Image Anal Stereol 2002;21:117-120
Original Research Paper

ESTIMATION OF THE NUMBER OF NEURONS IN THE HIPPOCAMPUS OF RATS WITH PENICILLIN INDUCED EPILEPSY

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(Accepted. February 17, 2002)

ABSTRACT

Epilepsy is a neurological disease arising from strong and uncontrollable electrical firings of a group of neurons in the central nervous system. Experimental epileptic models have been developed to assess the physiopathology of epileptic seizures. This study was undertaken to estimate the number of neurons in the rat hippocampus with penicillin induced epilepsy, using a stereological method, 'the optical fractionator". In the experimental group, 500 IU penicillin-G was injected intra-cortically, and in the control group, the same volume of saline was administered. A week later, the animals were decapitated and their brains were removed by craniatomy. Frozen brains were cut with a thickness of 150 µm in a cryostat. Sections were collected by systematic random sampling and stained with hematoxylen-eosin. Microscopic images of pyramidal cell layers from hippocampus CA1, CA2 and CA3 subfields were then transferred to a monitor, using a 100x objective (N.A. = 1.25). Using the optical disector method, the neurons were counted in the frames and determined with a fractionator sampling scheme. The total pyramidal neuron number was then estimated using the optical fractionator method. The total pyramidal neuron number was found to be statistically lower in the experimental group (mean = 142,888 ± 11,745) than in the control group (mean = 177,953 ± 10,907) ($p < 0.05$). The results suggest that a decrease in the hippocampal neuronal number in a penicillin model of epilepsy can be determined objectively and efficiently using the optical fractionator method.

Keywords: experimental epilepsy, hippocampus, optical fractionator, rat, total neuron number.

INTRODUCTION

Epilepsy is a neurological disease arising from abnormal and uncontrollable electrical firings of a group of neurons appearing in the central nervous system (Fletcher, 1987; Lindsay *et al*., 1997). Experimental epilepsy models were developed to assess the physiopathology of epileptic seizures (Biziere and Chambon, 1987). Animal models with epilepsy can be classified as: 1) experimental seizures induced by chemical convulsants or by electrical stimulation, 2) reflex epilepsies, 3) idiopathic epilepsies (Biziere and Chambon, 1987).

Chemical convulsants are widely used as simple and rapid seizures (Biziere and Chambon, 1987). The common antibiotic, penicillin, is a chemical convulsant (Biziere and Chambon, 1987; Fisher, 1989). If penicillin is injected into the brain intra-cortically, it develops epileptic seizures (Sagratella *et al*., 1985). No previous studies of the changes in the neuronal number of the rat hippocampus in the penicillin model of experimental epilepsy have been made. Other experimental models have shown a decrease in the neuronal number in the hippocampus, but none of them has used a stereological method. The aim of this study was to estimate the number of neurons in the hippocampus of penicillin induced epileptic rats using the optical fractionator. The optical fractionator is an unbiased stereological method for counting neurons (West, 1993). It combines a three-dimensional probe for counting neuronal nuclei, i.e. the optical disector, with a systematic random sampling scheme, the fractionator as (Gundersen, 1986; Gundersen *et al*., 1988; West *et al*., 1991, 1996). The optical disector produces unbiased estimates of neuron number, i.e., estimates free of assumptions about neuron size and shape, unaffected by lost caps and overprojection. The fractionator involves systematic sampling of a known fraction of a structural component (West *et al.*, 1991).

MATERIALS AND METHODS

Twelve Sprague-Dawley female rats aged 16-20 weeks were used in this study (n = 6 experimental group, n = 6 control group). The animals were fully anesthetized with a combination of Xylazine (10 mg/kg) and Ketamine (90 mg/kg) intra-peritoneally.

In the experimental group, 500 IU (0.1 cc.) penicillin-G was injected intra-cortically (2 mm. posterior and 2 mm lateral from the bregma, and 2 mm into the right cortex). The seizures happened 2-3 minutes after injection, and the epileptiform activity was recorded by EEG. In the control group, the same volume of saline (0.1 cc., 0.9% NaCl) was administered and no changes of electrical activity in EEG was observed.

A week later, all animals were decapitated, their brains were removed by craniotomy and frozen in a cryostat (Leica CM3050) at -50°C. Frozen brains were cut in the horizontal plane with a thickness of 150 µm by the cryostat at -15^{0}C. Sections were collected via systematic random sampling and stained with hematoxylen-eosin.

Microscopic images obtained from pyramidal cell layers in CA1, CA2, CA3 subdivisions of the hippocampus using 100×oil objective (N.A. = 1.25) with a microscop (Nicon Eclipe E 600) were transferred to a monitor (Sony Trinitron Color Video Monitor PVM - 14N1MDE) using a video camera (Hitachi OSP Color Video CameraVK - C22OE).

NEURONAL COUNTS AND OPTICAL FRACTIONATOR

Sections

The sampled sections were chosen according to the systematic random sampling scheme. The first section in the series to be analyzed was chosen randomly from the first 2-4 sections. This section and every 2th-4th section thereafter was stained for use in the analysis. The section sampling fraction, ssf, is thus 1/2 - 1/4.

Sectional Area

In each of the sections to be sampled, neurons were counted with the optical disector at regularly predetermined x, y axis within the CA1, CA2 and CA3 subdivisions. Step "x" was predetermined to 300 µm and step "y" to 300 µm. Microscopic images obtained from pyramidal cell layers in CA1, CA2 and CA3 subdivisions of the hippocampus using 100×oil objective were transferred to a monitor. An unbiased counting frame (Gundersen's unbiased counting frame) (West *et al*., 1991) was then super-imposed on the monitor image of the section. The area of the counting frame of the disector, a (frame), was known (25 µm ×25 µm = 625 µm^2 or 20 µm ×20 µm = 400 µm^2) relative to the area associated with each x, y movement, a (x, y step). Thus, the areal sampling fraction is (asf) = a (frame) / a (x,y step).

Section Thickness

At each step of the sampled pyramidal cell layer where the neuronal nuclei were observed under the frame, the plane of focus was moved 10 µm from the surface into the section. The counting frame was then focused through 30 µm of the thickness of the section and the number of neuronal nuclei was counted with unbiased counting rules (Q^-). With the optical disector, it is only necessary to determine the first recognizable profile of the nucleus to come into focus within the counting frame. The height of the disector was 30 µm for this study (h = 30 µm).

At each step in the sampled pyramidal cell layer, the distance between the top and the bottom surfaces i.e., the positions at which the neuronal nuclei first came into focus from above and below the section, was determined (Korkmaz and Tümkaya, 1997), indicating that shrinkage is homogeneous within each studied section. The mean thickness of each section was calculated. The fraction of the sampled section thickness is referred to as the thickness sampling fraction (tsf) = h (the height of the disector)/t (the mean thickness of the section).

Neurons were directly counted in the known fraction of CA1, CA2 and CA3 subdivisions of the hippocampus. The number of neurons in the CA1, CA2 and CA3 subdivisions (**N**) was estimated as:

$$\mathbf{N} = Q^- \times (1 / ssf) \times (1 / asf) \times (1 / tsf),$$

Q^-: The total number of neurons counted in the disectors on the sampled sections.

RESULTS

Neurons were counted by applying the optical disector method in the frames determined with the fractionator sampling scheme. We observed that the pyramidal neuron number in hippocampus CA1, CA2 and CA3 subdivisions was significantly lower in the experimental group (mean ± standart error = 142,888 ± 11,745) (Table 1) than in the control group (mean ± standart error = 177,953 ± 10,907) (Table 2), (Mann Whitney U test, $p < 0.05$) (Fig. 1). Our results for the control group differ from those obtained by West *et al*. (1991) for normal Wistar rats using the same stereological strategy. This may be explained by different animal species and age of the rats, different tissue handling protocols and probably different delineation of hippocampal regions between this study and the study by West *et al*.

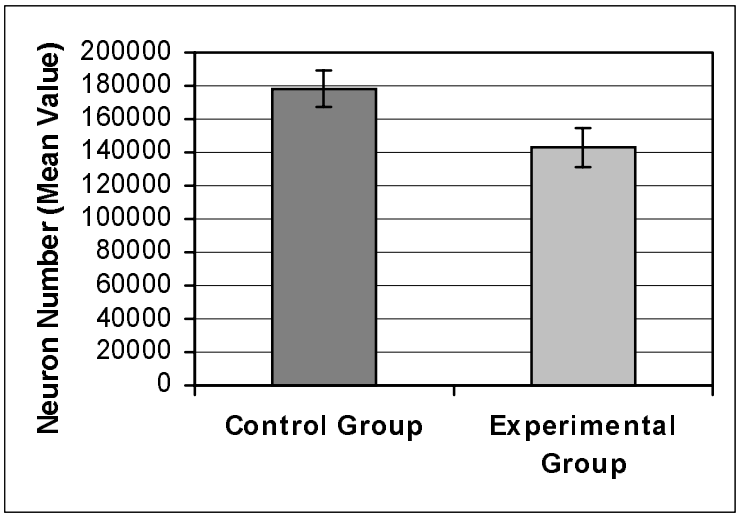

Fig. 1. *Total pyramidal neuron number of CA1, CA2, CA3 subdivisions of the left hippocampus in control and experimental group rats. For both groups n = 6; % difference = %19.71; p < 0.05.*

Table 1. *Total number of neurons of CA1, CA2 and CA3 subdivisions in the left hippocampus in the experimental group (penicillin induced epileptic rats) and parameters estimating total number of neurons.*

	Experimental 1	Experimental 2	Experimental 3	Experimental 4	Experimental 5	Experimental 6
Q^-	128	140	125	117	111	132
1 / ssf	4	2	4	4	4	3
1 / asf	144	225	144	144	144	144
1 / tsf	1.78	2.07	2.14	2.34	2.30	2.40
t (µm)	53.60	62.20	64.40	70.40	69.00	72.00
h (µm)	30	30	30	30	30	30
a (area of the unbiased counting frame) (μm^2)	625	400	625	625	625	625
N (The total number of neurons)	131,235	130,410	154,080	157,697	147,052	136,857

Table 2. *Total number of neurons of CA1, CA2 and CA3 subdivisions in the left hippocampus in the control group rats and parameters estimating total number of neurons.*

	Control 1	Control 2	Control 3	Control 4	Control 5	Control 6
Q^-	179	144	151	147	138	134
1 / ssf	2	4	4	4	4	4
1 / asf	225	144	144	144	144	144
1 / tsf	2.05	2.01	2.07	2.30	2.29	2.32
t (µm)	61.78	60.40	62.20	69.00	68.80	69.60
h (µm)	30	30	30	30	30	30
a area of the unbiased counting frame (μm^2)	400	625	625	625	625	625
N (The total number of neurons)	165,127	166,717	180,040	194,745	182,027	179,066

DISCUSSION

Using the optical fractionator design based method, we showed that the total number of neurons in the CA1, CA2 and CA3 pyramidal cell layers of the hippocampus of penicillin induced epileptic rats is statistically lower than in the control group. This tendency has been acknowledged previously (Dam, 1982). However, because the numerical cell densities reported by Dam (1982) are likely to be 'biased' by tissue shrinkage or swelling (West *et al*., 1991; Oorschot, 1994; Howard and Reed, 1998), we believe that our results are more reliable. Furthermore, our design based method does not require such correction factors (Abercrombie correction factor) as it is the case for the model based method reported by Boss *et al* (1987).

Based on the sole knowledge of the sampled fraction of the CA1, CA2 and CA3 pyramidal layers of the hippocampus, the optical fractionator allowed us herein to estimate the total number (N) of neurons. This design based stereological strategy avoids the bias that may be introduced in approaches where the calculation of N relies on the estimation of a numerical cell density (Nv) and of a reference volume (Vref) as expressed by the formula given in Coggeshall and Lekan (1996) and Gundersen *et al*. (1988): N = Nv x Vref. Indeed, Vref is nearly always estimated on un-embedded tissue section which may not be shrunken, whereas Nv is evaluated on embedded histological sections which typically are shrunken. In such a case, knowing how difficult it is to correct for shrinkage, the calculation of N would be biased.

This leads us to conclude, that the optical fractionator shall be used to estimate objectively and efficiently the neuronal number of the central nervous system.

ACKNOWLEDGEMENTS

Authors thank to Atilla OGUZHANOGLU, Turker SAHINER (Department of Neurology, Medical School of Pamukkale University) and Ender DUZCAN (Department of Pathology, Medical School of Pamukkale University, TURKEY).

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