[Frontiers in Bioscience 12, 1034-1040, January 1, 2007]

Brain nitric oxide synthases and mitochondrial function

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1. ABSTRACT

Nitric oxide is a small signaling molecule, which may act as a neurotransmitter and neuromodulator, exerting a regulatory effect on neuronal function. It can diffuse from its site of synthesis to different intra and extracellular compartments, being therefore present in the pre-synaptic, synaptic and post-synaptic spaces. Recently, a NOS located in the mitochondria (mtNOS) has been observed in different brain regions, responsible for the production of NO in these organelles and identified as nNOS. A regulatory effect of NO on mitochondrial function was described in brain mitochondria, where NO acts mainly by inhibiting cytochrome oxidase activity. Hippocampal mitochondrial dysfunction and decreased mtNOS activity and expression were reported in association with ultrastructural damage in an experimental model of hepatic encephalopathy. Enriched environment exposure preserved the aged animals from spatial cognition impairment; also environment and training modulated neuronal plasticity in pre-pubertal rats through NO-dependent mechanisms. In addition, brain cortical mitochondrial respiration and mtNOS activity and expression were analyzed as function of age. Mitochondrial NO production showed a decreasing tendency as a function of age. These results are in accordance with the protein expression analyzed by Western Blot of mitochondrial fractions which was 6.5 times higher in 1 month aged rats as compared with 14 old animals. Concomitant with these results, a clear increasing oxygen uptake tendency in state 3 respiration was observed, meanwhile only a slight increase was observed in state 4. All these results seems to be clearly related with the reversible and concentration-dependent attenuation of the respiratory chain by NO.

2. INTRODUCTION

Nitric oxide (NO) may act as a neurotransmitter between neurones, as a paracrine signal in different types of cells, or as an autocrine signal inside a given cell. Nitric oxide is produced in post-synaptic neurones, following stimulation of NMDA receptors and the subsequent influx of calcium which results in the activation of the neuronal nitric oxide synthase (nNOS). This molecule can affect neuronal plasticity including roles of neurite outgrowth, synaptic transmission and long-term potentiation (1). In addition, NO has been reported to be both neuroprotective and neurotoxic, depending on cell type, intracellular concentration, and cellular redox state. Recently, the presence of a nitric oxide synthase in mitochondria named mitochondrial nitric oxide synthase (mtNOS) gave a new insight to the comprehension of the role of NO in mitochondrial function.

3. BRAIN NOS ACTIVITY AND EXPRESSION

Nitric oxide is an important diffusible molecule involved in the regulation of different aspects of brain function. It is synthesized from L-arginine by the NOS (2,3) present in almost all cellular brain types, neurons, astrocytes, glyal cells as well as endothelial cells. It plays an important role in the modulation of neuronal activity, due to its presence in the pre-synaptic, post-synaptic and synaptic regions. If its production is highly increased in the post synaptic region, it will diffuse to the presynaptic space, exerting a retrograde effect on neurotransmitters release (Figure 1). In the post- and pre-synaptic regions it activates the soluble guanylyl cyclase (sGC) resulting in the formation of cGMP inducing different actions (3). In the brain there are two main types of nitric oxide synthases which are constitutively expressed and calcium dependent: the endothelial nitric oxide synthase (eNOS), and the neuronal nitric oxide synthase (nNOS), and another subtype which is inducible and calcium independent named inducible nitric oxide synthase (iNOS) (4) responsible for massive NO release. Recently, another NOS located in the mitochondria has been observed, responsible for a production of NO in these organelles, in different brain

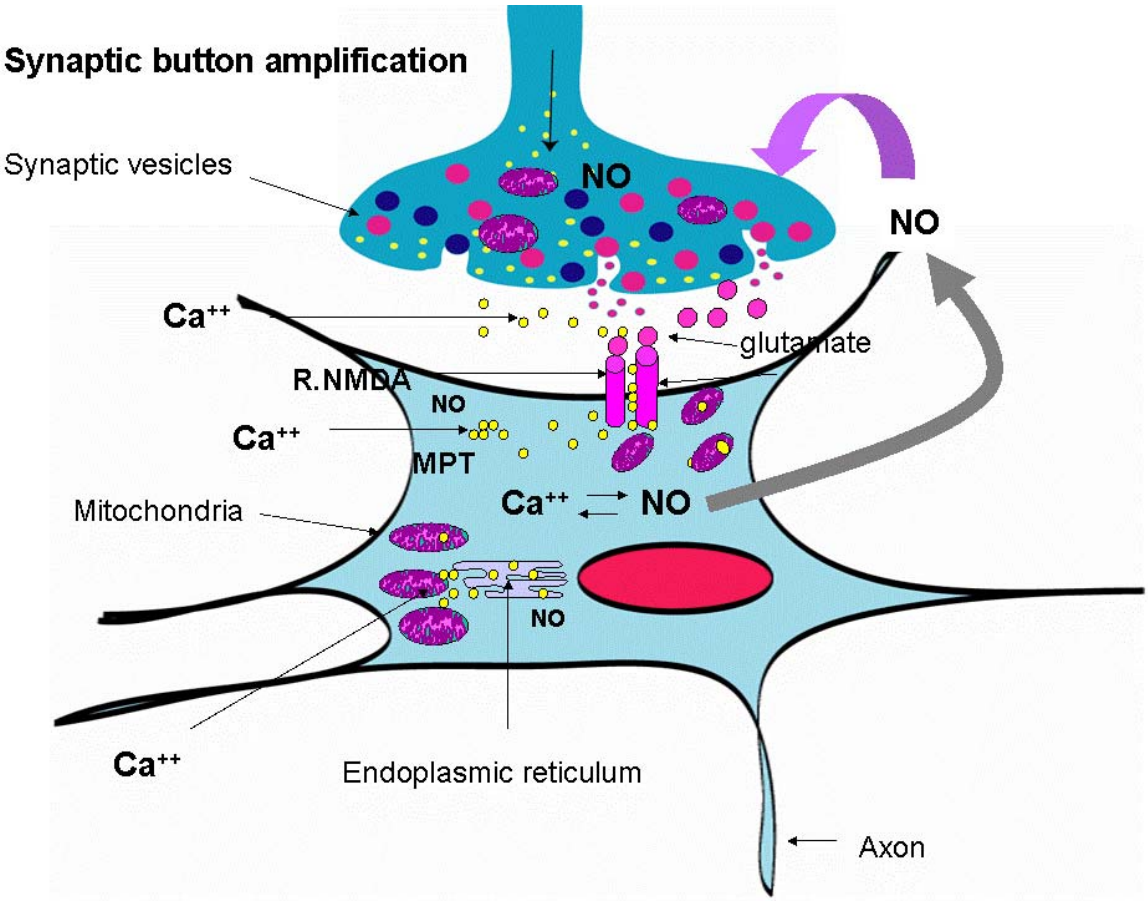

Figure 1. This synaptic buttom scheme shows the different compartments where NO can exert its effects and interact with Ca^{2+}. In the presynaptic space it can be produced mainly by nNOS exerting its effects on the neurotransmitter release, also it is present in mitochondria where it contributes to maintain mitochondrial function. NO can also reach this compartment by diffusion from the post-synaptic and synaptic space, acting as a retrograde messenger. The effect of NO in the synaptic extracellular space can be directed to modulate different receptor activities such as NMDA, depending on the presence of different neurotransmitters. In the post-synaptic space NO could be differently distributed in the neuronal cytoplasm, depending on the level of NO production, generating specific intracellular "microdomains", as has been accepted for Ca^{2+}. In addition, NO can be present in post-synaptic mitochondria; these organelles in turn regulate Ca^{2+} concentrations, when the sub-micromolar cytosolic concentrations of this molecule, are increased in mitochondrial environment, either by coming from outside through the NMDA receptor or by its endogenous release from the endoplasmic reticulum. Thus we can state that NO and Ca^{2+} interactions regulate different cytosolic and synaptic neuronal signals.

regions (5,6). A clear protein expression of nNOS type of 157 kDa reacting with anti-nNOS antibodies (amino and carboxy terminus) has been detected by Western blot analysis in brain cortex (5) and hippocampal mitochondria (6). In addition, this type of analysis also identified the same protein in brain submitochondrial membranes, cytosolic fractions and synaptosomes. An enriched band corresponding to cytosolic fractions compared with the other subcellular fractions was observed (Figure 2), which was in agreement with the increased NO production in cytosolic fractions as compared with the production in mitochondrial and synaptosomal membranes.

As well as calcium microdomains, we can suggest the existence of specific cellular microdomains where NO transient levels could be regulated by specific interacting mechanisms. In addition, actually it is not possible to differentiate clearly the NO effects on the pre-synaptic, post-synaptic and synaptic regions. To sum up, the high compartamentalization of this molecule makes more complex the interpretation of its neuronal effects.

4. NITRIC OXIDE EFFECT ON MITOCHONDRIAL FUNCTION

In brain mitochondria, NO is capable of regulating the electron transport chain mainly by acting as an inhibitor of cytochrome oxidase (complex IV) activity (7). Another effect of NO on the mitochondrial respiratory chain, is the inhibition of ubiquinol-cytochrome c reductase activity (complex III) with a simultaneously increased O_2^- and H_2O_2 production (8). A third effect, inhibition of NADH-ubiquinone reductase (Complex I) which is exerted through peroxynitrite formation has also been described (9).

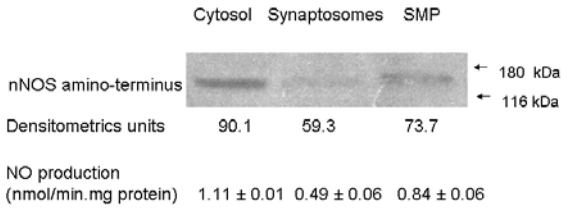

Figure 2. Western blots analysis of nNOS from mouse brain submitochondrial particles (SMP), synaptosomes and cytosolic fractions. Chemiluminescence ECL technique was used for final detection of nNOS bands. NO production values are shown for each of the different subcellular fractions.

A functional regulatory activity of NO on brain mitochondrial respiration has been described in different experimental models, for example, inhibition of mitochondrial NO production associated with an increased O_2 consumption in state 3 after treatment with chlorpromazine (10) and after incubation of brain mitochondria with deprenyl (11). Moreover, the addition of L-NNA, an inhibitor of the NOS, markedly increased the respiratory rate of state 3 normal brain mitochondria, but this effect was not observed in brain mitochondria with low mtNOS activity. L-arginine addition to brain mitochondria in state 3 condition did not significantly change the respiratory rate, confirming the existence of an intramitochondrial arginine pool as previously reported (10).

Mitochondrial respiratory chain activity has been measured in different brain areas. Hippocampal mitochondria showed succinate dependent state 4 respiratory rates of 24 $\pm$ 2 ng-atom O/min.mg protein and state 3 of 74 $\pm$ 3 ng-atom O/min.mg protein. Respiratory controls for hippocampal mitochondria were 3.1 $\pm$ 0.1. Similar respiratory controls were obtained using malate-glutamate as substrates (4.2 $\pm$ 0.3), being the ratio ADP:O estimated in 3.2 $\pm$ 0.1. L-NNA addition increased the state 3 respiratory rate by 19% in hippocampal mitochondria from control rats.

Brain cortex mitochondria showed state 4 respiratory rates of 32 $\pm$ 4 ng-atom O/min.mg protein and 29 $\pm$ 3 ng-atom O/min.mg protein for succinate and malate-glutamate respectively. State 3 respiratory rates were 168 $\pm$ 6 ng-atom O/min.mg protein and 156 $\pm$ 20 ng-atom O/min.mg protein for succinate and malate-glutamate respectively. L-NNA addition increased the state 3 respiratory rate by 56% in rat brain cortex mitochondria. Thus, it could be stated that mitochondrial NO can exert a regulatory effect on electron transport chain both in hippocampus and in cerebral cortex.

Similar results have been obtained in synaptosomal mitochondria from mouse brain cortex, isolated by Ficoll gradient centrifugation, and respiratory control ratios of 3-4 were obtained. L-NNA addition to mouse cortex synaptosomes increased state 3 oxygen consumption by 93%, as previously shown for hippocampal and brain cortex total mitochondria.

5. ROLE OF NO IN MITOCHONDRIAL DYSFUNCTION AND BRAIN PATHOLOGY

Mitochondria can be considered dysfunctional when they present a statistically significant decrease in state 3 respiration, respiratory control, or ADP: O ratio (12). State 3, the state of active respiration, represents the maximal physiological rate of O_2 uptake and ATP synthesis, while state 4 corresponds to a controlled respiration in the presence of mitochondrial substrates in the absence of ADP. The respiratory control ratio is the most sensitive indicator of mitochondrial oxidative phosphorylation coupling and is calculated as the ratio between state 3/state 4 respiration rate. The respiratory control is associated with the dependence of the rate of respiration on the availability of phosphate and phosphate aceptor, and is related to the ability of living cells to adjust their respiration to the actual need of energy under various physiological and pathological conditions.

Mitochondrial dysfunction has been shown to play a key role in the pathogenesis of several neurodegenerative processes such as Parkinson and Alzheimer's disease (13) and in the aging brain (14). Selective loss of complex I activity has been reported in Parkinson and Huntington disease (15). Recent studies have shown that a dysfunction of complex I respiratory chain enzyme and mitochondrial ultrastructural damage in the hippocampus are associated with prolonged epileptic seizure during experimental temporal lobe status epilepticus (16).

Mitochondrial dysfunction can also be associated with alterations on the membrane permeability, which are associated with a pore formation leading to mitochondrial permeability transition (MPT), typically induced by calcium overload and inhibited by cyclosporin A (17). Not only the type of stimulus, the NO concentration, and production rate but also the chronological relation of NO exposure to a potentially excitotoxic stimulus, could determine the extent of the subsequent intracellular Ca^{2+} response and the consequences for cell survival (18).

Also, mitochondrial permeability changes can occur by MPT independent mechanisms, mostly associated with Bax protein interactions with the outer membrane. Both processes are associated with a loss of mitochondrial transmembrane potential and in addition with a release of different proteins to the cytosol, such as cytochrome c, AIF and other intermembrane proteins capable of triggering apoptosis.

Increased plasma ammonia in human hepatic encephalopathy has been associated with mitochondrial damage, energy impairment, and increased free radical formation (19,17). Acute hyperammonemia produces a decrease in brain energy due to an inhibition of the α-ketoglutarate dehydrogenase and the activation of the

Table 1. Oxygen consumption and mtnos activity of hippocampal mitochondria from rats with portal hypertension

	Control	**Portal hypertension**
State 4 O_2 consumption (ng-atomO/min.mg protein)	24 ± 2	17 ± 1[1]
State 3 O_2 consumption (ng-atomO/min.mg protein)	74 ± 3	43 ± 1[1]
RC	3.1 ± 0.1	2.5 ± 0.1
(1) State 3 + L-Arg	70 ± 3	39 ± 2
(2) State 3 + L-NNA	83 ± 3	43 ± 1
mtNOS functional activity (2-1)	13 ± 4	4 ± 2[1]
mtNOS biochemical activity (nmol NO/min.mg protein)	0.28 ± 0.02	0.15 ± 0.03[1]

Values represent the mean ± SEM of 3 experimental points, each corresponding to hippocampal mitochondria obtained from pools of 4 rats, Mitochondrial respiratory rates were measured in a reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl, 1 mM EDTA, 4 mM $MgCl_2$, 5 mM phosphate and 0.2% BSA (pH 7.4), at 37 ^oC using 7 mM succinate as substrate to measure state 4 respiration rate and adding 1 mM ADP to measure state 3 respiration rate (12), The biochemical activity of mtNOS was determined in rat hippocampal submitochondrial membranes by the spectrophotometric oxyhemoglobin method (44). [1] $p < 0.05$, as compared with control. Data reproduced with permission from 6.

NMDA receptors (17). Evidence has been given that acute hyperammonemia leads to brain mitochondrial dysfunction with changes in Ca^{2+} homeostasis leading to a reduced intramitochondrial calcium capacity and uptake (20).

Results from this laboratory showed that moderated hyperammonemia produced mitochondrial dysfunction in the hippocampus in an experimental model of prehepatic portal hypertension in a two-week period. Portal hypertensive rats showed 29% and 42% decreased rates of succinate-dependent state 4 and state 3 respiration rates in the hippocampal mitochondria when compared with control rats. Portal hypertensive rats also showed a 20% decrease of respiratory control indicating the presence of dysfunctional mitochondria (6). The mtNOS biochemical activity was 46% lower in portal hypertensive rats than in control animals (Table 1). The difference in O_2 uptake between the situations of low mtNOS activity, i. e. after the addition of the competitive inhibitor L-NNA, and of high mtNOS activity, i. e. after arginine addition, is referred to as mtNOS functional activity or mtNOS-regulated cytochrome oxidase activity (21). Control hippocampal mitochondria exhibited a mtNOS functional activity of 13 ± 4 ng-atom O/min.mg protein, which was 69% decreased in portal hypertensive rats (Table 1). Structural damage of the endothelium and astrocytes was observed by electron microscopy of the hippocampal area from portal hypertensive rats in the anatomic area that corresponds to the blood-brain barrier. The damage consisted of mitochondrial swelling, decreased matrix electron density, loss and disruption of cristae, and disruption of the inner and outer mitochondrial membranes (6).

Exposure of cultured rat cortical astrocytes to 5 mM ammonia for 4 h induced a collapse of the mitochondrial membrane potential as determined by flow cytometry and the voltage-sensitive dye TMRE, being blocked by cyclosporin A in a dose-dependent manner (22). In our study, membrane potential was decreased by amonium addition to hippocampal isolated mitochondria, as measured by a fluorometric method after mitochondrial loading with TMRE (data not shown). This observation suggests that in portal hypertensive rats, amonium elevation could be responsible for mitochondrial dysfunction.

Several studies have shown that NO can be a mediator of MPT induction (23). However, in portal hypertension, mitochondrial dysfunction and alterations in mitochondrial permeability occur associated with a reduction in mtNOS activity and expression. Accordingly, a decreased NO production was found in this laboratory in association with the induction of MPT by calcium overload in mouse cortex mitochondria (unpublished results).

6. NITRIC OXIDE IN PLASTICITY AND AGING

NO has been implicated in the process of brain development and in an increasing number of experimental models of plasticity, in the formation of long term memory and in spatial learning in rats. Nitric oxide has been also proposed as a retrograde messenger during long term potentiation (LTP) induction. Deletion of the genes that encode nNOS and eNOS isoforms reduce the inducibility of LTP, and selective NOS inhibitors impair spatial learning (24).

Kolb (1998) showed that the brains of rats reared in an enriched environment have a higher number of synapsis by neuron, a greater size of synaptic connection zones, more dendritic density, higher proportion of glial tissue by neuron and a higher production of neurotrophic factors, than those of animals reared in an impoverished environment (25). In our laboratory, studies using pre-pubertal and young rats randomly assigned to enriched or standard environments, showed that the activity of mtNOS was 90% increased in pre-pubertal rats kept in enriched environments and subjected to 8 days of handling and shaping in the 8 arms radial maze, as compared with standard rats (Figure 3). This was correlated with cognitive performance, measured as the ratio number of correct selected arms/total arms from day 1-8 of shaping, which resulted significantly improved in pre-pubertal rats kept in enriched environments. In the young group, both enriched and standard rats showed a random performance from day 1-8 of shaping, and no significant differences in mtNOS activity was found, giving support to the hypothesis that age, environment and training modulate neuronal plasticity through NO-dependent mechanisms in defined development periods (26).

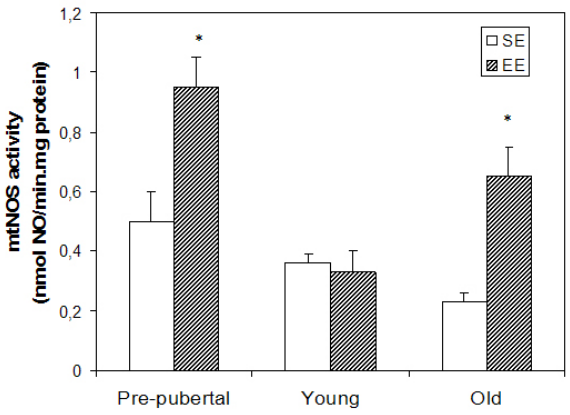

Figure 3. Brain mtNOS activity after enriched environment exposition. Pre-pubertal (40 days) and young (90 days) rats were kept in enriched (EE) or in standard (SE) environments for 17 days. EE rats were handled (8 days) and trained (8 days) in shaping in an 8-arms radial maze with 8 external cues. Old (27 months aged) rats were kept in enriched environments (EE) or in standard cages (SE) during all its life; EE animals were trained several times during their lifetime. NO production was measured using a spectrophotometric method based on the oxidation of oxyhemoglobin to methemoglobin at 37ºC, at 577-591 nm ($E = 11.2\ mM^{-1}\ cm^{-1}$) in a double-beam double-wavelength spectrophotometer (Beckman-Coulter Serie DU) Reproduced with permission from 44.

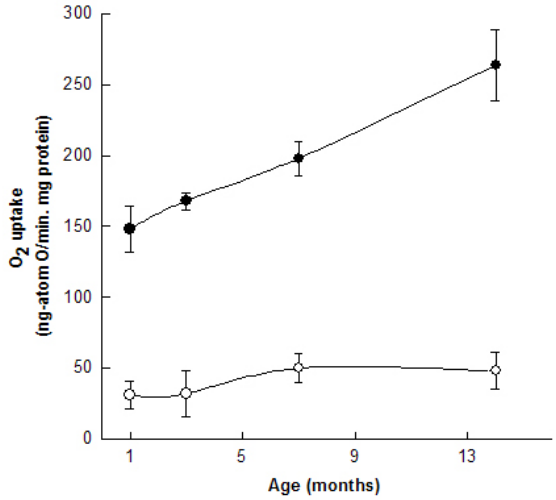

Figure 4. Brain mitochondrial respiration rate, as a function of rat age. Oxygen uptake from brain cortical mitochondria (0.8 mg/ml) was measured in state 4 and state 3, using 7 mM succinate as substrate and 1 mM ADP, with a two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria).

Moreover, the impairment of spatial cognition and mtNOS declined activity associated with the aging process can be prevented by rat exposition to enriched environments. Results from this laboratory have shown that 27 months aged rats reared in enriched environments show higher mtNOS activity than standard reared animals (Figure 3), as well as an increased area of NADPHd reactive neurons in the parietotemporal cortex and CA1 hippocampal region (27).

Aging is a gradual and spontaneous change which leads the organisms and its cells to a maturation, childhood, puberty and young adulthood declining to the middle and late age (28). Different hypotheses concerning the decline in physiological functions associated with aging have been stated; however, the free radical theory of aging is the most accepted. This theory is based on the close relationship between mitochondrial dysfunction and increased oxidative damage during aging (29-31). It has been observed that mitochondrial DNA during aging accumulate mutations generating protein alterations which can produce mitochondrial dysfunction (32-35). In addition, several studies have suggested that derangement of the controlled production of NO could be a factor of damage and impairment of brain during aging (36-39) establishing the so-called NO hypothesis of aging (40).

The presence of a NOS in mitochondria (41) and the ability of NO to inhibit the activity of complex I, III and IV of the mitochondrial respiratory chain (42,8,9) suggest that this molecule could be associated with the physiological inhibition of mitochondrial function and energy supply, which are central for neuronal function and survival. Studies from our laboratory showed that age related decreases in mitochondrial function and in the activity and protein expression of mtNOS in the rat brain cortical tissue are closely related. We found that NO production by mtNOS was 71% decreased in rat brain cortex submitochondrial particles (SMP) from 14 month rats as compared with 1 month animals. The NO production of SMP from rats aged 3 and 7 months showed values in between those of 1 and 14 months. Similarly, NO production was 54% decreased in whole brain SMP from 27 month-aged rats, as compared with 40 days aged animals. This was in accordance with previous reports from Navarro et al. showing a significant decrease in brain mtNOS activity in mice aging (43). Mitochondrial respiratory function in terms of O2 uptake in state 4 and state 3 respiration was studied in these groups of animals by using malate-glutamate and succinate as substrates. We found a 78% increase in state 3 respiration in aged rats as compared with the youngest group of animals (aged 1 month) in the presence of succinate. Rats aged 3 and 7 months showed intermediate increases in O2 uptake in state 3, 13% and 34% respectively (Figure 4). No differences were observed in respiratory state 4 in mitochondria in rats of different age (Figure 4). Similar results were obtained when malate –glutamate was used as respiratory substrate (data not shown). The mtNOS protein expression analyzed by Western blot also showed a clear difference between the youngest and oldest groups of animals (1 month and 14 months). Densitometric analysis showed that nNOS protein expression was 6.5

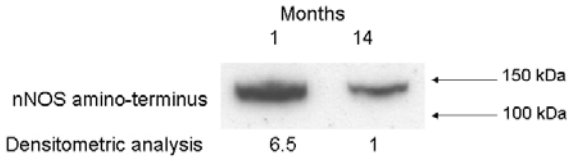

Figure 5. Western blot analysis of mtNOS. Protein expression of mtNOS was performed in brain cortical SMP from 1 and 14 months old rats using antibodies against nNOS amino terminus.

times higher in young rats as compared with the protein expression observed in old animals (Figure 5).

7. PERSPECTIVES

The complex effects of NO production in the central nervous system seems to be defined in each case by NO levels, neuronal energetics, cellular redox state, nervous system plasticity and apoptosis. The understanding of the inhibitory roles of NO in mitochondrial electron transfer certainly will contribute to clarify the role of mitochondrial dysfunction in the onset and progress of neurodegenerative diseases. The design of new clinical strategies able to prevent mitochondrial dysfunction and oxidative damage to the neuron will help to diminish the consequences of neurodegenerative processes during human aging.

8. AKNOWLEDGEMENTS

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad de Buenos Aires, Argentina.

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Key Words: Nitric Oxide Synthases, Brain Mitochondria, Mitochondrial Dysfunction, Neuronal Plasticity, Aging, Review

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