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QUANTITATIVE ANALYSIS OF PARVALBUMIN-IMMUNOREACTIVE CELLS IN THE HUMAN EPILEPTIC HIPPOCAMPUS

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Abstract—Hippocampal sclerosis is the most frequent pathology encountered in mesial temporal structures resected from patients with intractable temporal lobe epilepsy and it mainly involves hippocampal neuronal loss and gliosis. These alterations are accompanied by changes in the expression of a variety of molecules in the surviving neurons, as well as axonal reorganization in both excitatory and inhibitory circuits. The alteration of a subpopulation of GABAergic interneurons that expresses the calcium binding protein parvalbumin (PV) is thought to be a key factor in the epileptogenic process. We investigated the distribution and density of parvalbumin-immunoreactive (PV-ir) neurons in surgically resected hippocampal tissue from epileptic patients with and without sclerosis. Using quantitative stereological methods, we show for the first time that there is no correlation between total neuronal loss and PV-ir neuronal loss in any of the hippocampal fields. We also observed higher values of the total neuronal density in the sclerotic subiculum, which is accompanied by a lower density of PV-ir when compared with non-sclerotic epileptic and autopsy hippocampi. These findings suggest that, the apparently normal subiculum from sclerotic patients also shows unexpected changes in the density and proportion of PV-ir neurons. © 2007 Published by Elsevier Ltd on behalf of IBRO.

Key words: calcium-binding protein, epilepsy, inhibition, interneurons, subiculum.

Hippocampal sclerosis is the most frequent pathology encountered in resected mesial temporal structures from patients with intractable temporal lobe epilepsy (TLE; Honavar and Meldrum, 1997). In general, it is characterized by gliosis and neuronal loss, most prominently in the cornu ammonis (CA) 1 field of the hippocampus, followed by the hilus, CA4, CA3 and CA2 fields, and the dentate granular cell layer. Furthermore, there is often a dispersion of the dentate granular cell layer with ectopic neurons being found in the molecular layer (Houser, 1990; Houser et al., 1992; Arellano et al., 2004). This neuronal loss and gliosis is accompanied by changes in the expression of a variety of molecules in the surviving neurons, as well as axonal reorganization involving both excitatory and inhibitory circuits (e.g. de Lanerolle et al., 1989; Sutula et al., 1989; Babb et al., 1991, 2000; Sloviter et al., 1991; Mathern et al., 1995; Muñoz et al., 2002, 2007; Wittner et al., 2002; Arellano et al., 2004). The basic mechanisms inducing seizure activity still remain unclear, although most hypothesis are based on alterations of GABAergic circuits (reviewed in Avoli, 1983; Prince et al., 1997; DeFelipe, 1999; Bausch, 2005). However, as recently emphasized there is significant variability in the alterations of the GABAergic circuits in the epileptic hippocampal formation, which include the loss of GABAergic perisomatic innervation and aberrant GABAergic hyperinnervation (Arellano et al., 2004). In addition, hippocampal sclerosis per se is not epileptogenic, nor is it necessarily associated with the primary epileptogenic region (reviewed in DeFelipe, 1999). These features are the main reasons why there is still no unifying hypothesis to explain the relationship between changes in GABAergic circuits and the development of seizure activity.

The activity of hippocampal excitatory neurons, granular cells of the dentate gyrus and pyramidal cells of the hippocampus proper, is regulated by the inhibitory influence of a heterogeneous population of GABAergic interneurons (reviewed in Freund, 2003). In particular, the somata, the proximal dendrites, and the axon initial segments of pyramidal cells are innervated by the so-called basket cells and chandelier cells, respectively. These two domains are thought to be strategic in controlling pyramidal cell excitability and thus, chandelier and basket cells are considered crucial to control the output of pyramidal cells (Stuart and Sakmann, 1994; Colbert and Johnston, 1996; Miles et al., 1996). Immunocytochemical studies in the cerebral cortex of rodents, monkeys and humans have shown that the calcium-binding protein parvalbumin (PV) can be used as a valuable and selective marker of a subpopulation of GABAergic cells (Celio, 1986), which includes a subpopulation of large basket cells and chandelier cells (DeFelipe et al., 1989; Hendry et al., 1989; Lewis and Lund, 1990; Sloviter et al., 1991; Williams et al., 1992; Andressen et al., 1993; Ribak et al., 1993).

Immunocytochemistry for PV has been used to study possible alterations of these GABAergic neurons in cortical tissue resected from TLE patients (Sloviter et al., 1991; Zhu et al., 1997) and extensive loss of PV-immunoreactivity (-ir) has been described in both the hippocampus and in certain regions of the lateral neocortex. Accordingly, these alterations could be related to the genesis and/or maintenance of seizure activity (DeFelipe, 1999). For example, when compared with normal autopsy tissue a reduction in

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Abbreviations: CA, cornu ammonis; ir, immunoreactivity/immunoreactive; PV, parvalbumin; PV-ir, parvalbumin-immunoreactive; TLE, temporal lobe epilepsy.

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PV-ir has been described in the CA1 and hilus of the human epileptic hippocampus (e.g. Sloviter, 1989, 1991b; Arellano et al., 2004; Wittner et al., 2005). However, to date there have been no detailed regional studies that have quantified the decrease in PV-expressing neurons in the hippocampal formation and compared this with the neuronal loss observed in Nissl-stained sections.

The aim of the present study was to investigate whether the population of interneurons expressing PV is regionally and selectively affected in the epileptic hippocampal formation, and to examine to what extent the loss of this GABAergic subpopulation of interneurons parallels the overall neuronal loss in the epileptic hippocampus. For this purpose, we studied the hilus of the dentate gyrus, the CA fields and the subiculum from the hippocampus of autopsy and epileptic patients with and without hippocampal sclerosis using Nissl staining and immunocytochemistry for PV. The total neuronal density, as well as the distribution and density of PV-ir neurons, was estimated using stereological techniques in these regions.

EXPERIMENTAL PROCEDURES

Human brain tissue was obtained from two sources: from autopsies (supplied by Dr. R. Alcaraz, Forensic Pathology Service, Basque Institute of Legal Medicine, Bilbao, Spain) and postoperative tissue from 11 patients, suffering pharmacoresistant TLE (Department of Neurosurgery, Hospital de la Princesa, Madrid, Spain).

According to the Helsinki Declaration, the patient's consent was obtained in all cases (BMJ 1991;302:1194) and all protocols were approved by the institutional ethical committee (Hospital de la Princesa).

Tissue from epileptic patients was obtained from six men and five women suffering from partially complex and secondarily generalized seizures (age range: 21–65 years, average: 39.8; age of onset range: 1–50 years, average: 13.9; duration range: 7–40, average: 25.9). The hippocampal formation from these patients has been used in previous studies (Arellano et al., 2004; Arion et al., 2006). Briefly, tailored temporal lobectomy plus amygdalohippocampectomy was performed in all cases under electrocorticography guidance. After surgery, the lateral neocortex and mesial structures were subjected to standard neuropathological assessment. The surgical outcome of epileptic patients was evaluated after 18 months and patients were classified following the Engel scale: eight patients as grade I; 2 as grade II; and one as grade III (Engel, 1987). For further information on the material see Arellano et al. (2004) and Arion et al. (2006). The tissue obtained at autopsy (2–3 h postmortem) was from four normal males who had died in traffic accidents (aged 23, 49, 63 and 69 years). The brains were cut into 1.5 cm thick coronal slices and immersed in a cold solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24–36 h.

Histopathological analyses

Standard histopathological assessment was carried out on sections from the hippocampal formation of surgically removed tissue from 11 epileptic patients and from the four autopsy cases ($n=4$). Epileptic patients were selected when the resected hippocampus included all fields. Nine out of these 11 patients exhibited hippocampal sclerosis with no other significant pathological alterations. Thus, we classified the epileptic hippocampi as sclerotic ($n=9$) and hippocampi without histopathological alterations ($n=2$). In the autopsy cases no histopathological alterations were detected.

Immunocytochemistry

Sections from the hippocampal formation of autopsy and biopsy tissue were batch-processed using standard immunocytochemical techniques with an antibody against mouse PV (1:4000; Swant, Bellinzona, Switzerland). Briefly, the sections were processed by the avidin–biotin method, using secondary horse anti-mouse biotinylated antibodies (1:200; Vector Laboratories, Burlingame, CA, USA) and the Vectastain ABC immunoperoxidase kit, with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) as the chromogen. The sections were mounted, dehydrated, cleared with xylene and coverslipped. In control sections, processed either with primary antibodies preadsorbed with PV protein (Swant) or without the primary or secondary antibodies, no significant staining was detected. Furthermore, no qualitative differences were observed in the immunocytochemical staining of sections from autopsy material and sections from the control biopsies. Adjacent sections stained with Toluidine Blue were used to reveal the borders between the different hippocampal fields.

Estimation of neuronal densities

We have analyzed sections taken from similar parts of the hippocampus of both autopsy and biopsy material, in order to compare equivalent regions. The total neuron density and that of PV-ir neurons in the hilus, CA4–CA1 fields and subiculum was estimated by counting neurons from randomly selected areas (35,652 μm^2) in randomly selected PV-immunostained sections, and by counting nucleoli in two Nissl-stained sections per case. Neurons with more than one nucleolus were rare but in such cases, only one nucleolus was counted. Counting was performed on a computer screen at a final magnification of 1288 (using a 40× objective) and with the aid of an Olympus DP-70 digital camera attached to an Olympus BX51 microscope controlled by Castgrid software (Olympus Denmark A/S, Albertslund, Denmark). In each section, six randomly selected fields were analyzed in the hilus, CA4 and CA3 regions, whereas four fields were analyzed in CA2 and 14 in CA1 and the subiculum. Furthermore, in CA1 and the subiculum, the neuronal densities were analyzed in both the superficial and deep aspects of the pyramidal layer. Finally, CA1 was subdivided in three equal portions: proximal, medial and distal with respect to the dentate gyrus, and plots of the distribution of PV-ir somata were obtained with the aid of Neurolucida (MicroBrightField Inc., version 6.0, Williston, VT, USA). Statistical analysis of the data was performed using an ANOVA with Bonferroni post hoc comparisons. Correlation between the total and PV-ir neuronal densities was studied with Spearman's non-parametric test. Statistical analyses were carried out using SPSS software (SPSS 10.0, SPSS Science, Chicago, IL, USA).

To generate the figures, all the images were captured with a digital camera (Olympus DP50) attached to an Olympus microscope and Adobe Photoshop 7.0.1 software (Adobe Systems, San Jose, CA, USA) was used to generate the figure plates.

RESULTS

The cytoarchitectonic division of the hippocampal fields (Fig. 1A) was established on basis of the descriptions in Amaral and Insausti (1990), but also considering the CA4 field according to the definition of Rosene and Van Hoesen (1987).

The distinction between CA1 and the subiculum was established by the presence of clusters of modified pyramidal cells in the superficial aspect of the subiculum (Fig. 1). The CA1 field was divided in three subfields to account for the regional differences in neuronal density: proximal, intermediate and distal to the dentate gyrus. The