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Research Report

# Relationships between neurons expressing neuronal nitric oxide synthase, degree of microglia activation and animal survival. A study in the rat cortex after transient ischemia

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ABSTRACT

The focal ischemia obtained in an animal model of middle cerebral artery occlusion (MCAo) causes the "core" of damage in the striatum and the "penumbra" of damage in the fronto-parietal cortex. The latter is mainly functionally affected and shows changes in nNOS and iNOS expression during the acute phase of ischemia. With the aim to study possible relationships between these changes and the affection entity during the animal recovery, we investigated from 24 up to 144 h after reperfusion the expression and content of these two NOS isoforms in the neurons and microglia and the degree of microglia reactivity in the fronto-parietal cortices of rats undertaken to transient MCAo. Evaluation of motor-sensory performances and survival allowed dividing the animals into two groups. Immunohistochemistry, western blot and quantitative analysis demonstrated, both in the ischemic and contralateral cortex of the rats with longer survival, wellness and significantly increased number of the nNOS-IR neurons at 24 h and moderately activated microglia up to 144 h. In the rats not recovering, injured and significantly decreased nNOS-IR neurons, intensely activated microglia and appearance of iNOS-IR were seen at all time points. In conclusion, since the recovery occurs when nNOS-IR neurons are greatly increased, we presume nNOS protect the tissue likely controlling the passage from the state of reactive to that of activated microglia. Moreover, the morphological signs of wellness and the two-fold increase in number of the nNOS-IR neurons appear to be characteristic of the "penumbra" area and could explain why this region is mainly functionally affected.



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*Abbreviations:* CNS, central nervous system; DAB, diaminobenzidine; EDTA, ethylenediaminetetraacetic acid; iNOS, inducible nitric oxide synthase; IR, immunoreactive/immunoreactivity; MCAo, middle cerebral artery occlusion; N.E., neurological evaluation; NGS, normal goat serum; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline containing 0.1% Tween 20

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## 1. Introduction

In the mammalian central nervous system (CNS), nitric oxide (NO) is produced by neuronal nitric oxide synthase (nNOS) that is expressed by aspiny interneurons scattered in the entire brain (Bredt et al., 1991; Blottner et al., 1995; Norris et al., 1995). NO acts as a neurotransmitter and is also commonly involved in trophic actions, such as the development of the CNS and synaptic plasticity. However, NO also plays a role in neurodegenerative diseases, epilepsy, inflammation and ischemia. In these conditions, either the expression and/or the activity of nNOS was seen to increase (Wu et al., 1994; Iadecola et al., 1995) and the nNOS-expressing neurons are reported to be more resistant to neurotoxic insults (Thomas and Pearse, 1964; Uemura et al., 1990; Morton et al., 1994; Vannucchi et al., 2005). Proinflammatory stimuli or ischemia are usually followed by the appearance of the inducible (i) NOS isoform in various CNS cell populations (Iadecola et al., 1995; Iadecola, 1997; Nogawa et al., 1998; Wiesinger, 2001). The large quantities of NO generated by this isoform are considered to be responsible for the neuronal damage (Dawson et al., 1994; Iadecola, 1997; Heneka et al., 1998), while it has not yet been clarified whether the increased production of NO due to a nNOS over stimulation protects from or, on the contrary, favours the neurodegeneration (Schultz et al., 1995; Chabrier et al., 1999; Shen and Gundlach, 1999).

The ischemic stroke, one of the first causes of death and disability in the industrialised countries, is greatly studied in an animal model of middle cerebral artery occlusion (MCAo), which results in a focal ischemia with the "core" of the damage in the striatum (Zoli et al., 1997; Eliasson et al., 1999) and the "penumbra" area in the fronto-parietal cortex. By using this animal model, the possible role of NO in the evolution of cellular damage was largely investigated (Zhang et al., 1994a,b, 1996; Zhang and Iadecola, 1994; Huang et al., 1996; Yoshida et al., 1994; Iadecola et al., 1995; Iadecola, 1997; Nogawa et al., 1998; Eliasson et al., 1999; Holtz et al., 2001; Cash et al., 2001; Suzuki et al., 2002), but results obtained yielded conflicting conclusions, since both a protective and a detrimental role have been attributed to NO in tissue damage, and the NO source, whether neuronal or glial, has not been completely clarified (Iadecola, 1997; Holtz et al., 2001; Eliasson et al., 1999). We recently demonstrated in the striatum of rats treated with MCAo that the number of the nNOS-IR neurons was increased when the tissue damage was smaller and animal survival longer and we hypothesised that this increase protect the tissue from the ischemic damage (Vannucchi et al., 2005).

It is well known that the fronto-parietal cortex is differently affected by MCAo compared to the striatum. In particular, the affection of the cortex should be mainly functional, especially when the occlusion is followed by the reperfusion (Muller et al., 1994). Changes in nNOS and iNOS expression in the cortex have been reported during the acute phase of ischemia, i.e. up to 24 h after the reperfusion (Holtz et al., 2001). A relationship between these changes and the affection entity and recovery is possible. At this aim, we investigated the expression and content of these two NOS isoforms in the neurons and microglia of the fronto-parietal cortices of rats undertaken to a transient ischemia induced by MCAo, from 24 up to 144 h after reperfusion. Moreover, we also evaluated whether there was a correlation between the changes in nNOS expression and the degree of reactivity of the microglia cells.

## 2. Results

### 2.1. *Animal survival rate and neurological evaluation (N.E.)*

Fifty-nine animals underwent the operation; six of them were sham operated. Among the 53 ischemic rats, 2 were discarded because they obtained a score >15 on the N.E. 24 h after reperfusion; 15 animals died before their examination. Therefore, the total number of ischemic animals used was 36. All sham-operated rats survived after the operation. All the animals that survived were evaluated at 24 h for their motor-sensory functions and a score was attributed to each of them. At this time point, the distribution of the scores allowed for dividing the ischemic animals into two groups: one group of 25 rats having a score ranging from12 to 15, and a second group of 22 animals having a score ranging from 4 to 10. In the first group, the time course of motor-sensory functions showed a slight tendency to recover at 144 h. In the second group, the time course did not show any significant amelioration at 72 h and none of the animals were alive at 144 h (data not shown).

### 2.2. *Infarct and oedema volumes*

The measurement of the infarct area confirmed that all the animals were ischemic. Indeed, a significant increase in the volume ($mm^3$) of the ischemic hemisphere compared to the contralateral was detected either in the animals with higher score (132.8±3.1 vs. 120.4±1.4, $p<0.006$) or in those with lower score (150.3±2.53 vs. 120.00±2.34, $p<0.001$). Moreover, the

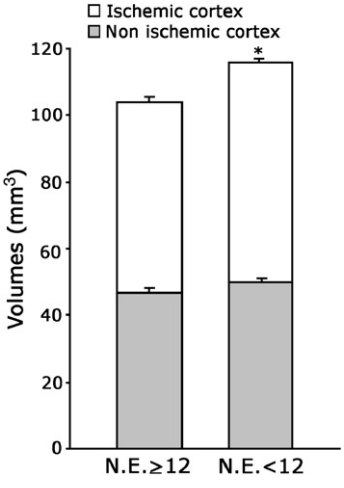


**Fig. 1 – Infarct volume. Non-ischemic tissue and cortical infarct volumes in the ischemic rats at 24 h after MCAo. Values ($mm^3$) are expressed as mean±SEM ($n=4$, each). Significant difference between the volumes of the ischemic cortex, $^*p<0.001$, vs. the ischemic rats with a score ≥12.**