Development of Nitric Oxide Synthase-Immunoreactive Nerves in the Cerebral Arteries of the Rat

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(Received 6 January 2004/Accepted 23 March 2004)

ABSTRACT. Development of cerebrovascular nitrergic nerves was investigated in the rat, using immunohistochemistry for nitric oxide synthase (NOS) and quantitative analysis. Cerebral perivascular NOS nerves usually appeared on the walls of both the intracranial part of the internal carotid artery (ICA) and the internal ethmoidal arteries (IEA) at birth. NOS nerves via the IEA grew more rapidly than those via the ICA. They extended over all the major arteries located more rostral than the middle part of the basilar arteries during the third postnatal week, while those from the ICA remained limited to the caudal segment of the anterior circulation and to the rostral segment of the posterior circulation throughout development. The appearance of NOS nerves on the vertebrate artery (VA) was not demonstrated before the third postnatal week, being apparently far late in development as compared to that of the same nerve type on the ICA and IEA.

KEY WORDS: cerebrovascular innervation, development, nitrergic nerve, rat.

The cerebral arterial tree is formed by the connection of the anterior and posterior circulations that develop independently from the internal carotid and vertebral arteries (ICA, VA). It has also been established that the two cerebral arterial systems in mammals are multiply and differentially supplied by noradrenergic and cholinergic nerves [21], and by various types of peptidergic nerves [23].

Nitric oxide (NO), a highly diffusible gaseous molecule, has been identified as an intercellular chemical messenger, first in the vascular endothelium and later in the central and peripheral nervous systems [14, 16]. Neuronal NO is synthesized from L-arginine by the constitutive enzyme NO synthase (NOS), of which the biochemical property is different from another constitutive NOS produced by vascular endothelial cells. NOS also exhibits diaphorase activity in addition to the synthesis of NO. Thus, neurons synthesizing NO, nitrergic neurons, can be visualized by immunohistochemical staining with an antibody against neuronal NOS, or by histochemistry for nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd). Actually, by mean of these two staining methods, the rich supply of nerves immunoreactive for NOS and positive NADPHd has been demonstrated for the cerebral arterial systems in a variety of mammals including man [1, 4, 8, 10, 12, 15, 22]. Some of these studies, in combination with denervation and/or retrograde tracing techniques, have also provided conclusive evidence that cerebral perivascular NOS and NADPHd nerves, as well as nerves positive for acetylcholinesterase (AChE) and immunoreactive for vasoactive intestinal polypeptide (VIP), are parasympathetic in nature and have their major origin at the sphenopalatine ganglion. Pharmacological experiments have shown that NO released from cerebral perivascular nerves plays a crucial role as a neurotransmitter for non-adrenergic and non-cholinergic vasodilatation in the cerebral vessels [6, 9, 11, 19].

As to the ontogenetic study on cerebrovascular innervation, relatively much work has accumulated to determine the age-related change of aminergic, AChE and peptidergic nerves [3, 7, 5, 13, 20]. These different types of cerebrovascular nerves display their own distinct patterns of development, with a peak density at one month of age. Information on the development of cerebrovascular NOS innervation has not yet been published up to now. The present study was undertaken therefore to investigate the sequence of morphological events in the development of nitrergic innervation in the anterior and posterior circulations of the rat cerebral arterial tree, using the combination of immunohistochemistry for NOS and quantitative analysis.
MATERIALS AND METHODS

Postnatal White Wistar rats of either sex, which ranged from one day to 30 days after birth, and their mothers aged at 120 days were used in this study. The day of birth was regarded as postnatal day one (PND 1). Mother and newborn rats were bred in the same cage until PND 20, under a room environment kept at a constant temperature (23 ± 1°C) and humidity (48 ± 3%), and then housed separately. Food and water were provided ad libitum. The animals were divided by age into the following nine groups: PND1, PND3, PND5, PND8, PND10, PND15, PND22, PND30 and PND 120. For each of these groups, seven animals or more were examined for immunohistochemistry for nitric oxide synthase (NOS). All experiment procedures were conducted in accordance with the National Research Council (NRC) publication guide for care and use of Laboratory Animals (copyright 1996, National Academy of Science).

Tissue preparation: Animals were perfused through the left ventricle with cold Ringer’s solution under ethyl ether anesthesia, followed by an appropriate volume of Zamboni’s fixative. The brains were removed immediately from the skull, and then postfixed in the same fixative for 18 hr at 4°C. The cerebral arterial tree was carefully dissected away from the brain. They were washed thoroughly with cold 0.01 M phosphate-buffered saline (PBS, pH 7.2). The materials were immersed sequentially in cold 0.01 M phosphate buffer (PB, pH 7.4) containing 10% sucrose for one day each, and then stored at 4°C in PBS containing 0.3% Triton X-100 (PBST) over one night.

Immunohistochemical protocol: Whole-mount preparations of the cerebral arterial trees from each age group were processed for immunohistochemistry by avidin-biotin immunoperoxidase technique as described previously [2]. Before the primary antigen-antibody reaction to NOS, the cerebral arterial trees were treated for 1 hr at room temperature with PB containing 0.1% hydrogen peroxide to inhibit endogenous peroxidase in tissue. The vessels were incubated with biotinylated anti-rabbit IgG and the avidin-biotin—peroxidase complex (Vectastain kit, Code no. R9223; Euro-diagnostica AB, Malmo, Sweden) at a dilution of 1:800. After washing with PBST, the materials were incubated with biotinylated anti-rabbit IgG and the with avidin—biotin—peroxidase complex (Vectastain kit, Vector Lab., Burlingame, U.S.A.) for 1 hr each at room temperature. They were visualized with 3, 3’-diaminobenzidine for 5–10 min, mounted on slide glasses, and examined under a light microscope.

Quantitative analysis: Micrographs of the respective major cerebral arteries in the different postnatal stages were taken on 35 mm film (Neopan 400, PRESTO) at a magnification of × 50 and then printed at × 400. In order to make a sharp distinction of NOS immunoreactive nerves from vascular walls, the profiles of nerves were elaborately outlined using a color pen. The photomicrograph images were displayed on a video monitor at final magnification of × 400. Using an image analyzer (Win Roof) linked to NEC computer system, the vascular area was first measured in relation to a given length (0.7 mm) of the vessels. Next, the profiles of nerve fibers covering the vascular wall were delineated and their total area was determined. Finally, the density of nerves in the major cerebral arteries at each developmental stage was expressed as a percentage of the total vascular area. A mean ± S.E.M. was calculated from seven values or more per each major artery.

RESULTS

Anterior circulation: Nerves immunoreactive for NOS were consistently found in the wall of the intracranial part of the ICA at postnatal day (PND)1. These immunoreactive nerves were very small in number and often appeared as fiber bundles (Fig. 1a). In most case, the axons from here entered the circle of Willis to advance rostrally toward the anterior ramus (AR), or sometimes extended further to the middle cerebral artery (MCA). In addition, a few fiber bundles displaying a faint NOS immunoreactivity were usually present on the wall of the internal ethmoidal artery (IEA) at birth (Fig. 1b). Their sprouts occasionally entered the circle of the Willis through the rostral part of the AR.

The density and distribution of NOS nerves ascending the AR from the ICA did not change greatly during the first postnatal week (Fig. 1c-1, f). Nevertheless, fiber bundles lying on the IEA exhibited a significant increase in staining intensity on PND3 (Fig. 1d). Their axon sprouting grew progressively along the anterior cerebral artery (ACA) in the rostral direction, or toward the MCA in the caudal direction. In some individuals, nerves ascending the right and left ACA were seen to spread toward the contralateral side through the anterior communicating artery (Fig. 1e). By PND 5, NOS nerves arising from the IEA reached the caudal part of the AR to meet with nerves via the ICA (Fig. 1f, g).

At this point in development, all the major arteries of the anterior circulation were systematically supplied with NOS fibers.

At the beginning of the second postnatal week, NOS fiber bundles, which were derived from the stem nerve bundles on the ICA, became much high in staining reaction, with a significant increase in number, and began to split rapidly into thin fibers. Consequently, NOS nerves supplying the anterior circulation on PND10, particularly its rostral segment such as the IEA, ACA and rAR, became abruptly high in density, and organized into rather elongated arboid pattern that was consisted of longitudinally-oriented fiber bundles and irregularly-oriented thin varicose fibers (Fig. 2a, Table 1).

On PND 15, the density of NOS nerves in the major cerebral arteries increased further as the result of the subsequent subdivision of fiber bundles (Fig. 2b). By PND 22, plexuses of NOS nerves now changed into fine and complicated meshworks consisting of abundant, thin varicose fiber, concomitant with a decrease in number of fiber bundles and a significant increase of fiber density (Fig. 2c). The nerve plexuses on PND 30 and 120 were composed of very dense and well-organized meshworks that were basically...
similar to those seen at PND 22 (Fig. 2d). Indeed, the relative densities of nerves measured from the corresponding arteries showed no clear difference among these three ages (Table 1). The supply of nerves to the anterior circulation was much more prominent in the rostral than in the caudal segment.

In addition, a small number (two to eight) of nerve cells with clear NOS immunoreactivity were found singly or in a small cluster on the vascular wall around the ICA in each one of the young rats examined at PND 3 and 5 (Fig. 1c-1). These intrinsic NOS neurons were mostly small in size (diameter 20–30). The immunoreactive products were homogeneously distributed throughout the cytoplasm, except the nucleus, and in some cases extended in the cell
processes.

Posterior circulation: At birth, there were no nerves with detectable level of NOS immunoreactivity in this brain arterial system, except for only a few nerves that were rarely localized at the posterior ramus (PR) near the intracranial part of the ICA. On PND3, NOS nerves descending the PR from the ICA could occasionally be followed up to the level where the PR ramifies into the posterior cerebral artery (PCA) and anastomoses with the posterior communicating artery (PCOMA) (Fig. 3a). On PND5, the immunoreactive nerves, though they were still sparse, were frequently observed in the PCA. During the first postnatal week, no nerves with NOS immunoreactivity were demonstrated in the major arteries located more caudally than the PCOMA. By PND8, NOS nerves via the IEA, together with those via the ICA, descended the posterior circulation through PR. Ten days after birth, the number of nerves projecting to the PCA increased significantly (Fig. 3b). In some individuals, NOS nerves were seen to penetrate into the upper part of the basilar artery (BA) through the PCOMA. At this stage, the walls from the caudal two third of the BA to the VA were equipped with no nerves immunoreactive for NOS.

On PND15, NOS nerves descending the PR became much larger in amount, formed a coarse and elongated plexus along the PCA and the rostral part of the BA (rBA) (Fig. 3c,d). The caudal part of the BA (cBA) received a poor or no supply of nerves with NOS immunoreactivity (Fig. 3e). In the VA at this stage, extremely few immunoreactive axons, which were confined to the rostral part, were observed only in one of the eight rats examined.

Table 1. The relative density of nerves immunoreactive for NOS in the major cerebral arteries at different postnatal day (PND)

<table>
<thead>
<tr>
<th></th>
<th>PND1</th>
<th>PND3</th>
<th>PND5</th>
<th>PND8</th>
<th>PND10</th>
<th>PND15</th>
<th>PND22</th>
<th>PND30</th>
<th>PND120</th>
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<tr>
<td></td>
<td>n=12</td>
<td>n=8</td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
<td>n=12</td>
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<td>n=7</td>
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<tr>
<td>IEA</td>
<td>0.22±0.05</td>
<td>0.78±0.07</td>
<td>1.26±0.06</td>
<td>3.27±0.35</td>
<td>4.12±0.57</td>
<td>6.78±0.51</td>
<td>10.95±0.38</td>
<td>11.18±0.32</td>
<td>1.18±0.47</td>
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<tr>
<td>ACA</td>
<td>0.35±0.02</td>
<td>1.67±0.14</td>
<td>3.80±0.59</td>
<td>4.62±0.43</td>
<td>7.58±0.67</td>
<td>11.28±0.55</td>
<td>11.64±0.45</td>
<td>12.08±0.64</td>
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<tr>
<td>MCA</td>
<td>0.25±0.07</td>
<td>2.25±0.43</td>
<td>3.88±0.35</td>
<td>4.16±0.84</td>
<td>6.87±0.74</td>
<td>7.12±0.48</td>
<td>7.65±0.41</td>
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<tr>
<td>cAR</td>
<td>0.16±0.05</td>
<td>0.41±0.15</td>
<td>2.56±0.54</td>
<td>4.02±0.82</td>
<td>5.89±0.39</td>
<td>8.22±0.73</td>
<td>8.48±0.59</td>
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<tr>
<td>PCA</td>
<td>0.25±0.07</td>
<td>0.88±0.14</td>
<td>1.36±0.28</td>
<td>1.91±0.64</td>
<td>2.43±0.39</td>
<td>2.78±0.52</td>
<td>2.83±0.29</td>
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<tr>
<td>rBA</td>
<td>0.16±0.05</td>
<td>1.47±0.31</td>
<td>2.97±0.55</td>
<td>3.28±0.36</td>
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<tr>
<td>cBA</td>
<td>0.21±0.07</td>
<td>1.48±0.92</td>
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<td>3.41±0.23</td>
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<tr>
<td>VA</td>
<td>0.76±0.05</td>
<td>2.11±0.53</td>
<td>3.08±0.62</td>
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The values represent the mean (%) ± SEM. n=number of the individuals examined. IEA: internal ethmoidal artery, ACA: anterior cerebral artery, MCA: middle cerebral artery, cAR: caudal part of the anterior ramus, PCA: posterior cerebral artery, rBA: rostral part of the basilar artery, cBA: caudal part of the basilar artery, VA: vertebral artery.

Fig. 2a-d. Photomicrographs of whole mounts showing NOS nerves in the rostral segment of the anterior circulation on PND 10 (a), 15 (b), 22 (c) and 30 (d). rAR: rostral part of the anterior ramus, ACA: anterior cerebral artery, IEA: internal ethmoidal artery. × 50.
NOS nerves in the PCA increased, to some degree, on PND22. The rBA generally contained a moderate or relatively less number of nerves (Fig. 4a). The density and distribution of nerves in the cBA varied considerably among the individuals. In some cases, NOS nerves were markedly sparse, or occasionally absent from the vessel wall (Fig. 4d), while in others, a substantial amount of nerves, of which the density was slightly lower than that seen in the rBA, was arranged in a network-like fashion (Fig. 4e). At this stage, NOS nerves were mostly visualized in the VA, but were still sparse, and mainly restricted to its rostral part (Fig. 4e, f).

Although the density of NOS nerves did not change much in the PCA and the rBA during the process of aging from PND 22 to 120 (Fig. 4b,c, Table), it tended to increase gradually in the cBA and the VA with age. Thus, the cBA on PND 30 usually had a moderate number of nerves, and rather less density was observed in the VA from most of the individuals (Fig. 4g). The same arterial regions in many of four month-aged rats were almost entirely covered by NOS nerves that were somewhat much larger in amount than those at PND30 (Fig. 4h,i). All through the postnatal stages, there was consistently more predominant supply of NOS nerves to the anterior than to the posterior circulation.

DISCUSSION

The present study is the first description of the age-related change in the cerebrovascular nitricergic innervation of the anterior and posterior circulations during postnatal development of the rat. The appearance of NOS nerves on both the ICA and IEA in almost all of newborn rats (PND1) indicates that cerebral perivascular nitricergic nerves usually enter the cranial cavity along these two vascular routes before birth.
The subsequent growth of NOS nerves arising from the ICA mostly remains limited to the level of the MCA in the anterior circulation, and does not extend as far as the BA in the posterior circulation. This finding is in good agreement with the developmental pattern of cerebral perivascular AChE and VIP nerves from the same vascular route [3]. Since similar pattern of VIP and AChE neuronal projection to the two cerebral arterial systems from the otic ganglion and the internal carotid mini ganglia via the ICA has been delineated in the adult rat [17, 18], these two parasympa-
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thetic ganglia may also contribute to the cerebrovascular NOS innervation through this neuronal pathway. Indeed, the observation that some of the neurons in the otic ganglion, which are labeled with the application of a retrograde axonal tracer, True Blue, to the MCA, are also immunoreactive for NOS has recently been reported for the rat [8]. Accordingly, a few NOS nerve cells, which were localized in the ICA and its neighborhood of each one individual at PND 3 and 5, may be interpreted as being an accidental outflow tract from these cranial facial ganglia.

NOS nerves via the IEA, unlike those via the ICA, grow rapidly after PND 5. At the beginning of the second postnatal week, cerebral perivascular NOS fiber system starts to reorganize abruptly into arborized pattern toward the mature form of innervation, with a marked increase of thin varicose fibers. This developmental stage agrees well with a specific time when cerebrovascular sympathetic, parasympathetic and sensory innervations are improved dramatically [3, 13, 20]. In adult rat, it has been corroborated that NOS neurons in the sphenopalatine ganglion path through the IEA, and then spread widely over the all major arteries more rostral than the middle BA [12, 15]. The present study has accumulated an additional information on this cerebrovascular nitrergic pathway by revealing that such wide distribution of NOS nerves over both the anterior and posterior circulations via the IEA is accomplished at the beginning of the third postnatal week.

We failed to demonstrate the presence of nerves with NOS immunoreactivity on the walls of the cBA and the VA before PND15. Even on the PND22, the immunoreactive nerves are still sporadic in the VA and mostly lack in its caudal half. Thus, the appearance of NOS nerves on the VA is distinctly far late in development as compared to that of the same nerve type on the intracranial part of the ICA and the IEA. It also lags apparently behind the time of appearance for AChE and VIP nerves via the VA that has been ascertained at PND3 [3].

In rat, cerebral perivascular VIP nerves attain a peak density at one month of age that is maintained until the fourth month [13]. This seems to hold in the case of NOS nervous system in the present study. Namely, the innervation is not fully developed until one month of age, owing to insufficient or no supply of nerves to the cBA and the VA. There is no appreciable difference in the fiber density between the corresponding arteries at one and four month-aged rats, except for a rather larger amount of nerves in the cBA and the VA of the latter age. In the cerebrovascular VIP innervation, it has also been pointed out that the nerve density declines substantially at 8 month, but is almost recovered at 27 month of age [13]. It remains to be elucidated whether such significant decrease and increase of the innervation also occur in the cerebrovascular NOS fiber system of older rats.

In conclusion, the present immunohistochemical study has provided an anatomical base that NOS nerves projecting to the cerebral arterial tree from the ICA and IEA and possible from the VA develop with their own characteristic sequence. It is a matter of interest as to which factors and mechanisms are responsible for the difference in the growth rate and density of this population of cerebrovascular nerves from different vascular routes, and lead to changes in the innervation pattern relevant to the respective stages of development.

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