Loss of NADPH Diaphorase-Positive Neurons in the Hippocampal Formation of Chronic Pilocarpine-Epileptic Rats

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ABSTRACT: Recent evidence suggests an important role for NO in cholinergic models of epilepsy. Nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd), a marker of NO containing neurons, was shown to intensely colocalize with GABA in double-labeling studies performed in the hippocampal formation (exception made for the pyramidal cell layer) (Valtschanoff et al., J Comp Neurol 1993:331:111–121). In this sense, it further characterizes an extremely important cell category due to the relevant involvement of inhibitory systems in the mechanisms of genesis and propagation of seizures. Here, we assessed the histochemistry for NADPHd in the hippocampal complex of chronic pilocarpine-epileptic animals. NADPHd-positive cells were lost in almost every hippocampal subfield in pilocarpine-treated rats. The central portion of the polymorphic layer of the dentate gyrus (hilus) presented one of the highest losses of NADPHd-positive cells (55–79%) in the hippocampus. A significant loss of NADPHd-positive cells was seen in *strata oriens, pyramidale,* and *radiatum* CA1, CA2, and CA3 subfields. NADPHd staining in the subicular pyramidal cell layer was not different from that observed in controls. A significant loss of NADPHd-stained cells was observed in entorhinal cortex layers II and III in the epileptic group. For entorhinal cortex layers V and VI, however, results varied from an almost complete tissue destruction to an overexpression of NADPHd-positive cells, as well as an increase in neuropil staining.

In summary, loss of NADPHd staining was not uniform throughout the hippocampal formation. There has been a growing support for the notion that GABAergic neurons in the hippocampal formation are not equally sensitive to insults. Our results suggest that, as a marker for a subpopulation of GABAergic neurons, NADPHd helps in further refining the characterization of the different neuronal populations sensitive to epileptic activity. *Hippocampus 1999;9:303–313.*

KEY WORDS: nitric oxide; GABAergic; epilepsy; hippocampus; entorhinal cortex

INTRODUCTION

Nitric oxide (NO) has recently been suggested to play important roles in the physiology and pathology of the nervous system. Substantial knowledge regarding cerebral NO distribution has emerged from the characterization of nitric oxide synthases (NOS). NOS have been purified from brain (nNOS) (Bredt et al., 1991; Mayer et al., 1990, 1991; Schmidt et al., 1991, 1992a,b; Schmidt and Murad, 1991; Vincent and Kimura, 1992), macrophages (Hevel et al., 1991; Stuehr et al., 1991), and endothelial cells (eNOS) (Pollock et al., 1991). In the brain, the histochemical method is based on the conversion of a tetrazolium salt into a visible formazan by the reduced cofactor nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd). NADPHd has been reported to colocalize with NOS (Bredt et al., 1990; Dawson et al., 1991, 1992; Schmidt et al., 1992a). Immunocytochemical studies have indicated that most NADPHd-positive neurons contain GABA in the hippocampus (Valtschanoff et al., 1993). Moreover, NADPHd has been reported to colocalize with neuropeptide-Y and somatostatin (Vincent et al., 1983). Damage to GABAergic neurons in epilepsy has been a contentious issue (Ribak et al., 1979; Babb et al., 1989), but recent studies suggest that specific subpopulations of GABAergic neurons might be damaged in epilepsy (Sperk et al., 1992; Obenaus et al., 1993; Houser and Esclapez, 1996).

In addition to the inhibitory mechanisms, excitatory aminoacids, mostly glutamate, have also been postulated to play a crucial role in the initiation and propagation of seizures (Fisher, 1991). The stimulating actions of glutamate-mediated epileptiform activity might occur via the NMDA receptor subtype, with a subsequent activation of NO (Snyder and Bredt, 1991). Moreover, NO produced in the vascular endothelium might be important for the regulation of blood flow associated with the increased metabolic demand observed during epileptic seizures (Penix et al., 1994). In spite of these facts, controversy has been reported concerning the experimental findings relating NO and animal models of epilepsy (Penix et al., 1994), particularly the cholinergic ones (Bagetta et al., 1992; Starr and Starr, 1993; Penix et

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al., 1994). Inasmuch as N^{W}-nitro-L-arginine methyl ester (L-NAME), a NO antagonist, has been suggested as a proconvulsant when injected in conjunction with low doses of pilocarpine (Starr and Starr, 1993), other studies found that systemic injections of this same agent had no effect on the latency for the onset of pilocarpine-induced seizures, although they markedly increased mortality (Penix et al., 1994). The pilocarpine (PILO) model of epilepsy is of particular interest as it follows a sequence of chronological events, from an acute episode of status epilepticus (SE), through a latent period to the development of spontaneous recurrent seizures that mimic those observed in human temporal lobe epilepsy (TLE) (Leite et al., 1990).

Despite much work relating NO and epilepsy, histochemical and immunohistochemical data are still lacking. Due to the similarities described above between the pilocarpine model and human TLE and the importance of inhibitory mechanisms to epileptogenesis, we have decided to investigate the histochemical expression of NADPHd, which might serve as a marker for subpopulations of GABAergic neurons in the hippocampal formation of chronic pilocarpine-epileptic rats.

MATERIALS AND METHODS

Pilocarpine Injections

Adult male Wistar EPM-1 rats (200–250 g) were housed in groups of 6 to 7, on a standard light-dark cycle, and fed with rat chow pellets and tap water ad libitum. Animals were injected with methyl-scopolamine (1 mg/kg, i.p.), followed 30 min later by pilocarpine (320–350 mg/kg, i.p.). Approximately 30 min after PILO injections, most animals had developed SE, which lasted for 5–24 h. To diminish the otherwise high mortality rates particularly associated with tonic seizures, thionembutal (25 mg/kg, i.p.) was injected 90 min after SE onset. Every animal undergoing SE was given oral saline and sucrose for the next 2–3 days. Both naive (saline injected, n = 2) and pilocarpine-treated animals that did not develop SE (n = 4) were used as controls. Previous studies (Mello et al., 1993) and current preliminary results have shown that the latter group did not differ from the former regarding NADPHd staining, electroencephalographic as well as behavioral features.

Starting the day after PILO injections, animals were observed for approximately 3–6 h/day, 5 days/week for the occurrence of partial and generalized spontaneous recurrent seizures (SRS, similar to kindled stages III, IV, and V seizures) (Racine, 1972).

Histochemistry for NADPHd

Within 150 and 275 days after SE, six epileptic and six control rats were deeply anesthetized with 10% chloral hydrate (400 mg/kg, i.p.) and transcardiacally perfused with 0.9% saline, followed by a fixative solution containing 2% glutaraldehyde and 0.5% paraformaldehyde in phosphate buffer (PB). After removal from skull, their brains were postfixed for 3 h at 4°C in a 10% sucrose solution and subsequently immersed overnight in a 20% sucrose/PB solution. Coronal, 60-μm-thick sections were cut on a cryostat, collected in PB, and incubated in 0.1 M Tris-HCl, pH 7.2; 0.2% Triton X-100; 0.2 mM nitroblue tetrazolium and 1 mM NADPH for 15–45 min at 37°C. After the histochemical procedure, sections were mounted on glass slides and counterstained with neutral red.

Quantitative and Qualitative Analysis

Quantitative and qualitative analysis of NADPH-diaphorase expressing cells in the hippocampal formation were performed in three different anteroposterior levels: corresponding to plates 29, 36, and 42, of Swanson's Atlas (1992). Considering that these plates obey a septotemporal sequence, results relative to plate 29 are referred in the text as "septal," to plate 36 as "median," and to plate 42 as "temporal" levels of the hippocampal formation. Representations of cell counts have superscribed correspondent abbreviations for each of the different levels in the upper right corner of the numbers (sep. for septal; med. for median; temp. for temporal). The following text might serve as an example: "Cell densities of epileptic rats, when compared to controls, were statistically significant ($P = 0.036^{sep}$ and $P = 0.02^{med}$)." As the epileptic animals presented tissue shrinkage in several cerebral regions (see Results for detail), we have chosen the sections that more closely resembled the Atlas plates mentioned above, based on structures outside the hippocampus that did not present cell loss, such as the medial habenula and some hypothalamic nuclei.

Cell counts were performed under a reticulate grid with 10× or 40× objectives, depending on the neuronal density of the cell layer to be analyzed. Three sections including both sides of the brain were counted per animal: One septal, one median, and one temporal. Cell counts were performed three times for each section and the average values were considered. The entorhinal cortex and the subiculum were assessed only in median and temporal levels, respectively.

The area for hippocampal and entorhinal cortex formation subfields in each section was achieved with the stereologic point-counting method and the density of cells per volume was assessed with the formula Nv = Q/At, where *Nv* is the neuronal density, *Q* is the number of neurons counted, *A* is the area considered, and *t* is the thickness of the section (West and Gundersen, 1990). Quantitative results are described either as absolute values per 60-μm-thick section or as the percentage of NADPHd cell loss obtained in epileptic animals relative to age-matched controls.

Nonparametric Mann-Whitney U-test was used for the statistical analysis of cell counts (our two independent samples did not seem to follow a normal distribution). Possible relations between cell counts in various areas of the hippocampal formation and the frequency of SRS were evaluated by correlation analysis. Differences were considered significant when $P \leq 0.05$.

RESULTS

The behavioural changes that regularly follow pilocarpine injections were similar to previous descriptions (Turski et al., 1989, 1983a,b,c, 1984). On the days following SE, animals entered a latent period during which no behavioral epileptiform activity was noticed. After this period, which varied from 26 to 72 days in the present study, a state of chronic epilepsy, characterized by the occurrence of spontaneous recurrent seizures (SRS), ensued. Most spontaneous seizures observed in our study were similar to stage-V-kindled seizures (Racine, 1972). Once initiated, SRS occurred for all the time animals were allowed to survive (in the present study up to 9 months after SE induction). The total number of observed SRS for each animal varied from 4 to 25. As previously reported (Mello et al., 1993), due to the observational nature of the monitoring used in this work, problems of sensitivity and accuracy considering seizure recording may arise. It is possible that the true seizure frequency has been underestimated, especially if nonconvulsive and nighttime seizures are considered.

Qualitative Analysis

Two classifications of NADPH diaphorase-positive neurons based on their staining intensity have been described: lightly or intensely stained (Mufson et al., 1990). The majority of neurons observed in our analysis were lightly stained, although in some cases an intense staining pattern resembling Golgi impregnation was seen.

Qualitative aspects of NADPHd-positive cells in the hippocampal formation of our control rats were similar to previous descriptions (Valtschanoff et al., 1993). Accordingly, here the great majority of stained cells were interneurons. In most cases, it was difficult to characterize specific cell types based exclusively on the morphology of their cell bodies, especially when those were lightly stained. Still, some basket and chandelier cells could be distinctly identified when intensely impregnated. In epileptic animals, the morphological appearance of NADPHd-positive neurons was often found to be altered and their classification, when possible, was performed not only in terms of cell body and initial dendritic segment's morphology, but also based on anatomical location (Fig. 1). Most frequently observed alterations were distortion and shrinkage of proximal dendrites, fewer or absent spines, and cellular pyknosis, in agreement with previous descriptions in Golgi-processed tissue of TLE patients (Scheibel et al., 1974).

Quantitative Analysis

Quantitative analysis of our results evidenced a widespread loss of NADPHd-positive neurons in the hippocampal formation of epileptic rats when compared to age-matched controls (see Table 1). No statistically significant correlations were observed between SRS frequency and NADPHd cell loss for any of the hippocampal formation regions considered in the present study. Data in the following text is expressed as absolute cell count values per 60-μm sections (means ± SD).

Dentate gyrus

In the granular layer of the dentate gyrus (DG), most stained cells were located near the subgranular border, being denser in the superior blade. No statistically significant differences between epileptic animals and controls were found in this layer. In the DG polymorphic layer (PoDG), areas closer to the superior blade (between the CA4 pyramidal cell layer and the superior blade DG granule cell layer) showed significant loss of NADPHd-stained cells, (controls 12.9 ± 2.9^{sep}; 17.2 ± 5.9^{med}; epileptics 5.7 ± 2.6^{sep}; 10.2 ± 4.7^{med}; $P \leq 0.01^{sep/med}$), whereas areas closer to the inferior blade (between CA4 pyramidal cell layer and the inferior blade of the DG granule cell layer) did not achieve statistical significance. Even though loss of NADPHd-positive cells was observed in most parts of the dentate gyrus polymorphic layer, NADPHd-positive cells located in the immediate vicinity of the granule cell layer were preserved in epileptic animals (Fig. 2).

The central portion of PoDG (Obenaus et al., 1993), showed one of the highest losses of NADPHd-positive staining in the hippocampal complex (controls 10.8 ± 5.1^{sep}; 8 ± 3.8^{med}; epileptics 2.2 ± 1.2^{sep}; 3.6 ± 2.4^{med}; $P \leq 0.002^{sep/med}$). Sparse cell staining was observed in the DG molecular layer of control animals. Pilocarpine rats presented a statistically significant ($P = 0.007^{sep}$) loss of NADPHd staining in anterior hippocampal levels but not in more caudal ones. Several types of basket cells were identified in the dentate gyrus as a whole (the most frequent being dentate pyramidal basket cells). Granule cells were not stained with NADPHd.

Ammon's horn and subiculum

Reduced numbers of NADPHd-positive neurons in pilocarpine rats were also found in the Ammon's horn *stratum oriens*, with the majority of the stained neurons being closer to the alveus. In epileptic animals, CA3 *stratum oriens* presented a significant loss of NADPHd-positive cells in rostral levels (controls 10 ± 4.2^{sep}; epileptics 5.3 ± 4.6^{sep}; $P \leq 0.02^{sep}$), whereas in more caudal planes no differences were found; both CA2 ($P \leq 0.0005^{sep/med}$) and CA1 *strata oriens* (controls 18.5 ± 7.8^{sep}; 24.2 ± 9.9^{med}; epileptics 6.6 ± 4.6^{sep}; 14.2 ± 6.7^{med}; $P \leq 0.01^{sep/med}$) presented significant NADPHd-positive cell loss. Sparse lightly stained neurons in CA1 *stratum oriens* had their cell bodies in close vicinity to blood vessels, resembling morphologically and spatially the backprojection neurons described elsewhere (Sik et al., 1995).

In the pyramidal cell layer, NADPHd-positive neurons were mostly radially oriented and bipolar, with CA1 neurons being smaller than CA3. Few basket and chandelier cells were characterized in controls and, in some cases, in epileptic animals as well. NADPHd-stained pyramidal neurons exhibited a very weak staining (particularly in CA1 areas close to the subiculum) and thus, were not counted. Loss of NADPHd-positive neurons in *stratum pyramidale* was significant for CA3 (controls 19.6 ± 3^{sep};

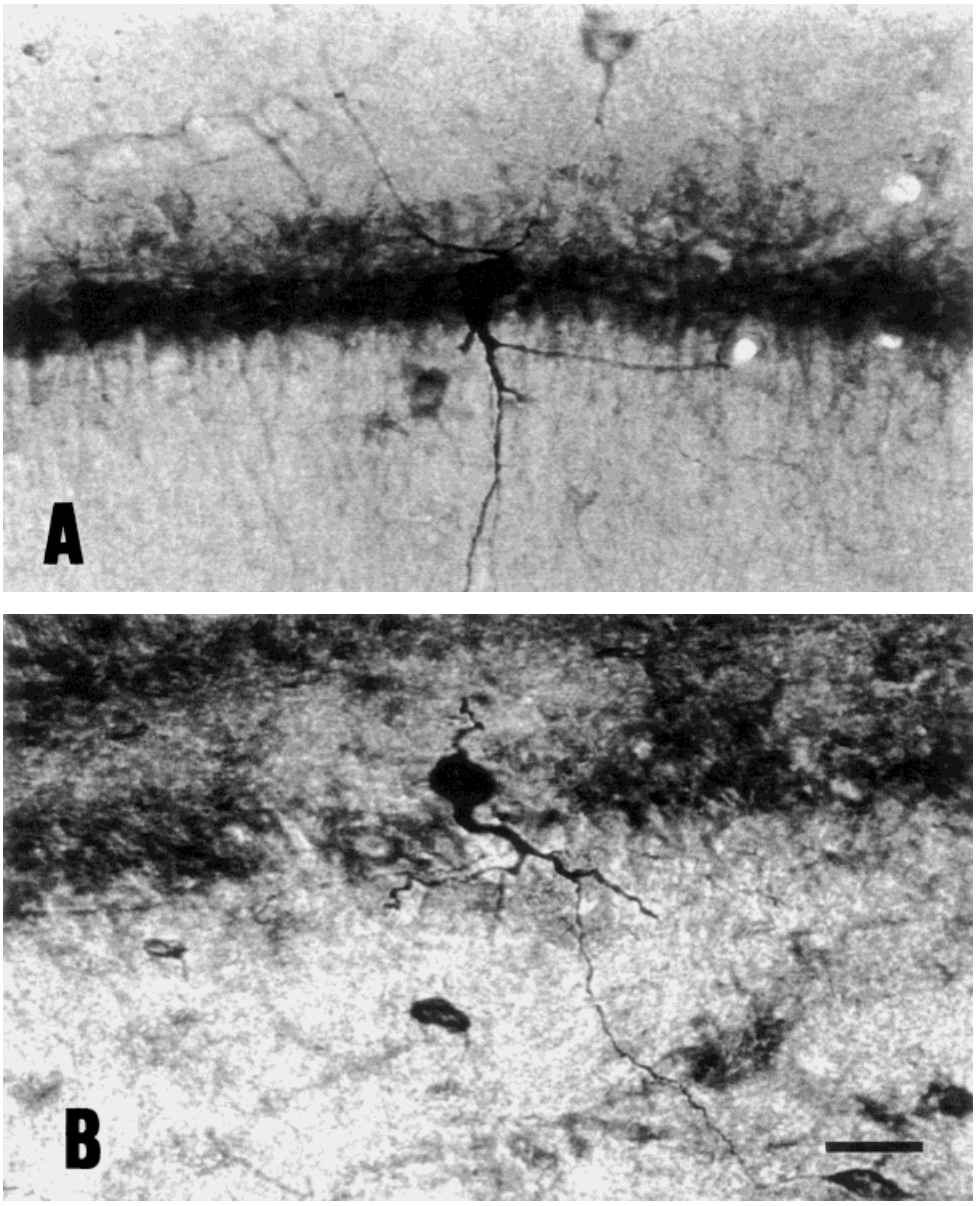

FIGURE 1. Intensely stained CA1 NADPHd-positive interneurons of the same morphological type, in a control (A) and a pilocarpine-treated rat (B). Note the morphological alterations present in the latter (see text for details). Scale bar = 50 μm.

73.7 ± 14.3^{med}; epileptics 11.5 ± 5.6^{sep}; 33.4 ± 11.5^{med}; $P \leq 0.003^{sep/med}$), CA2 ($P \leq 0.001^{sep/med}$), and CA1 (controls 42.5 ± 16.1^{sep}; 65.5 ± 8.4^{med}; epileptics 19.4 ± 7.8^{sep}; 39.2 ± 10^{med}; $P = 0.0005^{sep/med}$).

Stratum radiatum cells were predominantly multipolar or fusiform. A homogenous group of NADPHd-positive neurons was observed in the border between *stratum radiatum* and *lacunosum-moleculare*. These neurons had their dendrites branching in both of these *strata* and in rare occasions penetrating the molecular layer of the dentate gyrus, resembling interneurons described elsewhere (Lacaille and Schwartzkroin, 1988). Loss of NADPHd-positive cells in the *stratum radiatum* of epileptic rats was significant for CA3 (controls 19.2 ± 6^{sep}; 72.8 ± 17.1^{med}; epileptics 12.5 ± 4^{sep}; 38.1 ± 17.4^{med}; $P \leq 0.001^{sep/med}$), CA2 ($P \leq 0.05^{sep/med}$), and CA1 (controls 29.6 ± 7^{sep}; 62.7 ± 14.7^{med}; epileptics 21.8 ± 4.3^{sep}; 45.5 ± 8.5^{med}; $P \leq 0.01^{se/med}$). *Stratum lacunosum-moleculare* was subdivided according to the corresponding subfield (e.g., CA1, CA2, or CA3) in rostral, but not in caudal hippocampal levels. In rostral levels, CA1 *stratum lacunosum-moleculare* presented a significant ($P \leq 0.02$)^{sep} loss of NADPHd-positive cells, whereas no statistically significant differences were found for CA2 nor CA3. Statistically significant loss of NADPHd-positive cells was also found ($P \leq 0.001$)^{med} in more caudal hippocampal levels.

TABLE 1.

Loss of NADPHd Staining in Pilocarpine-Treated Rats as Compared to Age-Matched Controls†

	Dentate gyrus		CA3	CA2	CA1
Molecular layer	19%^{sep**} / 13%^{med}	Stratum oriens	46%^{sep*} / 37%^{med}	76%^{sep**} / 72%^{med**}	64%^{sep**} / 41%^{med**}
Granule cell layer	−8%^{sep} / 5%^{med}	Stratum pyramidale	41%^{sep*} / 55%^{med**}	49%^{sep**} / 58%^{med**}	54%^{sep**} / 40%^{med***}
Polymorphic, superior blade	55%^{sep***} / 40%^{med*}	Stratum radiatum	35%^{sep**} / 48%^{med**}	40%^{sep*} / 66%^{med**}	26%^{sep*} / 27%^{med*}
Polymorphic, inferior blade	37%^{sep} / 23%^{med}	Stratum lacmoleculare[a]	−17%^{sep} / 38%^{med**}	52%^{sep} / 38%^{med**}	32^{sep*} / 38%^{med**}
Polymorphic central	79%^{sep***} / 55%^{med*}				

	Subiculum		Entorhinal cortex
Stratum oriens	70%^{temp}	Layer I	−50%^{med}
Stratum pyramidale	16%^{temp}	Layer II	57%^{med**}
Stratum radiatum	36%^{temp*}	Layer III	77%^{med**}
		Layers IV, V, VI	−76%^{med}

†Percentages were obtained with the formula 1 (mean cell counting values obtained for epileptic animals / mean cell counting values obtained for controls) × 100. Representation of the percentages have superscribed in the upper right corner abbreviations correspondent to each of the different levels evaluated. sep, septal; med, median; temp, temporal. Statistical significance for each area is indicated in the main text; Negative values represent situations where the values found in epileptic animals were higher than the control ones.

[a]In the median level, stratum lacunosum was assessed as a whole.

*$P < 0.05$.

**$P \leq 0.001$.

***$P \leq 0.0001$.

The inner third of the subicular pyramidal layer of control and epileptic rats showed the greatest number of NADPHd-stained cells in the whole hippocampal formation, whereas in the parasubiculum and presubiculum an almost complete absence of stained cells was observed. Loss of NADPHd-positive cells in the subiculum *stratum radiatum* was statistically significant ($P \leq 0.03$)^{temp}. No differences were found in *strata oriens* and *pyramidale*, where an intensely stained neuropil could be observed. A high variability of NADPHd-positive cell counts was obtained for the subicular *stratum oriens* of both epileptic and control animals (controls 10.8 ± 12.6; epileptics 3.2 ± 1.2^{temp}).

Entorhinal cortex

In the entorhinal cortex, layer I showed an almost complete absence of stained cells in both control and epileptic rats and, therefore, no statistically significant differences were found. NADPHd-positive cells of all epileptic rats presented significant reductions for layers II (controls 11.3 ± 4.9; epileptics 4.8 ± 3.7; $P \leq 0.001^{med}$) and III (controls 57.2 ± 57.4; epileptics 13.1 ± 8.5; $P \leq 0.0006^{med}$). Cell counts were especially difficult to perform in layers IV, V, and VI due to extensive tissue destruction and loss of the normal laminar organization in some epileptic animals. Therefore, we quantitatively assessed these layers as a single unit. In layer IV, small numbers of NADPHd-positive neurons were found even in control animals. Animals subject to pilocarpine-induced SE presented an interesting pattern of NADPHd expression in the deep layers of the entorhinal cortex, in which either an almost complete cell destruction or the overexpression of NADPHd-positive cells, in conjunction with an intense neuropil staining, were observed (Fig. 3). Therefore, no significant differences were found when the average cell count in this area for the epileptic animals was compared to the control one. Overexpression of NADPHd-positive cells in epileptic animals was never encountered in the superficial layers. The animals with increased NADPHd expression did not differ in any other significant way (e.g., NADPHd staining in other areas, seizure frequency) from the remaining animals. As our analysis was performed in coronal sections, only the medial entorhinal cortex was counted.

DISCUSSION

Our results have shown a significant widespread NADPHd-positive neuronal loss in almost all hippocampal and entorhinal cortex subfields in the pilocarpine-treated rats when compared to age-matched controls. NADPHd was shown to intensely colocalize with GABA in double-labeling studies performed in the hippocampal formation (exception made for the pyramidal cell layer) (Valtschanoff et al., 1993). In this sense, it further characterizes an extremely important cell category due to the relevant involvement of inhibitory systems in the mechanisms of genesis and propagation of seizures. However, we cannot discard

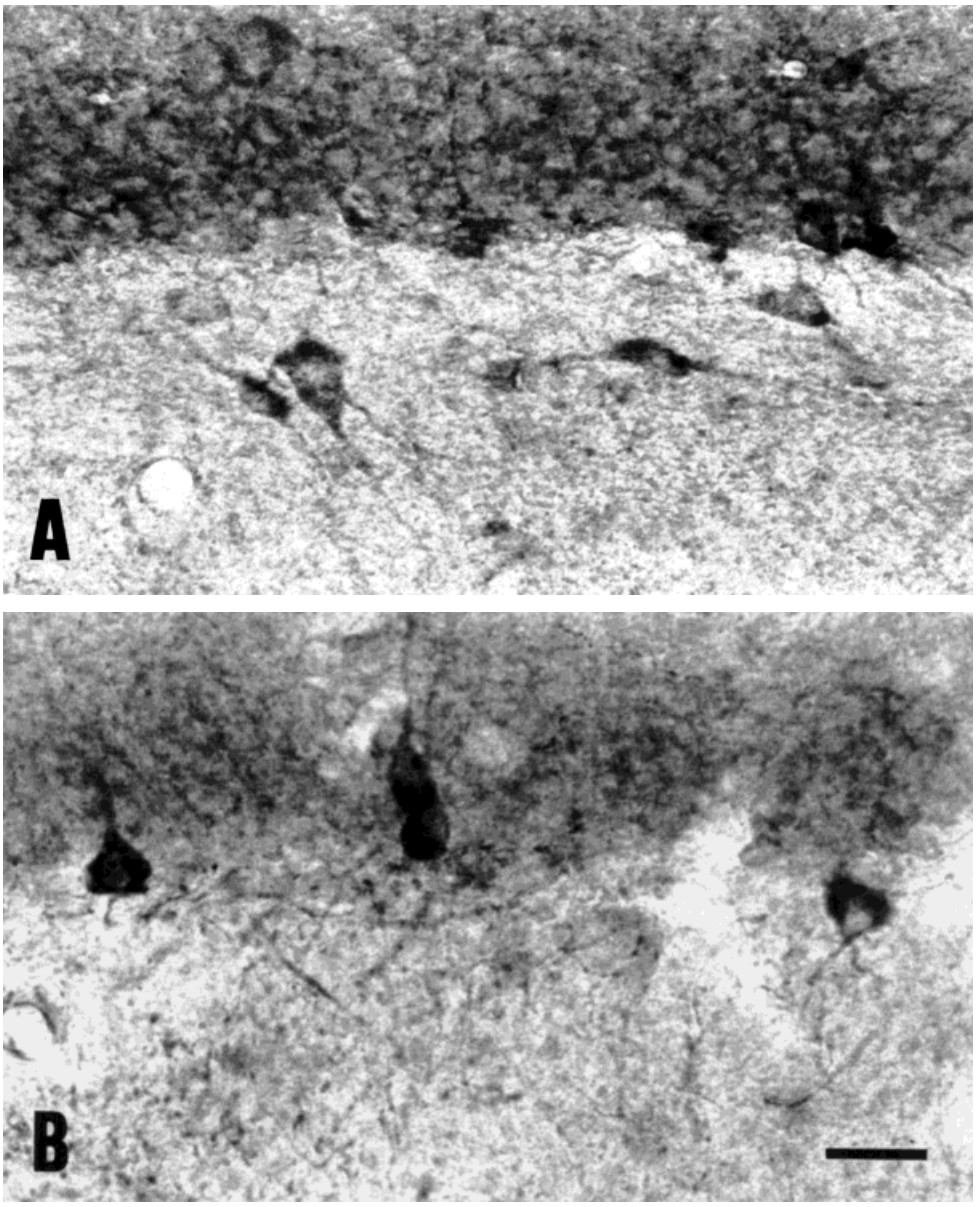

FIGURE 2. NADPHd-positive neuronal expression in the dentate gyrus of a control (A) and a pilocarpine-treated rat (B). Note the lack of positively stained NADPHd cells in the central region of the polymorphic layer and the preservation of NADPHd-positive cells in the vicinity of the granule cell layer. Scale bar = 50 μm.

the hypothesis that previously NADPHd-positive neurons have just altered their pattern of diaphorase expression after the animals became epileptic.

Dentate Gyrus

The use of different techniques for the detection of GABAergic neuronal loss in the PILO model of epilepsy has led to controversial results. Glutamate decarboxylase-(GAD) immunoreactive neurons were reported to be preserved in the hippocampus of rats with SRS after the systemic administration of PILO (Cavalheiro, 1990). In situ hybridization studies, on the other hand, have demonstrated a selective loss of GAD mRNA-containing neurons, particularly observed in the central portion of the dentate gyrus polymorphic layer but not in areas adjacent to the granule cell layer (Obenaus et al., 1993). Corroborating with the latter study and partially with our results, kainic acid-treated rats sacrificed 2 weeks after injection presented a reduction in NADPHd-positive cells in the hilus, but not in other regions of the dentate gyrus (Miettinen et al., 1995).

It has been postulated that hilar GABAergic neurons close to the inferior border of the granule cell layer differ from other cells in the hilus in regard to their capacity to buffer calcium molecules and, as a consequence, to withstand excitotoxicity (Sloviter, 1989). It has been reported that 32–38% of the axon terminals

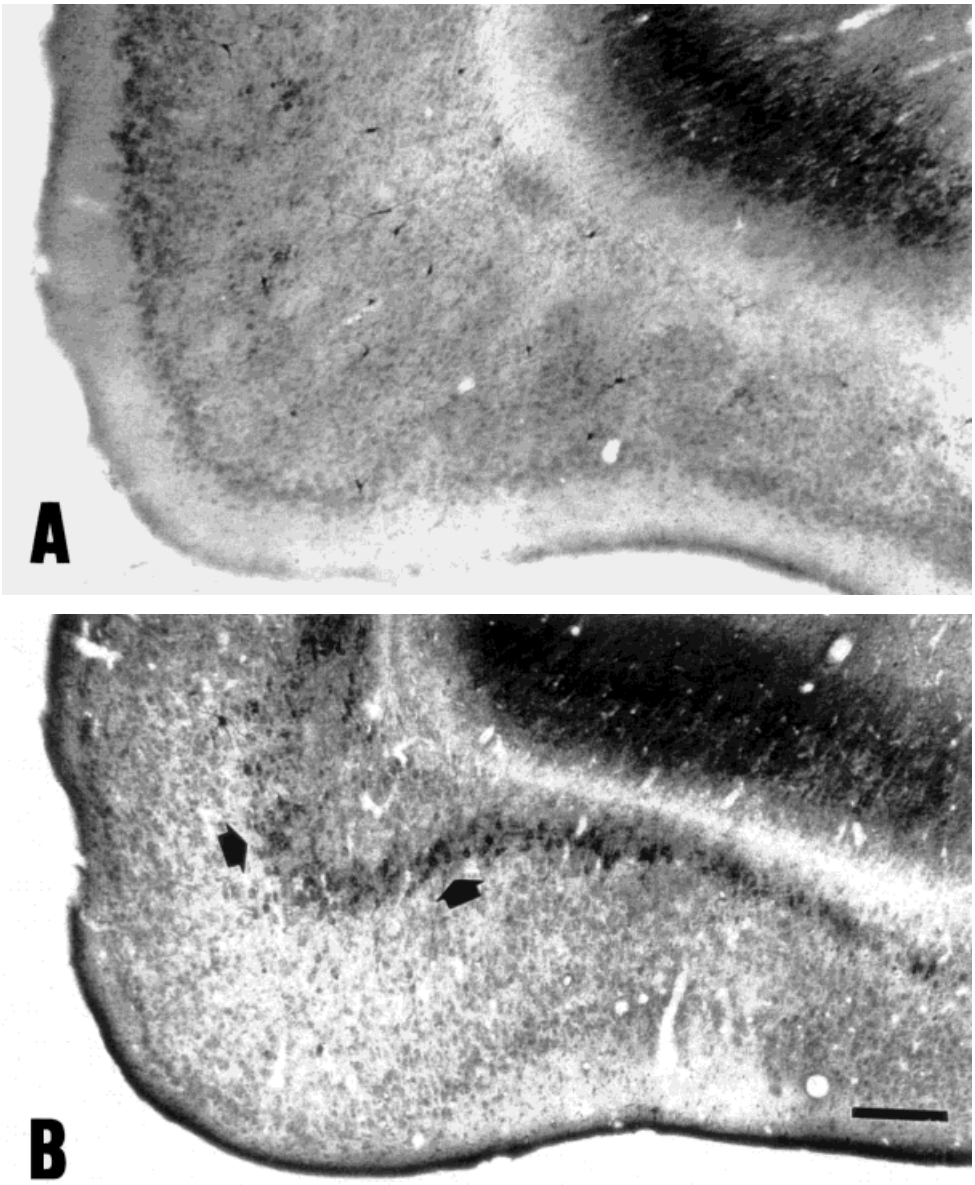

FIGURE 3. NADPHd-positive neuronal expression in the entorhinal cortex of a control (A) and a pilocarpine-treated rat (B). Note the overexpression of NADPHd-positive cells and intense neuropil staining in the deep layers of the entorhinal cortex in the epileptic animal (arrows). Scale bar = 500 μm.

involved in the inhibition of principal hippocampal cell types (pyramidal and granular) are GABA and parvalbumin (PARV) positive, whereas the larger remaining portion of the terminals contained GABA and neuroactive peptides (Kosaka et al., 1987). In the dentate gyrus, only 30% of hilar large basket cells were PARV positive, although all five types of basket cells have demonstrated immunoreactivity for this protein (Ribak et al., 1990). Injections of calcium chelators in cells devoid of calcium-binding proteins protected them from deterioration during prolonged stimulation (Scharfman and Schwartzkroin, 1989), which suggests that selective vulnerability of hippocampal neurons to seizure activity is dependent on their ability to chelate excessive amounts of calcium (Sloviter, 1989). Hence, particular subpopulations of neurons devoid of calbindin and parvalbumin (PARV) such as mossy cells, somatostatin-containing neurons (mostly located in the hilus), and some subclasses of GAD-mRNA positive cells, could be regarded as more susceptible when considering excitotoxic stimulation (Sloviter and Nilaver, 1987; Sperk et al., 1992; Obenaus et al., 1993). Colocalization of PARV and somatostatin in the same nonpyramidal cells was reported to be unusual (5.7%) (Nitsch et al., 1990).

Loss of neurons containing somatostatin neuropeptide Y (NPY) was observed in the inner portion but not in the subgranular border (possibly NPY-positive pyramidal-shaped bas-

ket cells) of the DG polymorphic layer after injections of kainic acid (KA) (Sperk et al., 1992). Nevertheless, despite neuronal loss, an increase in NPY-immunoreactivity was noticed in the terminal field of mossy fibers at the distal CA3 sector as well as in the inner molecular layer (Sperk et al., 1992). A similar increase in NPY staining was also reported within the region of mossy fibre terminals as well as the inner molecular layer after PILO injections (Lurton and Cavalheiro, 1997). Quantitative analysis showed an approximately 50% reduction in the number of NPY-positive neurons in the hillar region of animals that had been subject to pilocarpine-induced SE (Lurton and Cavalheiro, 1997). In contrast, pyramidal-shaped basket cells that contain GABA, colecystokinin (CCK), and vasoactive intestinal polypeptide (VIP), were spared after electrical stimulations of the perforant path (Sloviter and Nilaver, 1987). Vulnerable somatostatin-positive hilar neurons do not exhibit GABA, CCK, or VIP staining (Sloviter and Nilaver, 1987). Our findings of a selective loss of NADPHd-positive neurons, especially in the central portion of the dentate gyrus polymorphic layer, but not in the vicinity of the granule cell layer, are in agreement with many of the above-mentioned studies, lending further support to the notion of different subclasses of vulnerable GABA/NADPHd neurons in this region.

Several interneuron subpopulations, such as the basket cells and chandelier cells, could be morphologically characterized, particularly in control animals. Polymorphic interneurons that spatially and morphologically resembled HIPP cells (*hi*lar cell with its axon ramifying in the *p*erforant *p*ath terminal field) (Han et al., 1993; Halasy and Somogyi, 1993) were also observed. HIPP cells are not only similar in their features to somatostatin- and/or NPY-containing hilar neurons but also exhibit terminal axonal plexus concentrated in the outer third of the dentate gyrus molecular layer (equivalent to the spatial distribution of somatostatin and NPY-positive terminals) (Han et al., 1993; Halasy and Somogyi, 1993). Hence, loss of certain NADPHd-positive cells similar to HIPP, NPY, and somatostatin-positive neurons, could account in part for the decrease in the intralamellar inhibitory systems described by Sloviter (Sloviter, 1987, 1991) and, thus, increasing granule cell excitability via the perforant path in epileptic animals.

Ammon's Horn and Subiculum

In the Ammon's horn, virtually all NADPHd-stained neurons outside the pyramidal cell layer are GABA-positive (Valtschanoff et al., 1993). A reduced number of NADPHd-positive neurons was observed in almost all Ammon's horn subfields (i.e., CA1, CA2, and CA3) of pilocarpine-treated rats. In contrast, animals injected with kainate presented NADPHd cell loss only in the CA1 subfield, although a nonstatistically significant decrease could also be observed in the CA3 region (Miettinen et al., 1995). A possible rationale for these differences might be the consequence of the different models of epilepsy utilized as well as the time animals were allowed to survive after drug administration (120 days for PILO vs. 15 days for kainate-treated rats). However, perhaps the most important difference is that all of our epileptic animals entered status epilepticus after PILO administration, as opposed to the mild epileptic episodes (partial and forelimb clonus-generalized seizures) considered for the kainic acid-treated rats in the study of Miettinen and colleagues (1995).

Important levels of cell loss were seen in the *stratum oriens* of our epileptic rats. Decreased numbers of GAD-mRNA-labeled neurons were found within the *stratum oriens* as well as along the oriens/alveus border in pilocarpine-treated rats (Houser and Esclapez, 1996). The concordance between our results and that described by Houser and Esclapez (1996) is in agreement with the fact that the majority of *stratum oriens* NADPHd-positive cells contain GABA (Valtschanoff et al., 1993). Therefore, NADPHd/GABAergic neurons could be regarded as an eminently vulnerable cell group in this layer. A few neurons observed in CA1 *stratum oriens* of controls had their cell bodies in close vicinity to blood vessels, resembling morphologically and spatially the backprojection neurons described by Sik et al. (1995), which were postulated to exert an inhibitory feedback from the CA1 subfield to CA3 and hilar regions (Sik et al., 1994). Loss of these NADPHd-positive *stratum oriens* neurons could account for network synchronization alterations in epileptic tissue.

The few NADPHd-positive neurons in the Ammon's horn pyramidal cell layer exhibit only a weak staining for GABA (Valtschanoff et al., 1993). Controversy exists in the literature regarding the staining of this layer and the differences concerning localization of nNOS and eNOS. nNOS is cytoplasmatic and exclusively soluble, differing in this sense from eNOS, which is more particulate (Schmidt et al., 1992a). Glutaraldehyde fixation has been reported to better preserve the active form of eNOS by much effective crosslinking to other components of the plasma membrane (Dinerman et al., 1994). Recent evidence suggests that nNOS preferentially stains local circuit neurons while eNOS is responsible for staining pyramidal CA1 cells (Dinerman et al., 1994). Despite using the same fixative technique described by Dinerman et al. (1994) (paraformaldehyde 0.5% plus glutaraldehyde 2%), Ammon's horn NADPHd-positive pyramidal cells were seen only in CA1 areas close to the subiculum, and even then these were weakly stained. In this sense, almost all pyramidal cell layer NADPHd-stained neurons counted in our study were interneurons (the subicular pyramidal layer constituted an exception). Chandelier cells as well as basket cells were clearly identified. Pyramidal cell layer GAD-immunoreactive neurons comprise a numerically minute subpopulation of cells, with levels ranging from 1% (posterior CA1 sections) to 4% (anterior CA2 section), when compared to the total amount of neurons contained in different Ammon's horn subfields (Babb et al., 1988). This suggests that most of the pyramidal cell layer neurons are non-GABAergic (Babb et al., 1988). Only 4% of CA3, 1.6% of CA2, and 0.3% of CA1 pyramidal cell layer neurons exhibited NADPHd-positive staining (Valtschanoff et al., 1993). Good preservation of GAD-mRNA positive neurons was observed in the pyramidal cell layer of pilocarpine-treated rats (Houser and Esclapez, 1996). In contrast, our results revealed significant levels of NADPHd-neuronal loss in the pyramidal cell layer of the entire Ammon's horn, with an exception made for the subicular pyramidal cell layer.

A relatively high number of GABAergic neurons have been described in the Ammon's horn *stratum radiatum* (Babb et al., 1988; Woodson et al., 1989; Sloviter and Nilaver, 1987). Both parvalbumin and calbindin immunoreactivity (smaller number) were reported in CA3, while only the former protein was observed in CA1 (Ribak et al., 1990; Nitsch et al., 1990). No loss of GAD-mRNA labeled cells was observed in this layer in pilocarpine-epileptic rats (Houser and Esclapez, 1996). The percentage of *stratum radiatum* GABAergic and NADPHd-positive neurons was similar when compared to the total number of neurons in this layer (Valtschanoff et al., 1993; Babb et al., 1988). Furthermore, a strong GABA/NADPHd colocalization was observed in *stratum radiatum* in almost every hippocampal subfield (NADPHd subicular *stratum radiatum* cells were only weakly stained for GABA). Our results have shown a significant loss of NADPHd-positive cells in this layer in all analyzed fields. Nevertheless, it is worthy of note that *stratum radiatum* NADPHd cell loss observed in our PILO rats, although statistically significant, was one of the lowest found in the Ammon's horn (only the *stratum lacunosum moleculare* displayed a less intense cell destruction). Hence, *stratum radiatum* could be regarded as a partially resistant layer in most of the analyzed subfields. The discrepancy between the present results and that of Houser and Esclapez (1996) might imply that NADPHd-stained cells constitute a vulnerable subpopulation of GAD containing neurons in the *stratum radiatum*. Alternatively, this could represent only a loss of NADPHd phenotype but not of GAD phenotype for the neurons of this *stratum*.

Entorhinal Cortex

In the entorhinal cortex (EC) formation, studies performed in other species have shown high levels of NADPHd staining in layers II, III, V, and VI (Carboni et al., 1990). Somatostatin-labeled neurons were present mainly in layers II and III, whereas only a few cells were seen in the upper portion of layers V and VI (Carboni et al., 1990). Substance P immunohystochemistry demonstrated a scarcity of cells in layer III as well as in the upper portion of deep enthorinal layers (Carboni et al., 1990). Our results for control animals are in agreement with the above data (although performed in different animal species). NADPHd-positive cells were abundant in layers II, III, V, and VI, but not in layers I and IV.

EC superficial cell layers II and III have shown a significant loss of NADPHd-positive cells in pilocarpine animals. EC layer II neurons project to the dentate gyrus, composing the major portion of the perforant path fiber system, whereas layer III neurons project monosynaptically to hippocampal CA1 and subiculum (Hjorth-Simonsen and Jeune, 1972; Jones, 1993). Tight inhibitory control, mediated by the GABAergic system activity in layer II, might contribute to the high threshold for spike firing in dentate granule cells (Jones, 1993). Low frequency perforant path stimulations evoked suprathreshold responses in CA3 and CA1 and subthreshold responses in the dentate gyrus. Increments in stimulation frequencies significantly increased the probability of suprathreshold trisynaptic excitation (Yeckel and Berger, 1990). A preferential neuronal loss in EC layer III was demonstrated in several models of epilepsy as well as in human TLE (Du et al., 1993, 1995). Loss of NADPHd-positive cells in both of these layers, as reported here, could in this sense be a powerful contribution to epileptogenesis.

Deep EC layers in our epileptic animals presented either an almost complete tissue destruction or an overexpression of NADPHd-positive cells, in conjunction with an increase in neuropil staining. Due to this, no significant differences were seen when the average cell counting for the epileptic group was considered as a whole. Synaptic responses in layer V/VI, where neither $GABA_A$ nor $GABA_B$ mediated inhibition are pronounced, are characterized by fast AMPA/kainate and more prolonged NMDA components (Jones, 1993). In vitro studies have suggested that entorhinal cortex epileptiform discharge initiated in deep layers (relatively devoid of intrinsic inhibitory activity) might synchronously activate deeper and more superficial layers, where a strict inhibitory control provides refractoriness to the propagation of epileptic seizures (Jones and Lambert, 1990a,b). In this sense, deep EC layers could be regarded as another critical region concerning epileptogenesis. Benzodiazepine binding studies performed in TLE patients, have shown an increase in [^{11}C]flumazenil binding in the lateral temporal cortical laminae V and VI (Burdette et al., 1995). In agreement, the overexpression of NADPHd-positive cells observed in some of our epileptic rats might be regarded as a reactive adaptive process in which inhibitory mechanisms are enhanced to provide seizure blockade. In addition, NO might be an important element concerning tissue reorganization as well as synaptic modulation after the epileptogenic damage, since it has been suggested to mediate the formation of synaptic connections in developing and regenerating olfactory receptor neurons (Roskams et al., 1994).

CONCLUSION

A relevant question regarding epileptogenesis might be the relative loss of principal versus inhibitory neurons and thus, the net effect of such losses over neuronal circuits (Covolan and Mello, 1996). In any event, the analysis of the relative contribution of reorganized inhibitory vs. excitatory circuits in epilepsy has been the focus of recent studies (Mathern et al., 1995). From the above comparisons, it is tempting to see the great loss of NADPHd neurons as an important factor for epileptogenesis.

In conclusion, we have demonstrated loss of NADPHd staining in several layers of the hippocampal formation and EC in pilocarpine-epileptic rats. As NADPHd might serve as a marker for subpopulations of GAD expressing cells, the present results might contribute to characterizing the differential vulnerability of the various populations of GABAergic cells in TLE, and thus the potential effects resulting from the lack of such specific neurons.

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