumu u odnosu na obe grupe, i porastom superoksid dizmutaze u hipokampusu u odnosu na grupu koja je dobila NaCl. Telesna temperature je bila normalna kod svih pacova u oba vremena merenja.

Dokazano je da ne postoji paralelizam između kliničkog i biohemijskog oporavka posle operacije na mozgu pacova, izvedene u opštoj anesteziji uz primenu pentobarbitona. To je ispoljeno pojačanom lipidnom peroksidacijom sedam dana posle operacije u sve tri ispitivane strukture mozga koji su dobili NaCl. Porast lipidne peroksidacije je najverovatnije posledica mehaničkog oštećenja izazvanog operacijom, pre nego same anestezije. U ovim procesima, NO ima značajnu regulatornu ulogu, pri čemu njegovi efekti nisu podjednako ispoljeni u svim delovima mozga. Njegova snažna antioksidativna svojstva registruju se u hipokampusu i strijatumu, ali ne i u kori prednjeg mozga, što govori u prilog selektivne osetljivosti mozga.