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Alterations of the neocortical GABAergic system in the pilocarpine model of temporal lobe epilepsy: Neuronal damage and immunocytochemical changes in chronic epileptic rats

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ABSTRACT: A wealth of previous studies reported pathological alterations in extrahippocampal regions in mesial temporal lobe epilepsy. Previous experimental findings have also demonstrated that the entorhinal cortex and the neocortex are damaged in different animal models of acute limbic seizures. The present study was aimed at verifying possible alterations in neocortical areas, and, in particular, structural changes of GABAergic interneurons in the sensorimotor cortex, in pilocarpine-induced chronic epilepsy in the rat. Series of sections were Nissl stained and processed for immunocytochemistry using antibodies that recognize nonphosphorylated neurofilament (SMI311), glial fibrillary acidic protein (GFAP), the calcium-binding protein parvalbumin (PV) which is expressed by a subset of cortical GABAergic neurons, the GABA transporter (GAT1), and isoform 65 of glutamic acid decarboxylase (GAD65), the GABA synthetic enzyme. Epileptic rats showed decreased cortical thickness, and diffuse gliosis was observed with GFAP antibody. Neurofilament alterations were also detected in sections processed using SMI311 antiserum. In addition, a diffuse decrease of PV, GAD65, and GAT1 immunoreactivity was observed in the sensorimotor cortex. Altered expression of PV, GAD65, and GAT1 pointed out specific neocortical disturbances in GABAergic inhibition, which could play a crucial role in seizure generation and expression. Thus, the present findings indicate that damage of GABAergic interneurons could be strictly associated with neocortical hyperexcitability in temporal lobe epilepsy.

KEY WORDS: Seizures, Neocortex, Sensorimotor cortex, Parvalbumin, GABA transporter, Glutamic acid decarboxylase.

INTRODUCTION

Although extrahippocampal areas, such as the amygdala, entorhinal cortex, piriform cortex, and thalamus, have been extensively studied in patients and animal models of temporal lobe epilepsy (TLE) [2–4,6,11,14,19,25], limited information is available on temporal and extra-temporal neocortex in TLE. Neuropathological studies on surgical specimens of temporal neocortex resected from TLE patients have suggested an alteration of the intrinsic neocortical circuits [12,23]. Abnormal intracortical inhibition and facilitation was observed in adult TLE patients during extraoperative cortical stimulation, suggesting a remote effect of epileptic activity onto the motor cortex, that leads to an alteration in local inhibitory circuits [34]. Changes in cortical circuitry and GABA-mediated cortical inhibition have been observed in different epileptogenic lesions [10,15,16,18,20,21,30]. In TLE, abnormal staining for the calcium-binding protein parvalbumin (PV), which is expressed by a subpopulation of GABAergic local circuit neurons, was found in neocortical tissue [23], associated with an increased excitatory and decreased inhibitory synaptic density [12]. These findings are consistent with the hypothesis of a participation of the neocortex in TLE.

The pilocarpine model of epilepsy provides an effective experimental model of human TLE [4,31] and allows to investigate the basic mechanisms underlying the involvement of the neocortex in this type of epilepsy. The systemic administration of pilocarpine induces, in rats, a sequence of behavioral alterations and partial seizures leading to limbic status epilepticus. After a mean period of 15 days, the animals develop recurrent limbic seizures and show histopathological features similar to those of human mesial temporal sclerosis [4,31]. Some of the first reports on this model of TLE described, in chronic epileptic animals, anatomical alterations of the neocortical mantle including the sensorimotor cortex [6,31,32]. However, no further studies have been performed to assess the neocortical derangement in these animals. On this basis, the present investigation was performed to study the occurrence of structural changes in the sensorimotor area of chronic epileptic rats by means of an immunocytochemical approach. Markers of astrocytes and neurons were used. In particular, we utilized markers of the GABAergic system, such as PV, glutamic acid decarboxylase (GAD), which is the GABA synthetic enzyme, and the GABA transporter (GAT1). GAT1, a sodium-dependent plasma membrane protein modulating the extracellular GABA concentration, is present in axon terminals that form GAD-containing symmetric synapses [24].

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MATERIALS AND METHODS

Animal Treatment and Histology

Adult male Wistar rats (220–250 g) were kept under standard laboratory conditions with a 12 h light/dark cycle (light on at 0700 h) and food and water *ad lib*. The pilocarpine model of epilepsy was performed in rats as previously reported [5]. Briefly, the animals received a dose of 350 mg/kg of pilocarpine intraperitoneally (Sigma, St. Louis, MO, USA). To prevent peripheral cholinergic effects, scopolamine methylnitrate was injected subcutaneously (1 mg/kg) 30 min before pilocarpine injection. Moreover, assisted feeding and hydration were carried out during the initial recovery period to improve the general clinical state of the animal and likelihood of survival. The control group was composed of animals that received an injection of scopolamine methylnitrate plus saline. Each group of pilocarpine-treated and control rats was composed of five animals. The experiments were performed under institutional approval and all ethical requirements were respected.

The pilocarpine-injected animals used in the present study developed spontaneous recurrent seizures after the initial status epilepticus, followed by a silent period of 4–45 days. After a survival time of 60 days from the onset of chronic seizures, the animals were deeply anesthetized and perfused through the ascending aorta with heparinized 0.01 M phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in PBS. The brains were removed, briefly postfixed in 4% paraformaldehyde, cut coronally with a vibratome in 50 μm-thick sections, which were collected in series and processed for immunocytochemistry. At least three sections, regularly spaced through the anteroposterior extent of the sensorimotor cortex of each animal, were processed with each antibody. In all cases, sections adjacent to those processed for immunocytochemistry were stained with cresyl violet for cytoarchitectural control. After the staining procedures, the sections, mounted on slides, were dehydrated and coverslipped.

Immunocytochemical Procedure

The immunoperoxidase procedure was performed on free-floating sections using antibodies against glial fibrillary acid protein (GFAP, monoclonal, 1:300, Boehringer Mannheim, Mannheim, Germany), nonphosphorylated neurofilament (SMI311, monoclonal, 1:1000, Sternberger Monoclonal Inc., Lutherville, USA), PV (monoclonal, 1:10000, Swant, Bellinzona, Switzerland), isoform 65 of GAD (GAD65, monoclonal, 1:300, Chemicon, Hofhein, Germany), and GAT1 (polyclonal, 1:300, Chemicon). Paired sections from experimental and control animals at matched anteroposterior levels were processed in the same vial in order to minimize the intergroup differences during the immunocytochemical procedure.

The sections were pretreated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity, rinsed in PBS, preincubated for 45 min in 10% normal serum in PBS with 0.2% Triton X-100, and then incubated in primary antibodies overnight (SMI311, GFAP, and PV) or for 24 h (GAT1 and GAD65) at 4°C. Sections were then rinsed in PBS, incubated in biotinylated anti-rabbit or anti-mouse IgG (Vector, Burlingame, USA) at a dilution of 1:200 in PBS (1 h at room temperature), rinsed in PBS, incubated in avidin–biotin peroxidase complex (ABC; Vectastain, Vector) for 1 h, washed several times in PBS, and then incubated in 0.075% diaminobenzidine in 0.002% H_2O_2.

Data Analysis

The material processed for immunocytochemistry was analyzed qualitatively at the microscope, under bright-field illumination, independently by two investigators. In addition, the thickness of the sensorimotor cortex was evaluated in Nissl-stained sections from all the control and epileptic animals. To this purpose, 3–5 cresyl violet-stained sections per animal were sampled through the anteroposterior extent of the sensorimotor cortex. The sections were selected, using the rat atlas of Paxinos and Watson [26], from the most anterior (bregma: −1.8) to the most posterior (bregma: −3.8) levels of the sensorimotor cortex; special care was devoted to match the same levels in the control and epileptic animals. The linear dimensions of the total cortical thickness (layers I–VI), obtained with a 2.5× objective, were assessed in each section using the public domain software NIH 1.62 (National Institutes of Health, Bethesda, MD). Individual values generated for each linear measurement were averaged in each animal group. The significance of the differences between the control and epileptic groups was statistically evaluated using the Student's *t*-test.

RESULTS

The results will be described comparing sections from control and epileptic animals after Nissl staining or processing for immunocytochemistry with the same antibodies.

In cresyl violet-stained sections, a decrease in the thickness of the sensorimotor cortex of the epileptic rats (1.622 ± 0.17 mm) compared with that of control ones (1.786 ± 0.22 mm) was evident (Fig. 1A,A'). Such shrinkage was observed throughout the anteroposterior extent of the sensorimotor cortex and was statistically significant ($p < 0.05$).

In addition to an overall reduction of cortical thickness in the epileptic animals, in the sections derived from the latter rats, the laminar cortical organization was disrupted so that individual layers were difficult to discern precisely, especially in the superficial layers but with the only exception of layer I (Fig. 1A'). Moreover, although no cell counts were performed, a dispersion of cells, i.e., a lower neuronal density, was evident in the cortex of the epileptic animals.

The observations performed with Nissl staining were confirmed by the data observed in the sections processed for SMI311 immunoreactivity (Fig. 1B,B'). In the sensorimotor cortex of the epileptic animals, the latter material showed an increased immunostaining of labeled pyramidal neurons (Fig. 1B'). In addition, below layer I, intensely immunostained neurons were observed, and these cells exhibited apical dendrites distributing dense arborizations in the most superficial layer (Fig. 1B'). This peculiar pattern observed with SMI311 immunolabeling was not observed in the matched sections from control animals (Fig. 1B,B').

In the sections processed with anti-GFAP antibodies, a band of reactive gliosis was observed in the epileptic animals in the superficial third of the cortex. GAD65 immunoreactivity was markedly decreased in the cortical neuropil of the epileptic animals (Fig. 1C,C'). In the sections from epileptic animals compared with control ones, PV immunostaining also exhibited a diffuse decrease of the positive elements, both in terms of neuropil staining and density of immunostained neuronal cell bodies (Fig. 1D,D').

In control animals, GAT1 immunoreactivity corresponded to that described in previous studies [7,24]. Unlabeled neurons surrounded by GAT1-positive puncta, interpreted at least in part as labeled terminals, were observed, and these elements were especially dense and evident in layers II/III (Fig. 1E). GAT1-labeled puncta, although diffusely distributed throughout the neuropil, were especially evident around the soma and initial axon segment of cortical neurons (Fig. 1E, inset). In the epileptic animals, a decrease of GAT1 immunolabeling was observed (Fig. 1E'). The density of perisomatic GAT1-positive terminals was much lower than in controls, while periaxonal rows of labeled puncta were well evident, especially in the superficial layers (Fig. 1E', inset).

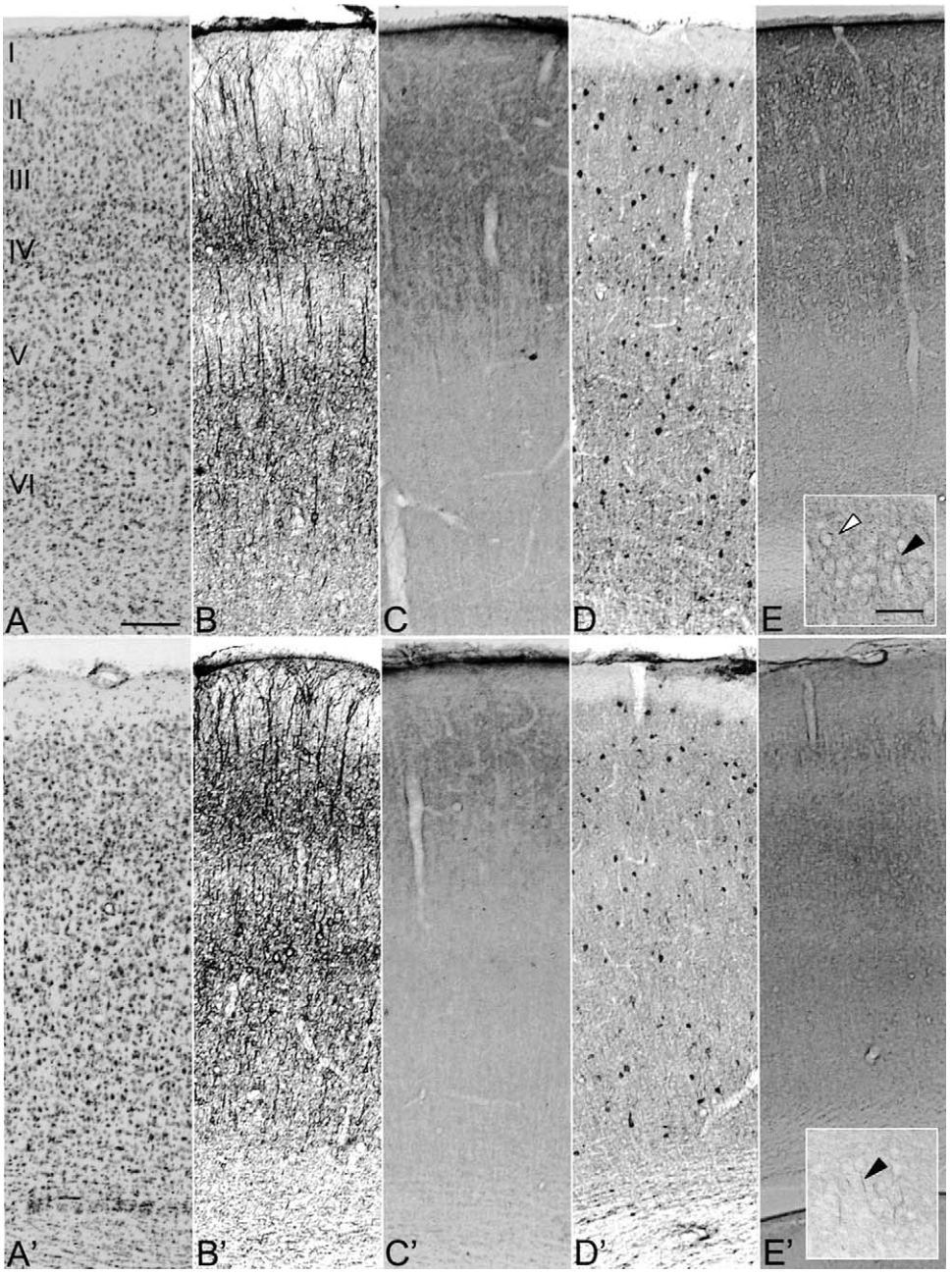

FIG. 1. Photomicrographs showing the cytoarchitectural (A and A') and immunocytochemical (B–E and B'–E') organization of the somatosensory cortex in control (A–E) and epileptic (A'–E') animals. A and A': thionin-stained sections; note the marked reduction of cortical thickness and the indistinct boundaries between most of the cortical layers. B and B': sections processed using SMI311 antibody; note, in the epileptic animal (B'), the increased staining of some neurons in the lower part of the cortex and the increased ramification of the apical dendrites invading layer I, as compared to the control animal (B). C and C': decreased immunostaining for GAD65 is evident in the lower part of the cortex from the epileptic animal. D and D': in sections processed using anti-PV serum, a decrease of both immunostained neurons and neuropil immunoreactivity is evident in the epileptic animals (D') as compared to the control (D). E and E': using GAT1 antibody, numerous immunoreactive puncta, interpreted as axon terminals, are evident in control animals around neuronal somata, especially in layers II and III (E). In addition to the perisomatic terminals, vertical rows of immunoreactive puncta are visible (inset), presumably representing GAT1-positive terminals contacting the proximal portion of the axon of pyramidal neurons. In chronic animals, a dramatic decrease of the perisomatic terminals is evident, while periaxonal-labeled terminals are still present (inset). Calibration bars: A, 150 μm (applies also to the other images); inset in E, 50 μm (applies also to the inset in E').

DISCUSSION

The present data showed structural and immunocytochemical alterations in the neocortex in the pilocarpine model of TLE. Chronic epileptic rats showed a significant reduction of cortical thickness, with laminar disorganization that especially evident in the superficial layers. Structural neuronal changes were also detected using the SMI311 antibody, showing an increased staining of the cell bodies of pyramidal neurons in the deep layers and increased apical dendritic ramifications invading layer I. These structural abnormalities were associated with structural alterations specifically related to the GABAergic system. In particular, a reduction of GAD65, PV, and GAT1 immunostaining was observed in the neocortex of the chronic epileptic animals. Taken together, these findings support an involvement of the neocortex in TLE, paralleling the neuropathologic observations in surgical specimens resected from patients [22,23].

The characteristic neuropathological features observed in pilocarpine-induced epilepsy has been reported to involve the hippocampal and parahippocamnpal regions: the thalamus, the amygdala, and the substantia nigra, whereas, as mentioned above, alterations of the neocortex were reported only occasionally [4,5,6,8,9,31,32]. The present study provided instead evidence of neocortical abnormalities, suggesting that in chronic epileptic animals spontaneous seizures could generate changes in neocortical excitability, particularly involving the GABAergic function.

Recent experimental data have shown an increased neocortical glucose consumption during interictal period in pilocarpine-induced epilepsy [29]. Moreover, electrophysiological experiments performed in neocortical slices from pilocarpine-treated chronic epileptic rats showed a pronounced hyperexcitability of cortical neurons, suggesting changes in the local network or intrinsic neuronal properties [28]. These findings and the present data could thus suggest that excessive metabolic activation induced by repetitive propagation of epileptic activity from limbic structures may lead to neuronal death and to a rearrangement of local circuitry in neocortical areas of chronic epileptic subjects.

Functional abnormalities in extratemporal motor areas have been described in epileptic patients using transcranial stimulation, suggesting that changes in cortical excitability could be due to inhibitory presynaptic abnormalities [34]. Inhibitory GABAergic neurons have a high metabolic rate and are more sensitive to anoxia than other types of cells [1,13]. These neurons could thus be especially vulnerable to anoxia resulting from excessive functional demands and from the increases in metabolic activity that occur during prolonged seizure activity. Destruction of GABAergic interneurons may be one of the major cerebral defects predisposing to seizures [27]. In the neocortex, damage of GABAergic interneurons seems to be critical to initiate epileptic phenomena, once GABA prevents runaway excitation leading to synchronous discharges [33].

Classical studies have described the relationship between loss of GABAergic neurons and the development of the epileptic condition [17,27]. Recent observations pointed out that specific populations of GABAergic neurons, particularly the PV-containing ones, could be involved in the establishment of epilepsy in humans [10]. The present report of a reduction of neocortical PV and GAD65 immunoreactivity in chronic epileptic rats suggests an impairment of the inhibition mediated by GABAergic interneurons. Because PV has been found to labeled primarily, if not exclusively, the basket and chandelier subpopulations of GABAergic interneurons [10,21], the present data indicate that these two cell types are especially affected in these epileptic animals.

The peculiar distribution of GAT1-positive terminals observed in the present experimental material suggests a preferential loss and/or reduced expression of GAT1 protein in perisomatic GABAergic terminals, which mostly derive from basket cells. These possibilities are not mutually exclusive because the rats exhibited a diffuse reduction of PV and GAD65-positive terminals throughout the neocortex. Reduced perisomatic expression of GAT1 could represent an adaptive mechanism to increase GABAergic function in residual terminals. Nevertheless, it has been demonstrated that increased potassium concentration can induce a “reverse operation” of GABA transporters (nonvesicular GABA release) and activates postsynaptic GABA receptors [16]. It is possible that this carrier-mediated GABA release occurs *in vivo* during seizures [12]. If this is the case, reduced GAT1 expression observed in pilocarpine chronic epileptic rats could represent an impairment of GABA-mediated control during epileptic activity.

In conclusion, the present study pointed out an altered expression of GAD and GAT1, involving especially basket and chandelier interneurons, which could lead in chronic epilepsy to specific neocortical disturbances in GABAergic inhibition. These changes could be strictly associated with neocortical hyperexcitability and could be relevant to seizure expression in TLE.

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