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In contrast to neuronal NOS-I, the inducible NOS-II expression in aging brains is modified by enriched environmental conditions

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With 2 figures

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Summary

Changes in the expression of the nitric oxide synthase isoforms (NOS) have been implicated in age-related neurodegeneration, although mechanisms responsible for specific differences such as reduced cognitive and motor functions are not well understood. Immunohistochemical examination of the age-dependent NOS-I and NOS-II expression in aging rats at 3, 12, 24, 36 months of age as well as in 36 months old rats who spent their last 3 months in an enriched environment, revealed a significant decrease in NOS-I expression with aging. In contrast, NOS-II increased at 36 months. Old rats kept under enriched environmental conditions, however, showed strongly reduced NOS-II expression. These results indicate that both NOS-I and II may contribute to age-related degenerative processes, but in contrast to NOS-I the age-dependent changes of NOS-II are reduced or even reversed by stimulating environmental conditions.

Introduction

Normal aging is affected by loss of function in cortical areas and decreased blood flow in the whole cortex, associated with diminished metabolic activity. These phenomena are accompanied by oxidative stress and aberrant calcium metabolism often associated with, or caused by, changes in excitability, in part directly linked to glutamate metabolism (GRIFFITH 1999; INADA et al. 1996; MCCANN 1997). Age-related changes in cerebrovascular regulation are currently discussed as one of the more important factors and nitric oxide (NO) is identified as major mediator of functional hyperemia (DREIER et al. 1998; FARACI et al. 1993; IADECOLA 1993; LINDAUER et al. 1999; OBRIG et al. 2000). NO derived from the constitutively expressed, calcium-dependent neuronal NOS-I and from the inducible, calcium-independent NOS-II has been suggested to be involved in neurodegeneration (VINCENT 2000). Therefore, the expression of these two enzymes was studied during aging either by NADPH-diaphorase histochemistry or by immunohistochemistry (BIDMON et al. 1997; LÜTH et al. 1995; VINCENT 2000). Individual differences in aging conditions have been addressed by housing one group of already aged animals within an enriched environment (COQ and XERRI 1998; DIAMOND 1988) instead of standard conditions.

The present study combined theses approaches in order to find out whether enriched environmental conditions may specifically prevent and reverse age-related degenerative processes by reducing NOS-II expression.

Material and methods

We used forty-seven male hybrid Fischer 344*Brown Norway (FBNF1) rats. The animals were divided into age-groups:

3 months (n = 3, young adult controls), 11–13 months (n = 9), 24 months (n = 9), 36 months (n = 8) kept under standard housing conditions, and 36 months old rats (n = 8) who spent their last 3 months (33–36 months) under enriched environmental conditions as previously described (SPENGLER et al. 1995). Groups of four to five rats were kept under standard conditions in littered standard Type IV cages (54 cm × 33 cm × 18.5 cm, length × wide × height).

The dimensions of the cages containing the "enriched environment" were 100 cm × 60 cm × 80 cm (l × w × h). The interior was changed weekly and consisted of large irregular-shaped styrofoam blocks of variable size, pasteboard boxes and several wooden ladders. Food-pellets and sunflower seed were supplied at variable locations to reinforce explorative behavior. Following ether anaesthesia, all rats were transcardially perfused with saline, followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and post-fixed for 2 h in the same fixative. The NADPH-d activity was visualized by the tetrazolium technique (LÜTH et al. 1995). Alternate brain sections were incubated with the primary polyclonal antibodies against NOS-I, or NOS-II (Transduction, diluted 1:100) respectively. Biotinylated goat-anti-rabbit (diluted 1:50, Vector Laboratories) served as secondary antibody, followed by an incubation in extravidin peroxidase (Sigma-Immunochemicals) for 1 h. After visualization of the reaction products with DAB/H_2O_2 the sections were mounted and coverslipped according to standard protocols. In order to compare the numbers of NOS-I immunoreactive neurons and NOS-II positive clusters between the different rat brains we used the Kontron Image Analysis system (Zeiss, Germany). The internal statistic program provided the data for the minimum, the maximum and the average ± S.D. In each brain, three slides containing the somatosensory cortex were analyzed. Two additional groups of male rats with an age range of 13 months (n = 5) and 34 months (n = 5) kept under standard conditions were decapitated. Their motor- and somatosensory cortices were dissected, homogenized and prepared for NOS-II detection by Western blotting according to standard protocols (XIE et al. 1994) using either macrophage lysate (Transduction Lab.) or homogenates from cultured, LPS-stimulated rat astrocytes (gift from Dr. R. Fischer, Dept. of Gastroenterology, University of Düsseldorf) as positive controls for NOS-II expression. Blots were quantitatively evaluated using image analysis (grey value analysis) and results were statistically evaluated using the ranking test of WILCOXON (WHITE 1992).

Results

In the cerebral cortex of adult animals 3–12 months of age both NADPH-d activity and NOS-I immunoreactivity was confined to non-pyramidal bi- and multipolar interneurons (fig. 1A) showing the typical pattern of intracortical distribution. NOS-II related glial NADPH-d activity was not detectable in these rats. At 24 months the number of NOS-I positive neurons had decreased and at three years of age, immunoreactivity and NADPH-d activity was only weak (figs. 1B, F, 2A). Conversely, NOS-II immunoreactivity in the walls of cortical blood vessels and their surroundings had increased and was strongest in rats which were housed for 36 months in standard cages (figs. 1D, E, 2B). The group of 36 months old rats kept for the last 3 months in an enriched environment showed much less cortical vascular and perivascular NOS-II immunoreactivity (fig. 1C) when compared to the age-matched group housed under standard conditions (fig. 1D). The quantitative differences are shown in figure 2. Ranking test of Western blots revealed a significant increase ($p = 0.05$, table by WHITE 1992) of NOS-II expression in three year old brains when compared to one year old ones (fig. 1G). Summarising, our data indicate, that in contrast to the age-related loss of NOS-I, the concomitantly occurring upregulation of perivascular (non-neuronal) NOS-II can be stopped or even reversed by stimulating environmental conditions.

Discussion

NO with its widespread effects may potentially contribute to consequences of aging (GARTHWAITE and BOULTON 1995) and age-related behavior. Transferring rats to an enriched environment forces them to new explorative behavior, which goes hand in hand with increased sensory input and locomotor activity. These changes usually reduce or even reverse age-related and use-dependent functional impairments (COQ and XERRI 1998; DIAMOND 1988; SPENGLER et al. 1995). Therefore it is probable that the observed cortical changes in NOS-II immunoreactivity and related NADPH-d activity were functionally related to the level of explorative activity. Neuronal NOS-I distribution shows comparable profiles during ontogeny and adolescence in rodent and human brains (OERMANN et al. 1999; OHYU and TAKASHIMA 1998; VINCENT 2000). Disturbance of glutamate homoeostasis and glutamate release followed by a rise in intracellular calcium and inappropriate NO production coupled with an impairment of endogenous oxidative defense systems have been discussed as the major mechanisms leading to cognitive deterioration during aging (INADA et al. 1996; KUO et al. 1997). Furthermore, an increase in neuronal calcium-dependent NOS-I has been reported (UTTENTHAL et al. 1998), whereas others found a significant reduction of NOS-I immunoreactive neurons in striatum and cortical areas of aged rats (CHA et al. 1998; INADA et al. 1996; KUO et al. 1997; LÜTH et al. 1995). The latter findings are in agreement with the present study demonstrating an age-dependent loss of NOS-I expression. The observation that the loss of NOS-I neurons was not affected by changes in environmental conditions seems to be of special interest, since NOS-I positive neurons innervate cerebral vessels (VINCENT 2000). NOS-I-derived NO is a major mediator of functional hyperemia and seems to play an important role during pathological cerebrovascular alterations in rats and humans (DREIER et al.1998; FARACI et al. 1993; IADECOLA 1993; LINDAUER et al. 1999; OBRIG et al. 2000). It is indicated that the loss of functional cerebrovascular reactivity during aging, which could include even partial ischemic episodes (MAYHAN et al. 1990), may be attributable to reduced nitrergic innervation, due to the loss of NOS-I positive neurons. Therefore, the reduction of age-related functional sequelae due to enriched environmental conditions may either be related to higher NO output by the few remaining NOS-I positive neurons or

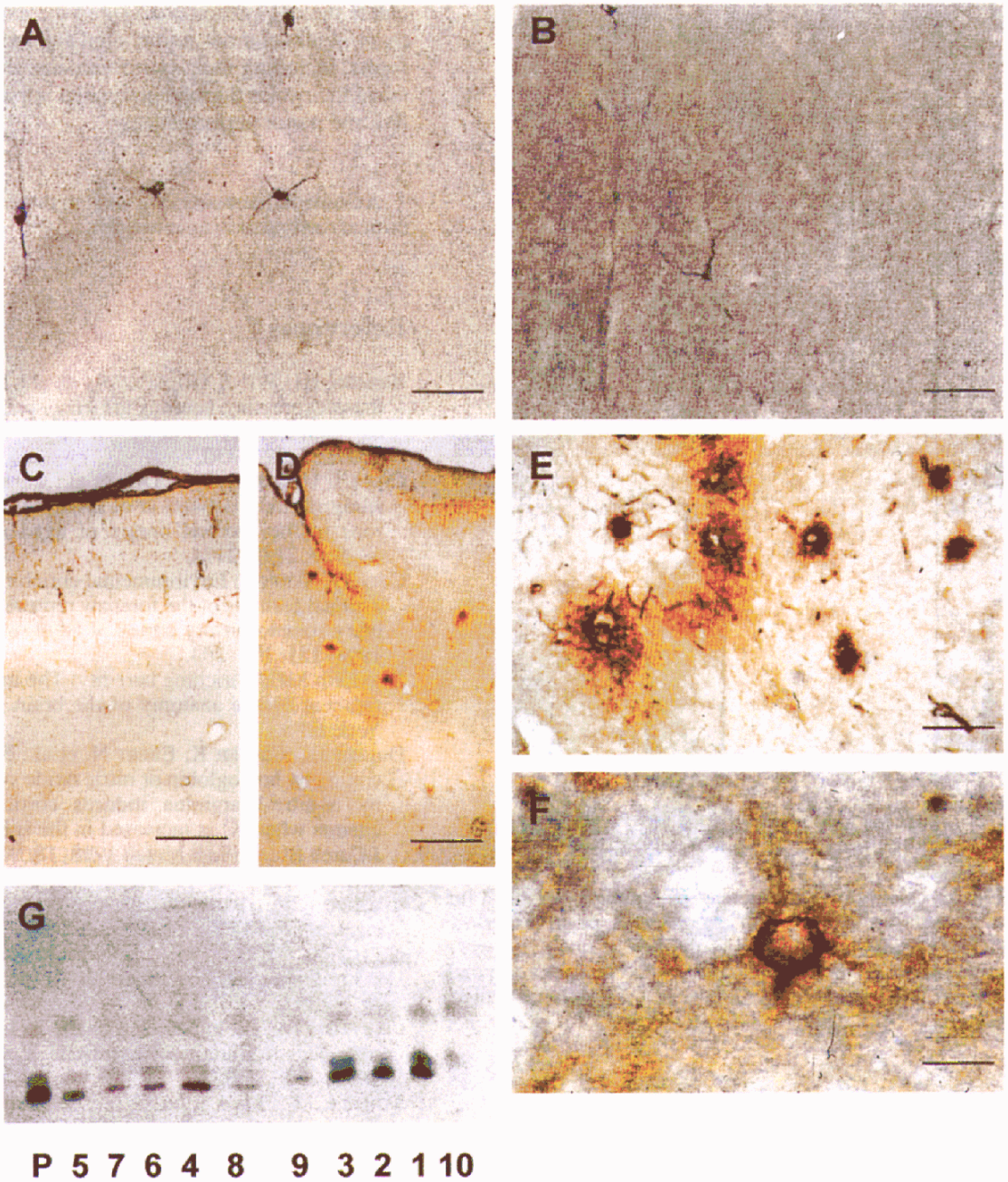

Fig. 1. Comparison of the NADPH- diaphorase-activity in cortical areas at the age of 1 year **(A)** or 3 years **(B)**. Note reduced NADPH-d-activity in **(B)**. Bars: 100 µm. Inducible NOS-II immunoreactivity at the age of 3 years is strongly increased within the cortex of rats housed under standard conditions **(D)** when compared to age-matched rats kept for 3 months (33–36 months) within an enriched environment **(C)**. Bars: 150 µm. **E:** High magnification of D showing NOS-II accumulations around cortical capillaries. Bar: 20 µm. **F:** High magnification of a cortical neuron at the age of 3 years shows reduced NOS-I immunoreactivity. Bar: 10 µm. **G:** Western blots of NOS-II and ranks of immunoreactivity, numbers 1, 2, 5, 6, 8 represents 34 months old brains, other probes of 13 months old rats, P: positive control.

caused by other compensatory mechanisms within the brain. The observed age-related upregulation of glial NOS-II expression is usually discussed as part of the age-related neuropathological cascade, which also involves reactive gliosis including disturbances of the blood brain barrier (McCann 1997). The loss of functional cerebrovascular reactivity due to the decrease of NOS-I may contribute to the vascular and perivascular degenerative changes including gliosis and glial NOS-II expression. The NOS-II activity could be reduced by

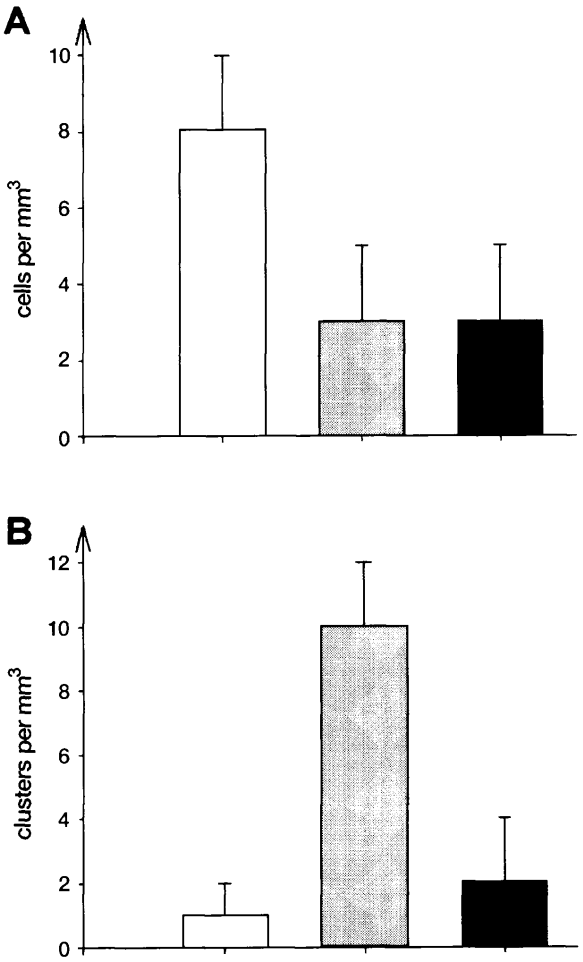

Fig. 2. Quantitative differences of numbers of NOS-I immunoreactive neurons **(A)** and NOS-II positive clusters **(B)** in cortical areas of rats at the age of one year (white column), three years (grey column) and three years spending the last three months in enriched environment (black column).

transferring the animals to an enriched environment, indicating that this type of NOS-II upregulation could be mediated or even regulated. Therefore, the question remains whether the perivascular NOS-II upregulation is the consequence of aging only or whether it is due to a mechanism by which the brain compensates for the loss of NOS-I, since the smooth muscle cells within the vascular walls may also respond to NOS-II derived NO. Of course, glial NOS-II activity alone may not be a functional regulator of cerebral blood flow, but it could contribute to a more permanent vessel dilatation in order to prevent hypoperfusion due to the loss of NOS-I derived NO. This compensatory part of the NOS-II expression would remain responsive to other functional stimuli and would become reduced, if the remaining NOS-I expressing neurons increased their NO output in response to functional demand. Further studies are warranted to elucidate the age-related changes in the expression and activity of all neuronal, endothelial and inducible NOS isoforms, including their splice variants, in order to understand their roles during age-related impairment of cognitive and motor performances.

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