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NITRIC OXIDE-PRODUCING NEURONS IN THE AMYGDALOID NUCLEAR COMPLEX OF RAT. AN IMMUNOHISTOCHEMICAL STUDY

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Abstract

The nitric oxide-producing neurons in rat amygdala (Am) were studied by means of immunohistochemical technique for detection of the specific synthetic enzyme nitric oxide synthase (NOS). Almost all nuclei of the Am contained NOS-positive neurons and fibres, but their density, somatodendritic morphology and the intensity of staining of different subpopulations varied. The strongly stained cells displayed labelling of the dendritic tree with Golgi impregnation-like quality, whilst the processes of the lightly stained neurons were less successfully followed. Numerous strongly stained neurons were present in the external capsule and in the intraamygdaloid fibre bundles. A large number of small, strongly stained cells was present in the amygdalostriatal transition area. Condensation of deeply stained cells occurred in the lateral Am nucleus. In the basolateral nucleus, the strongly NOS-positive neurons were few, and were located along the lateral border of the nucleus. These cells clearly differed from the large efferent, pyramidal neurons of the basolateral nucleus. The basomedial nucleus contained numerous positive cells, but most of them were only lightly labelled. A moderate number of strongly stained neurons appeared in the medial division of the central Am nucleus, and a larger accumulation of positive cells was present in the lateral and the capsular divisions. The medial Am nucleus contained numerous moderately stained small neurons and displayed the strongest diffuse neuropil staining. The anterior Am area contained numerous NOS-positive neurons; in its dorsal part the cells were moderately

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stained, whereas in the ventral part the neurons were strongly positive. The intercalated Am nucleus lacked NOS-positive neurons, but an appreciable plexus of fine tortuous axons was present.

Key words: basal forebrain, gaseous neurotransmission, limbic system, NADPH-diaphorase, nitric oxide synthase

Introduction. Nitric oxide (NO) is a gaseous neurotransmitter, apparently released as soon as synthesized (broadly reviewed in [1]). As a freely diffusible molecule, NO does not require a synaptic contact to exert its effects [1,2], although more recent evidence suggests that it may act preferentially at the synapse [3]. The formation of NO is carried out by nitric oxide synthase (NOS) [1,2]. Reduced nicotinamide adenosine dinucleotide phosphate diaphorase (NADPH-d) was introduced as histochemical marker that stains certain neurons with a Golgi impregnation-like quality ([4] and references therein). Following the discovery that neuronal NOS (NOS-I) is an NADPH-d [5] both reinterpretations of the already published neuroanatomical and neuropathological data, and numerous new findings appeared rapidly (see [6,7] for comprehensive reviews). Mappings of the CNS distribution of NADPH-d/NOS consistently show that this enzyme appears in groups of neurons that cannot be defined by other anatomical criteria (perikaryal size, somatodendritic morphology, neuronal connectivity, etc.). NOS-positive neurons are to be found throughout the neuraxis, from the cerebral cortex to the spinal cord, as well as in the sensory and autonomic ganglia. They might be robust projection cells, and small local circuit neurons (broadly reviewed in [7]).

The amygdaloid nuclear complex (Am) is a highly heterogeneous structure and consists of several nuclei, divided on the basis of cytoarchitectonic, hodological, histochemical, and immunohistochemical studies (reviewed in [8]). Am has diverse afferent and efferent connections throughout the CNS (reviewed in [9,10]). It is involved in the modulation of neuroendocrine functions, visceral effector mechanisms, and in complex patterns of behaviour: learning and memory, aggression and defence, pain modulation, reproduction, food intake, etc. ([10,11] and references therein).

Mappings of NADPH-d/NOS noticed positive neurons in Am, and several investigations were focused on Am: in the echidna [12], in the mouse [13], in the rat [7,14], in the sheep [15], in the dog [16], in the monkey [17], and in the human [18]. Most, if not all, of the studies quoted above preferred the simple, reliable, and effective NADPH-d histochemical technique instead of the laborious and expensive NOS immunocytochemistry. Recently, we studied the NOS-positive neuronal populations in the brainstem, associated with autonomic functions [19], utilizing both techniques, and some subtle differences were noticed. Thus, it appeared reasonable to us to reinvestigate the nitrergic neurons in Am by means of immunocytochemistry, and to compare them with our data obtained by means of the NADPH-d histochemical technique [7], and other data available.

Material and methods. Five young-adult male Wistar rats, weighing 260–300 g, were used. The experiments were carried out following the Bulgarian, Russian and German regulations on animal welfare in conformance to the National Institutes of Health Guidelines. Under deep ether anaesthesia, the animals were perfused transcardially with 150 ml 0.05 M phosphate buffered saline (PBS), followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. Two hours after the end of the perfusion, the brains were removed and stored in the fixative solution at 4 °C. The brains were blocked in the coronal plane and were immersed in 0.1 M PB containing 20% sucrose for 24 h. Serial 30 μm thick sections were cut on a Reichert Jung freezing microtome and were collected in PB. The immunohistochemical staining procedure was performed on free floating sections according to the ABC (avidin-biotin-horseradish peroxidase) method. Briefly, the sections were washed in PBS/0.3% Triton X 100 (Fluka, Buchs, Switzerland) and the background was blocked with 10% normal rabbit serum (NRS) in PBS for 1 h. The sections were incubated in monoclonal mouse anti-NOS primary antibody (Sigma, St. Louis, MO, USA) in concentration 1:1000 diluted in PBS/1% NRS for 24 h. After washing in PBS/Triton X 100 the sections were transferred in rabbit anti-mouse IgG (Sigma, St. Louis, MO, USA), 1:250 for 90 min. Following washing in PBS/Triton X 100, incubation in ABC complex (Vector Lab, Burlingame, CA, USA) was realized for 90 min. After rinsing in PBS and in 0.05 M Tris/HCl, the reaction product was visualized under microscopic control by SG (Vector) and DAB (Sigma) chromogenes for 5 min at room temperature. Control sections were processed by omission of the primary antibody and the results were negative. The sections were mounted on chrome-gelatin-coated slides, air dried, dehydrated in 100% ethanol, cleared in xylene and embedded in Entellan (Merck, Darmstadt, Germany). Every third from the NOS-immunostained sections was counterstained with 1% neutral red to enhance cytoarchitectonic orientation, and to compare the NOS-positive and negative neuronal populations. In addition, adjacent sections were stained with cresyl violet. The sections were observed with Zeiss Axioplan 2 microscope. Photomicrographs of selected fields were taken with a digital camera AxioCam MRc and saved in TIF format.

Results. The reaction product by the immunocytochemical demonstration of NOS is intensively blue, darker by the development with DAB, enhanced with nickel ammonium sulphate. The perikarya of the nitrergic neurons are filled with reaction product, except for the cell nucleus. Especially by the strongly immunostained neurons, the reaction product extends also in the dendrites, and they might be followed to distal arborizations. The nitrergic axons are also positive, so that the stem axons might be followed to the finest terminal arborizations. NOS-positive cells and fibres were present in almost all subdivisions of Am, as well as some parts of bed nucleus of stria terminalis (BST), but their number, cytological characteristics and intensity of staining varied significantly.

Most conspicuous were the so-called "border cells" located in the external capsule, separating Am from more superficial regions (cerebral cortex, claustrum

and endopiriform nuclei), as well as within the thin white matter laminae that separates individual nuclei of Am (Fig. 1*A*,*B*). The perikarya were medium-sized, elongated or multipolar. The dendrites were long, straight and branched infrequently.

A large condensation of NOS-positive neurons was observed in the amygdalostriatal transition area (Astr), wedged between the lateral (La) and basolateral (BL) nuclei laterally, and central lateral nucleus (CeL) medially (Fig. 1*C*). The cells were generally small, round or oval and always deeply stained. Often the slender, sparsely branched dendrites could be followed for a considerable distance. Apparently dendrites of these cells radiate in all directions because many stained dendritic fragments were present in the neuropil.

In the Am "proper" (Am excluding the transitional areas and the intraamygdaloid portion of BST), strongly stained and most densely arranged neurons were encountered in La (Fig. 1*D*). Weakly immunoreactive cells were rare in this nucleus. The neurons were small to medium-sized. The perikarya were oval and elongated and gave rise to wavy dendrites that could only rarely be followed for a long distance. Fragments of stained dendrites were common. The neuropil displayed a substantial number of stained axons that sometimes formed loose bundles.

BL contained a small number of NOS-positive neurons that were, however, always strongly stained. The great majority of them were located in the peripheral portions of BL close to the external capsule. Thus, in most cases it was difficult to evaluate whether this is a "true" BL neuron or a border cell as shown in Fig. 1*A*. Only occasional NOS-positive neurons were present in the central portion of the nucleus (Fig. 1*E*). The nitrergic cells were smaller than the majority of BL neurons that are the largest neurons in Am. The slender, wavy dendrites rapidly disappeared from the plane of the section. Probably they are considerably long because, contrary to the paucity of stained perikarya, numerous NOS-positive dendritic fragments appeared in the neuropil.

In the basomedial Am nucleus (BM) numerous NOS-positive neurons were present, regularly dispersed throughout the nucleus, but only few of them were deeply stained (Fig. 1*F*). Such neurons had an oval or elliptical perikaryon with several dendrites. The majority of the labelled perikarya displayed a pale staining of the thin cytoplasmic rim surrounding the unstained nucleus. The dendrites could usually be followed for a short distance. The neuropil contained a dense network of thin NOS-positive axons, as well as a diffuse staining.

All three divisions of the central Am nucleus (Ce), medial (CeM), lateral (CeL), and capsular (CeC), contained a moderate number of NOS-positive neurons. In CeM the labelled cells were medium-sized, multipolar, with several thin, long dendrites (Fig. 2*A*). Only few labelled thin axons were observed in the neuropil. A somewhat larger number of NOS-positive neurons were found in CeL. The cells were irregularly distributed; in some sections only occasional neurons

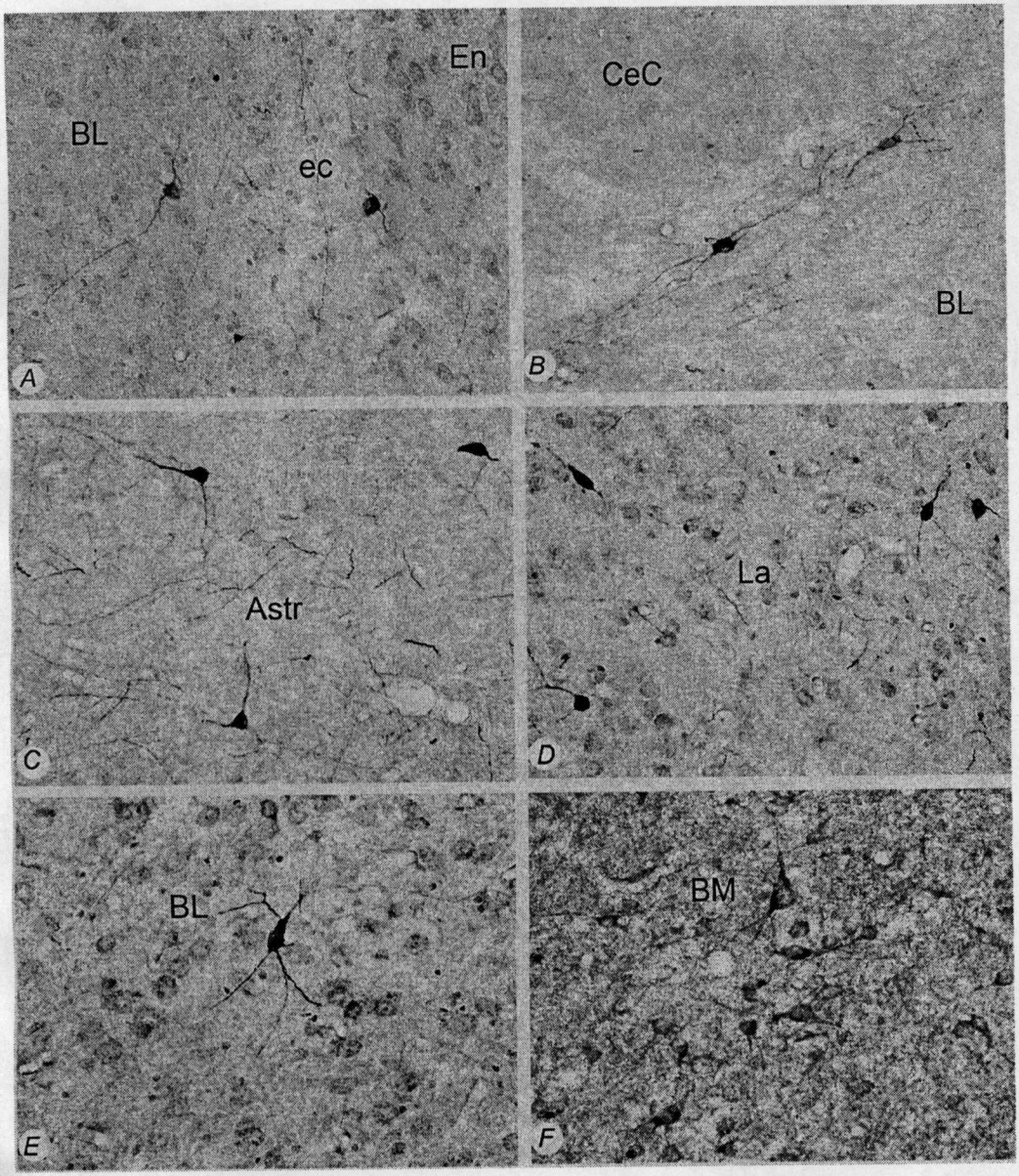

Fig. 1. NOS-immunoreactive neurons in the amygdaloid complex. *A*) Two border cells. To the left, a NOS-positive neuron in the most lateral part of the basolateral nucleus (BL). To the right – a similar neuron in the most medial part of the endopirifom nucleus (En). Vertically oriented NOS-positive processes in the external capsule (ec); *B*) Two typical border cells within a fibre lamina between the central capsular nucleus (CeC) and BL; *C*) Labelled neurons within the amygdalostriatal transitional area (Astr) with numerous labelled dendritic fragments in the neuropil; *D*) Strongly immunoreactive neurons in the lateral amygdaloid nucleus (La); *E*) Deeply stained multipolar neuron in the central portion of BL. Such neurons are very rare; *F*) Numerous NOS-positive neurons in the basomedial nucleus (BM). All magnifications × 372.5

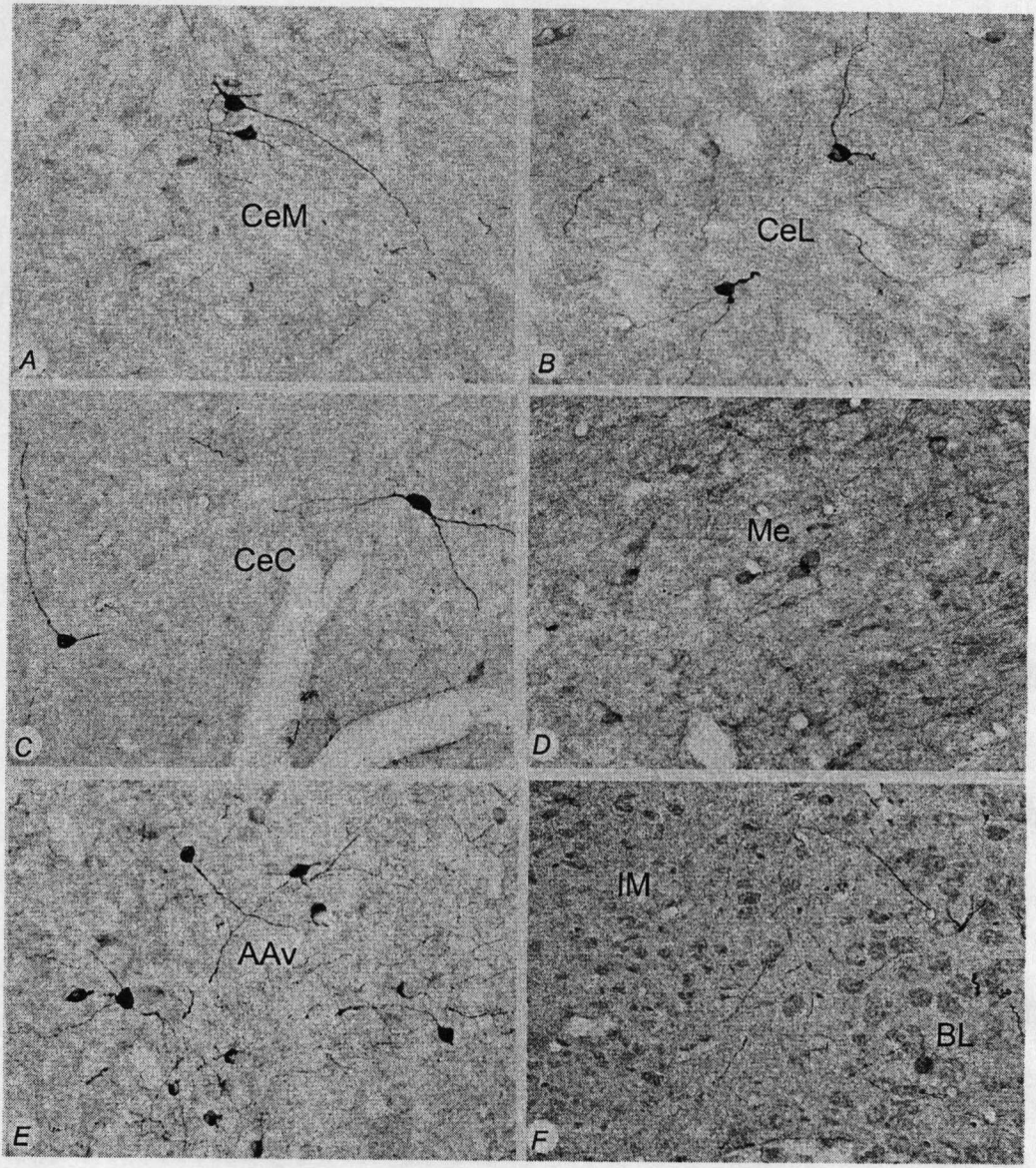

Fig. 2. NOS-immunoreactive neurons in the amygdaloid complex. *A*) Two adjacent strongly stained neurons in the central medial nucleus (CeM); *B*) NOS-positive cells in the central lateral nucleus (CeL)] *C*) Neurons with distinctly labelled dendrites in the central capsular nucleus (CeC); *D*) Numerous moderately stained neurons in the medial amygdaloid nucleus (Me). Strong diffuse neuropil labelling; *E*) A group of small, deeply stained neurons in the ventral part of the anterior amygdaloid area (AAv); *F*) The intercalated masses (IM) contain no NOS-immunoreactive perikarya, but nitrergic processes from adjacent regions invade the IM. All magnifications × 372.5

were labelled, whereas in other sections the NOS-positive neurons appeared in groups. The round or oval perikarya were small to medium-sized, and deeply stained (Fig. 2*B*). The dendrites were thin and with a wavy course. The neuropil contained a substantial number of fine varicose axons. Compared to the low density of the neuronal population in CeC, the number of the NOS-positive cells appeared not to be small (Fig. 2*C*). The small to medium-sized neurons were deeply stained. The perikarya emitted two to several dendrites. The secondary dendrites were thin, long, and branched infrequently. The neuropil contained a plexus of fine tortuous axons.

Numerous NOS-positive cells were observed in the medial Am nucleus (Me), especially at the border between the dorsal and ventral divisions of the nucleus. The cells were small, with round perikarya. Some neurons were strongly stained but the majority of cells displayed a moderate staining of the scant cytoplasm, and only the proximal portions of the primary dendrites were visualized. In the deep layer of Me, the strongest diffuse staining of the neuropil was present (Fig. 2*D*).

In the anterior Am area (AA) an appreciable number of NOS-positive neurons were encountered. In the dorsal part of AA the labelled population was heterogeneous: medium-sized to small, moderately stained multipolar and oval perikarya. The larger neurons had distinct primary dendritic trunks and thin, long, sparsely branched secondary dendrites. The NOS-labelled neurons in the ventral part of AA (AAv) were small and strongly stained. The dendrites of these cells were often varicose and wavy (Fig. 2*E*).

The intercalated Am nucleus (intercalated cell masses, "massa intercalata", IM) contained the smallest neurons in Am. IM contained no NOS-stained neurons but in the neuropil a plexus of labelled thin, varicose axons was seen (Fig. 2*F*). In addition, dendrites of adjacent cells in CeC were observed to invade IM.

Discussion. We here present a comprehensive description of nitric oxide producing-neurons in the nuclei of Am by means of the most unequivocal method, e.g. the immunohistochemical visualization of NOS. The results are nearly identical with our previous study [7] in which the mapping of nitrergic Am neurons was carried out by NADPH-d technique. The only more significant difference is that the diffuse neuropil staining in Me is less pronounced by the immunostaining than by the NADPH-d histochemical technique (compare Fig. 2*D* in the present report with Fig. 4*a*,*b* in [7]).

Most characteristic are the border cells. They are invariably strongly stained, indicating a significant production of nitric oxide. These cells are medium-sized, with several very long, sparsely branched, and spine-free dendrites. The border cells were first thoroughly described by McDonald et al. [14] and they noticed that the dendrites of the border cells invade both adjacent regions. The plottings of Pitkänen and Amaral [17] suggest that border cells are also common in the monkey. The transmitter characteristics and connections of the border neurons remain to be elucidated. The somatodendritic morphology of these cells resembles

the isodendritic neurons of the brainstem reticular formation. For decades LEONTOVICH [18] insists that reticular neurons are also present in the basal forebrain, and recently she described such neurons also in Am (see Fig. 5*a* in [18]).

A large density of perikaryal and axonal NOS staining was observed in the Astr. The NOS-positive neurons are small with divergently radiating dendrites, so that often the perikarya appear as "unipolar". When compared to the adjacent striatum, some of the NOS-stained striatal neurons are larger and regularly emit several strongly stained dendrites. In this structure the NOS-positive cells are the third type of aspiny interneurons that also contain several peptides (reviewed in [7]). In all probability, the NOS-positive cells in the Astr are local circuit neurons as well. Moreover, they are most typical non-pyramidal cells, as defined by McDonald et al. [14].

Several studies agree that La contains the highest number of strongly stained nitrergic neurons. It appears that this finding is common throughout the phylogenetic scale, since it was reported in echidna [12], mouse [13], rat [7,14] and the present study, sheep [15], dog [16], monkey [17], and human [18]. The cells observed by us were generally small and oval and, obviously belong to the group of non-pyramidal neurons of the basolateral nuclear complex, as defined in [14]. Probably these cells are GABAergic local circuit neurons that also contain calcium-binding proteins (reviewed in [7]). The production of nitric oxide in La appears to be higher in primates. Similar to McDonald et al. [14] we observed only a population of deeply stained neurons. However, in monkey La a significant number of weakly positive neurons is present as well [17].

In the BL a significant number of NADPH-d-positive cells were reported in the monkey [17], most of which are moderately stained. Similarly, ASHWELL et al. [12] described abundant lightly stained neurons in the BL of the echidna. In the rat both the number of NOS-positive neurons and the intensity of staining are different. We observed a limited number of positive neurons but all of them were deeply stained. Only very few NOS-positive cells were seen in the central part of BL, whereas most of the stained neurons were observed in the most peripheral portions of BL, close to the border cells.

McDonald et al. [14] plotted only few strongly labelled cells in BM. We observed a moderate number of deeply stained neurons. However, we observed numerous lightly stained small cells, too. There was also a diffuse neuropil labelling, although not so strong as in Me (see below).

We observed a moderate number of densely stained small to medium-sized neurons in CeM. According to McDonald et al. [14], only the initial dendritic segments were clearly visualized. However, in fortunate cases (Fig. 2*A*) we were able to follow the dendrites of CeM neurons for a long distance. McDonald et al. [14] suggest that the NADPH-d-positive neurons of CM might be cholinergic. We accept this proposal with some caution since the data available are extremely controversial (see [7,20] for extensive review and discussion). We encountered an

appreciable number of NOS-containing neurons and axonal plexus in CeL and CeC. Interestingly, Pitkänen and Amaral [17] reported that CeL of *Macaca fascicularis* is almost free of positive cells and fibres, and their statement was also confirmed by other studies on the primate Am (reviewed in [7]). This species difference is difficult to be explained. Several studies agree that CeL is closely related to the ventral putamen (reviewed in [8]), where numerous nitrergic neurons are present.

Similar to several previous studies, including ours [7], we found a large number of NOS-positive neurons in Me. Interestingly, these small cells are efferent – their axons run over the entire course of stria terminalis [7]. In Me the strongest diffuse neuropil labelling was noticed in NADPH-d investigations, and it was also observed in the present immunohistochemical study, although somewhat less prominent than in the diaphorase experiments. The nature of this strange phenomenon including electron microscopic observations [3] is a matter of debate, which is beyond the scope of the present study.

AA attracted very little attention in previous studies. The drawings of Pitkänen and Amaral [17] suggest that the monkey AA contains predominantly lightly stained cells, whereas our findings (Fig. 2*E*) demonstrate strong immunopositive neurons.

By IM there is a significant species difference. Pitkänen and Amaral [17] documented a large number of type 3 (lightly labelled) neurons in the monkey IM. Similar to our previous study [7] we never observed a nitrergic perikaryon on IM territory, but a plexus of tortuous fine axons and "invading dendrites" of cells in the neighbouring nuclei were invariably present.

In conclusion, many nuclei of Am in the rat contain NOS-producing neurons, but their number, somatodendritic morphology and the intensity of immunostaining of different subpopulation vary. Evidence is accumulating that nitrergic neurons of Am are profoundly involved in physiology and pathology of this key structure of the limbic system [7], such as normal aging, nociception, Alzheimer's disease and other neurodegenerative diseases, as well as epilepsy.

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