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Distribution of neuronal nitric oxide synthase-immunoreactive neurons in the cerebral cortex and hippocampus during postnatal development

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Summary

Although many reports have argued a role for nitric oxide (NO) during postnatal development, there has been no combined demonstration in the cerebral cortex and hippocampus. We have investigated the distribution and morphology of neurons and fibers expressing neuronal NO synthase (nNOS) in the cerebral cortex and hippocampal formation of rats during the postnatal development, and correlated these findings with developmental events taking place in these regions. In the cerebral cortex, the nNOS-immunoreactive cells could be divided into two classes: heavily stained neurons and lightly stained neurons. For the lightly stained nNOS-positive neurons, only the cell bodies were observed, whereas for the heavily stained neurons, the cell bodies and their dendrites were visible. During the postnatal days, heavily stained neurons reached their typical morphology in the second week and appeared in all layers except for layer I. In the hippocampus, there was a transient expression of nNOS in the pyramidal cell layer at P3–P7, and this expression disappeared during following days. The adult pattern of staining developed gradually during the postnatal period. This study suggested that these alterations might reflect a region-specific role of NO and a potential developmental role in the postnatal cerebral cortex and hippocampus.

Introduction

Nitric oxide (NO) is a gaseous neurotransmitter and neuromodulator that serves multiple roles in developing and mature brains (Vincent 1994). During embryonic and postnatal development, neuronal NO synthase (nNOS) is observed in different cell groups of the central nervous system (CNS) at different times (Li *et al.* 1997); it is expressed only transiently in some brain regions (Vercelli *et al.* 1999), and there is a progressive increases in others (Vercelli & Cracco 1994). It has been proposed that NO production in developing postsynaptic neurons contributes to the establishment and refinement of afferent connections via an activity-dependent mechanism (Cramer *et al.* 1995; Cramer *et al.* 1996). NO is believed to act as a retrograde signal that stabilizes synchronously active afferent terminations (Wu *et al.* 1996). NO has been reported to have a permissive role during long-term potentiation (LTP) of synaptic connections as well as in neuronal development and plasticity (Brenman & Bredt 1997).

NO seems to serve distinct functions during different stages of the ontogenesis in the forebrain, midbrain and cerebellum of rats (Iwase *et al.* 1998), which is also reflected in nNOS expression. Most authors have described the sequential appearance and transient increase of nNOS-immunoreactive (IR) neurons in different cerebral subdivisions until birth, followed by a decrease the number of nNOS-IR neurons towards later stages. In the prenatal human hippocampal formation, positive neurons for NOS or its marker, NADPH-diaphorase (NADPH-d), were sparse in the pyramidal layer of CA1-3 region in spite of the suggested role of NO as a messenger in LTP (Yan & Ribak 1997). In contrast to the well-documented distribution of NADPH-d containing neurons in the adult rat hippocampus, there is little information concerning the developmental pattern of NOS expression on this structure.

Although many reports have argued a role for NO during postnatal development, there has been no combined demonstration in the cerebral cortex and hippocampus. In the previous studies, we demonstrated the distribution of nNOS or nitrotyrosine in the cerebral cortex (Cha *et al.* 1998, Shin *et al.* 2002) and hippocampus (Shin *et al.* 2002) of aged rats. Based on these previous studies, we have investigated the distribution and morphology of neurons and fibers expressing

nNOS during the postnatal development of the cerebral cortex and hippocampal formation of rats, and correlated these findings with developmental events taking place in these regions. For the first time, we showed postnatal alterations in the number of nNOS-IR cells and the intensity of staining in the rat cerebral cortex and hippocampus.

Materials and methods

Sprague–Dawley rats ranging in age from postnatal day 3 (defined as P3) to the adulthood (4–6 months old), obtained from our breading colony, were used for these experiments. Ages used were P3 ($n = 5$), P7 ($n = 5$), P14 ($n = 5$), P21 ($n = 5$), P28 ($n = 5$) and adulthood ($n = 5$). The rats were treated in accordance with the 'Principles of Laboratory Animal Care' (NIH publication No. 86-23, revised in 1985). The animals were anesthetized with sodium pentobarbital (50 mg/kg) and perfused transcardially with cold phosphate buffered saline (PBS, 0.02 M, pH 7.4) followed by ice-cold 4% paraformaldehyde. Brains were cryoprotected in a series of cold sucrose solutions, and were cut at 40 μm in the coronal plane. Immunohistochemistry was performed using the free-floating sections and rabbit anti- nNOS antibody (1:1000; AB1632, CHEMICON International Inc., Temecula, CA) as primary antibody (Cha *et al*. 1998, Shin *et al*. 2002).

A sample of sections was reacted without primary antiserum, and a different sample was exposed to a primary antiserum that had been preincubated for 24 h with nNOS control peptide (AG1591, CHEMICON). Sections from these samples did not exhibit any of the immunoreactivity described in this report. Sections from each group were stained together eliminating conflicts between different experimental conditions. Visual assessment and densitometric measurement using a NIH image program (Scion Image) was used to determine the staining density.

Results

In the cerebral cortex, intensely stained fibers and neurons were rare in the differentiating cortical plate at P3 (Figure 1A–C). Diffuse staining was present in the surrounding neuropil at moderate levels. Several IR neurons were found with short dendrites in lower part of cortical plate (Figure 1B and C). From P7 to P28, nNOS-IR cells could be divided into two classes: heavily stained neurons and lightly stained neurons. For the lightly stained nNOS-positive neurons, only the cell bodies were observed, whereas for the heavily stained neurons, the cell bodies and their dendrites were visible. At P7, nNOS-positive cells were found in layers II–VI as well as white matter, as small undifferentiated neurons (Figure 1D–F). Diffuse neuropil staining was also present in layers II/III and IV, but the staining density was low in the upper part of layer V and in layer VI. Lightly stained cells were still found with neuropil staining in layers II–VI. Intensely stained neurons were more numerous throughout the cortical regions than those at P3 (Figure 1D), and nNOS-IR neurons were bipolar (Figure 1E) or multipolar (Figure 1F) under high magnification.

The pattern of staining did not change significantly until P14 when it started to take on an adult-like distribution. During the following postnatal days, heavily stained neurons reached their typical morphology in the second week and appeared in all layers except for layer I (Figure 1G and H). Perikarya of these neurons could be classified as multipolar, triangular, or bipolar, and some had dendrites with a clearly vertical or horizontal orientation. Vertically oriented neurons with fusiform cell bodies were detected in layers II/III and V (Figure 1G). The horizontally oriented, intensely nNOS-positive neurons were most common in the layer II, in deep layer VI (Figure 1H) and in the white matter. Faintly stained nNOS-IR neurons having non-pyramidal shapes were observed most frequently in layers VI (Figure 1H), and these neurons were found through adulthood. Each faintly stained neuron had nNOS immunoreactivity around the nucleus and the proximal dendrites.

From P21 to adult, the prominent orientations of the processes in layers III–VI seemed to run perpendicularly to the surface (Figure 1I–K). For the lightly stained nNOS-positive neurons, only the cell bodies were observed, whereas for the heavily stained neurons, the cell bodies and their dendrites were visible. The heavily stained neurons were morphologically diverse, but no pyramidal neurons were found (Figure 1I–K). Multipolar neurons were located throughout the depth of the cortex, including the white matter. Several intensely stained cells again took on a predominantly horizontal orientation in layers II–III (Figure 1I) and at the interface between layer VI and the white matter.

In the hippocampus, there was a transient expression of nNOS in the pyramidal cell layer at P3–P7, and this expression pattern disappeared during postnatal development. We found that the adult pattern of staining developed gradually during the postnatal period. At P3, a dense and diffuse staining was found in the pyramidal layer, while few nNOS-IR cell bodies were observed in the strata oriens and radiatum (Figure 2A,B). At P7, the number of IR cells and the intensity of the reaction increased dramatically compared to P3 animals (Figure 2C–E). The CA1-3 region displayed a darkly stained pyramidal cell layer. Stratum oriens and stratum radiatum had nNOS-IR cells

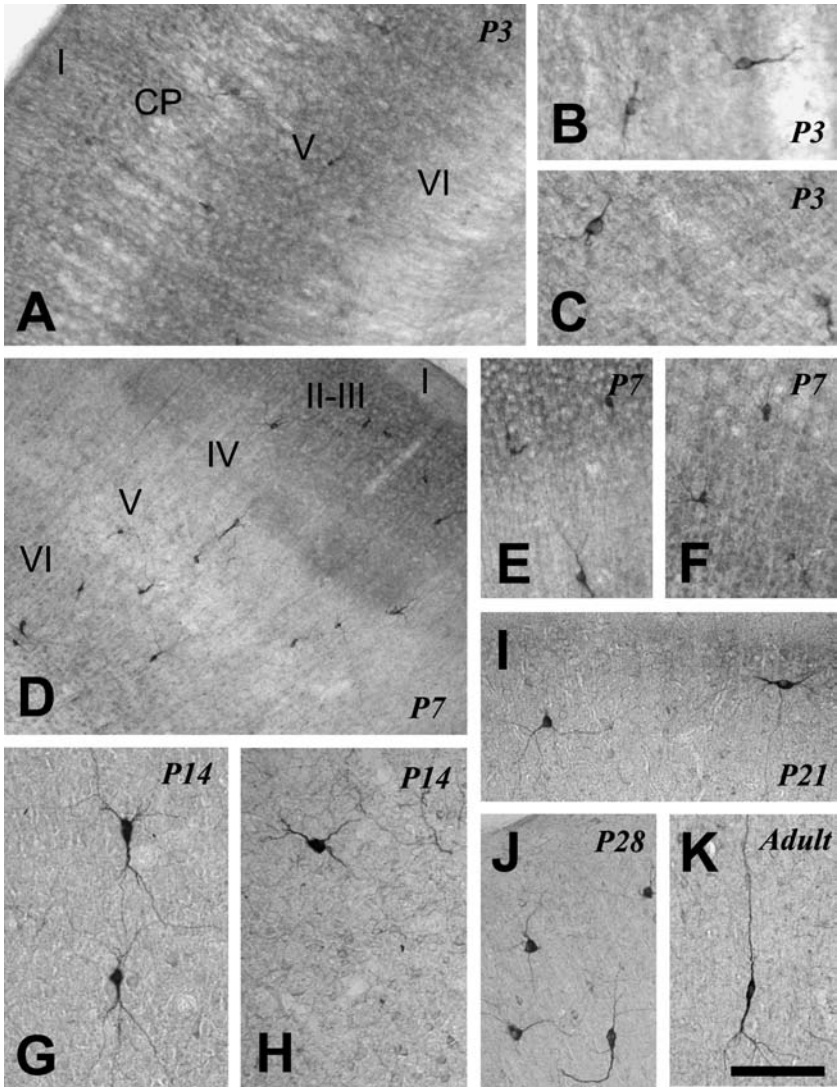

Figure 1. Localization of nNOS-IR neurons in the cerebral cortex during postnatal development. At P3, intensely stained fibers and neurons were rare, and diffuse staining was present at moderate levels (A). Several moderately stained neurons were found with short dendrites in lower part of cortex (B, C). At P7, nNOS-positive cells were found in layers II–VI as small undifferentiated neurons (D–F). Diffuse staining was also present in layers II/III and IV (D), and intensely stained multipolar or bipolar neurons were frequently observed (E, F). At P14, the pattern of staining started to take on an adult-like distribution (G, H). During following days, heavily stained neurons reached their typical morphology, which was multipolar, triangular, or bipolar with dendrites oriented vertically or horizontally (I, J). In adult rats, intensely stained neurons with the cell bodies and their long dendrites were found (K). I–VI, layer I–VI; CP, cortical plate. Scale bar: 150 μm (A, D); 30 μm (B, C); 50 μm (E–K).

with perikarya and short proximal processes in CA1-3 region (Figure 2C and D). Dentate gyrus showed an intensely stained granule cell layer, and nNOS-IR cells were scattered in the molecular layer and polymorphic layer (Figure 2C and E). In the hippocampus of P14, several darkly stained neurons can be observed with short dendrites in a region between the stratum oriens and the alveus (Figure 2F). The pyramidal cell layer showed less intense staining in CA1–3 compared to P7, and the stratum oriens and stratum radiatum had many stained cells (Figure 2F). The granule cell layer of the dentate gyrus was diffusely stained (Figure 2G), which pattern was maintained until P21 (Figure 2H and I).

The labeling pattern of P28 hippocampal formation showed similarities with the adult pattern (Figure 2J and K). The pyramidal cell layer presented less intense labeling than in the younger stages, but the number of strongly stained cells was much higher (Figure 2J). In the dentate gyrus, many labeled cells were scattered, and a diffuse and weak labeling was observed in the granule cell layer (Figure 2K). In the adult, the pyramidal cell layer exhibited weak diffuse staining throughout all regions of Amman's horn, and the stratum oriens and stratum radiatum displayed many stained cells (Figure 2L). In the dentate gyrus, the granule cell layer was diffusely stained and the lower part of molecular layer showed band-like distribution (Figure 2M).

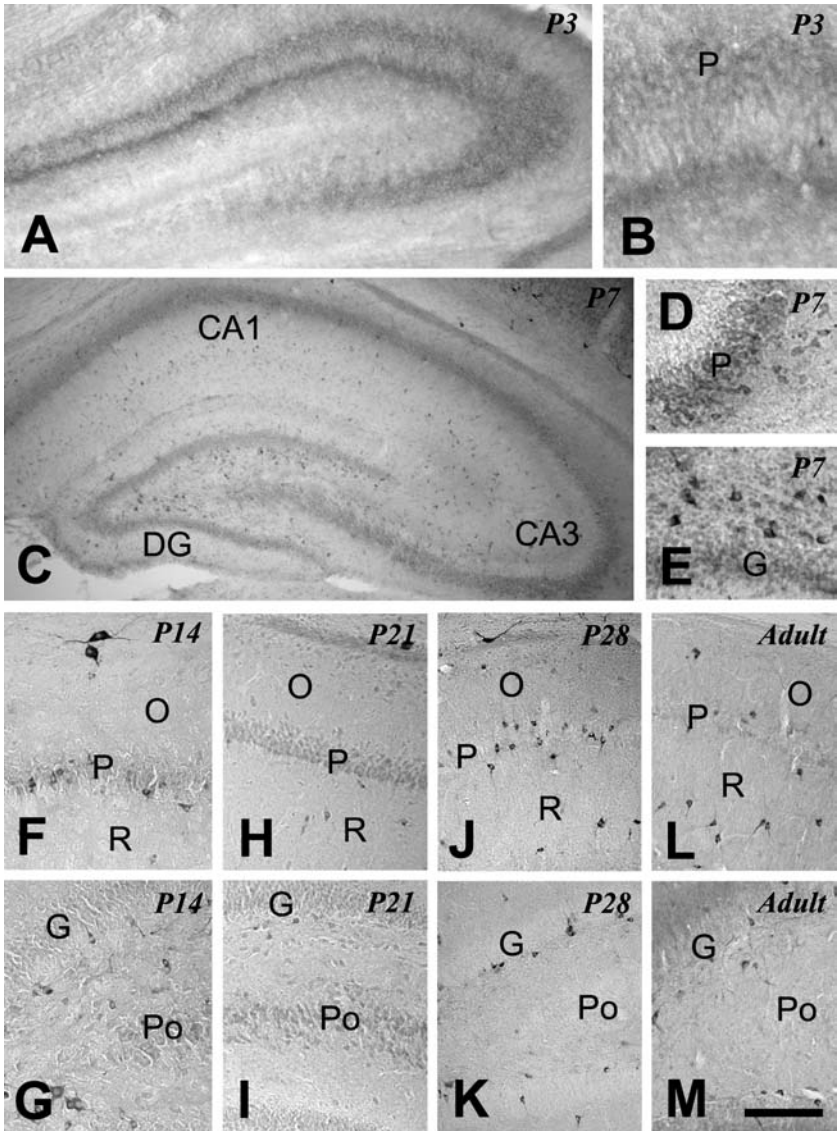

Figure 2. Localization of nNOS-IR neurons in the hippocampus during postnatal development. At P3, a dense and amorphous staining was found in the pyramidal layer (A, B). At P7, the CA1-3 region displayed a darkly stained pyramidal cell layer (C, D), and dentate gyrus showed an intensely stained granule cell layer (E). At P14, several darkly stained neurons can be observed with short dendrites in a region between the stratum oriens and the alveus (F). The pyramidal cell layer showed the diffuse type of staining, which was less intense in CA1-3 compared to P7 (F). The granule cell layer of the dentate gyrus was diffusely stained (G). The staining pattern was maintained until P21 (H, I). At P28, the pyramidal cell layer presented less intense labeling than in the younger stages (J). However, the number of cells strongly stained throughout the layers of the Ammon's horn was much higher. In the dentate gyrus, the granule cell layer presented a diffuse and weak labeling, and the molecular layer showed few labeled cells (K). The labeling pattern of adult hippocampal formation showed similarities with that of P28 (L, M). CA1-3, fields CA1-3 of Ammon's horn; DG, dentate gyrus. G, granule cell layer; O, stratum oriens; P, pyramidal cell layer; Po, polymorphic layer, R, stratum radiatum. Scale bar: 150 μm (A, C); 30 μm (B, D, E); 50 μm (F–M).

Discussion

In the present study, we demonstrated that there were various types of nNOS immunoreactivity in the postnatal rodent cortex and hippocampus. In particular, there was a transient expression of nNOS in the pyramidal cell layer of the hippocampus, and this expression pattern disappeared during postnatal development. Immunoreactivity for nNOS in the cerebral cortex and hippocampus showed three staining characteristics: diffuse staining distributed in bands overlying one or more layers; faint labeling of cells localized to specific layer; and intense labeling of several neurons. Each of these characteristics was changed region-specifically during postnatal days. Faintly stained cells were detected in the cortical regions from embryonic times (Bredt & Snyder 1994) to adulthood. The intensely stained neurons developed according to inside-out and lateral-to-medial gradients, as did the cortex in general (Erzurumlu & Jhaveri 1992). It has

been proposed that the transient elevation of NOS activity in the cortical plate coincides with the cessation of division of committed precursor cells and that it may be related to the control of cell proliferation (Peunova *et al*. 1996).

Our results on the morphology and distribution of the intensely stained nNOS-positive neurons in adult rats were in general agreement with previous reports (Cha *et al*. 1998, Vaid *et al*. 1996). In monkey cortex, the distribution pattern of nNOS-positive neurons in the neocortex was layer-specific, and nNOS-IR cells were most frequent in layers II/III and VI and at the layer VI–white matter interface (Dombrowski & Barbas 1996). In our study using rats, their distribution was similar, but nNOS-IR cells were also detected in the lower part of layer V. nNOS-IR neurons in the neocortex were reported to be involved exclusively in local circuits and, they may mediate the coupling of blood flow to neuronal activity in the cortex (Estrada & DeFelipe 1998). Thus, the heterogeneous patterns of NOS expression may reflect several different functions of NO in the adult mammalian neocortex.

NO was reported to influence the development of connections in several areas of the CNS as a retrograde messenger to stabilize synaptic connections via an activity-dependent mechanism (Williams *et al*. 1994). In the somatosensory cortex, transient NOS activity might play a role in the development of thalamocortical connections. However, NOS inhibition had no effects on the formation of ocular dominance columns in the ferret visual cortex or on barrel formation in the rodent somatosensory cortex (Finney & Shatz 1998). In the developing hippocampal formation of rats, most of the neurogenesis in Ammon's horn is over by E19 but it peaks at P7–P10 in the dentate gyrus (Bayer & Altman 1995). The low staining intensity in the dentate gyrus at P3 with high staining in the hippocampus proper may be interpreted as a reflection of the difference in neurogenesis between these two regions. In the human hippocampal formation, NADPH-d reactivity decreased during development and mature neurons displayed a faint staining for NOS (Yan & Ribak 1997). These results were in accordance with our findings in which pyramidal and granule cells exhibited high levels of nNOS expression transiently at early stages of postnatal development. Therefore, our data suggests that NO may play a region-specific role in neuronal development in the cerebral cortex and hippocampus by modulating neuronal differentiation and maturation.

This study demonstrated that nNOS was expressed at different levels of intensity and had varying distributions in the cerebral cortex and hippocampus during the postnatal development, suggesting that these alterations may reflect a role of NO in development (such as establishment of projections), in adulthood (such as a linkage between neuronal activity and blood flow), and in pathology. The mechanisms underlying the altered immunoreactivity for nNOS, and the functional implications of this change, require elucidation.

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