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Reduction of hippocampal Ca 1 neurons in Wistar rats following the administration of phenytoin for seven days

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Summary

This study evaluated the neurotoxic effect of parenteral Phenytoin on the *Cornu Ammonis 1* (CA 1) region of the Hippocampus in Wistar rats.Twenty wistar rats were randomized into two groups of ten animals each with the experimental group receiving intraperitoneal Phenytoin at a dose of 25mg/kg body weight per day for seven days while the control group had sham injection of normal saline at equivalent volume for the same period. Hippocampal sections were processed for histology using routine paraffin sectioning followed by Heamatoxylin and Eosin staining. There was a statistically significant reduction in the mean body weight of the experimental group compared to the control group. The cell density in the stratum pyramidale (per 0.11mm^2 area of the CA 1 region of the Hippocampus) was reduced in the experimental group when compared to the control group. (P<0.05). The mean brain weight in both groups did not differ significantly. Our findings reveal that the administration of parenteral phenytoin at a dose of 25mg/kg body weight per day for seven days in Wistar rats resulted in reduction of the cell density in the stratum pyramidale of the CA 1 subfield of the Hippocampus in Wistar rats and a reduction in the mean body weight.

Keywords: *Hippocampus, CA 1, phenytoin, neurotoxicity*

Résumé

Cette étude évaluait les effets neurotoxiques de l'injection intraveineuse de la phenytoine sur la région *cornée Ammonit* (CA1) de l'hippocampe chez les rats. Le groupe expérimental recevait la phenytoine intraperitoneale à la dose de 25mg/kg de poids corporelle par jour sept jours et l'autre groupe de contrôle sain recevait une injection d'eau salée normale de volume équivalent à la même période. Les sections d'hippocampe étaient collectés et préparés pour l'analyse histologique en utilisant la dissection routine de paraffine, suivi de la teinture à l'hématoxyline et à l'éosine. Les résultats démontraient une réduction statistiquement significative du poids corporel moyen du groupe expérimenté comparé au groupe de contrôle sain. La densité cellulaire dans le stratum pyramidale (0.11mm^2 d'hippocampe) était réduit au groupe expérimental comparé au groupe sain (P<0.05). Il n' y avait pas de différence significative sur le poids moyen du cerveau chez les 2 groupes. Nos données révèlent que l'administration de la phenytoine parentale à la dose de 25mg/kg de poids corporel pour sept jours aux rats résultait d'une réduction de la densité cellulaire dans la pyramide de l'hippocampe et d'une réduction du poids corporel moyen.

Introduction

The mammalian hippocampus is crucial for learning and memory [1]. The classical studies of Lorent de No [2] led to the characterization of the different sub-regions of the hippocampus into CA 1, (*Cornu Ammonis 1*) CA 2 and CA 3 on the basis of layer arrangement, neuronal size, neuronal shape and axonal growth patterns [2]. Many investigators have demonstrated that the Hippocampal region is one of the most vulnerable portion of the central nervous system during exposure to chemical and physical insults [3].

This vulnerability of the hippocampus is unique because the different sub-regions exhibit selective vulnerability with some regions being more reactive to injurious stimuli than others. Although the exact mechanism of this selective vulnerability is still

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subject of intense debate, the CA 1 subfield is perhaps the most vulnerable part of the hippocampus [4].This subfield provides the major cortical output of the hippocampus with its projections to the subiculum and lateral septal nucleus being well characterized [5].

Phenytoin is a widely utilized medication for the management of seizure disorders and its central nervous system concentration equals that of the unbound fraction in the plasma [6]. Potential neurotoxicity of Phenytoin has been demonstrated in the cerebellum of epileptic patients who were being managed with the drug [7]. Experiments using rodents have shown that the administration of Phenytoin at a dose of 25-50mg/kg body weight resulted in cerebellar Purkinje cell toxicity [8].

There is paucity of literature on the effect of Phenytoin on the Hippocampal CA 1 region. Considering the important function of hippocampus in learning and memory, this study was conducted to investigate the effect of parenteral Phenytoin on the Hippocampal CA 1 region of Wistar rats.

Materials and methods

Experimental animals

Twenty adult Wistar rats were obtained from the animal house of the Faculty of Basic Medical Sciences, University of Ibadan, Nigeria. The animals were randomly divided into two groups of 10 animals per group. The average weight of the animals ranged from 118gms to 142gms. The animals were left for forty-eight hours (48hrs) to acclimatize to animal room conditions and were maintained on standard rat pellet and water *ad libitum*.

All procedures on animal handling conformed to the acceptable guidelines on the ethical use of animals in research. [9].

Phenytoin Preparation and Administration

The experimental group of rats had parenteral phenytoin administered to them intraperitoneally (Parke-Davis pharmaceutical company-batch number 42851 /37/97415178) via a hypodermic syringe with a 28 gauge needle (Tyco Healthcare group LP-28 G×1/2") at a dose of 25mg/kg body weight in a volume of normal saline which corresponds to a dose volume of 2ml/kg body weight. The control group had an intra-peritoneal sham injection of normal saline at a dose volume of 2mls/kg per body weight [8]. The animals had their weights measured at the commencement and the end of the experiment (Day one and seven respectively). The weight of the rats was obtained utilizing a Swiss microwa balance (type 7720)

Histology

At the end of the experiment, the animals were immobilized with Ketamine (10mg/kg, i.m) and their thoracic cavity was dissected out to expose the heart. Cannulation of the heart was subsequently done and intracardial perfusion of 10% formalin was conducted on the animals until they expired [10]. To dissect the perfusion fixed brains, the animal's head was positioned on a modified stereotaxic frame, the scalp was reflected and the brain were dissected out and washed in 10% formalin. These perfusion fixed brains were subsequently placed on a filter paper to mop them dry and weighed using a swiss microwa analytical balance (type 5540)

After weighing, the brains were immersed for 72 hours in 10% formalin for further fixation. The fixed brain tissues were processed with routine Paraffin wax techniques and serial coronal sections of 5 microns thickness were subsequently made using a rotary microtome (Leitz wetzlar TN). Neuroanatomical borders corresponding to the classically defined CA 1 region of the hippocampus were utilized. The sections were stained using the Heamatoxylin & Eosin (H & E) technique.

The density of the stratum pyramidale cells in the CA 1 region was evaluated by counting the cells using a microscope with a graticule attached to the eye piece as described by Blinkov *et al* [11] and modified by Osuagwu *et al* [12]. Neuronal cell count was done utilizing the eye piece of a Leitz wetzlar binocular research microscope at times forty magnification. The radius of the eye piece at times forty was calibrated with a graticule to be 0.19mm and the area of the view at times forty magnification was thus estimated as $0.11mm^2$

To count the neuronal cells in the pyramidal layer, a graticule was aligned along the length of the neurons in the pyramidale layer and counting was conducted along the length of the graticule starting from one end of the graticule to another so as to avoid duplicate counting. A total of 10 separate equidistant areas of graticule space were counted for each slide. The values obtained were expressed as a specified number of cells per $0.11mm^2$ which is the area of the eye-piece at times forty magnification. [12]

Statistical analysis

The results were presented as mean±S.E.M.The data was further analyzed with independent sample t-test and paired sample t-test. Values of $P < 0.05$ were considered to be significant. Computer software SPSS version 11 was utilized for the statistical analyses listed above.

Results

There was no mortality in both groups of animals through out the duration of the experiment and they all tolerated the procedures without any complications. The mean body weight of the control animals on day one of the experiment did not differ significantly (129±1.95gm) from the mean body weight of the animals in the experimental group (128.9±2.6gm) $P>0.952$. However, at the end of the experiment, there was a significant reduction in the mean body weight of the experimental group animals (124.5±2.1gm) when compared to their control group peers (130.5±1.82gm) on day seven.($P<0.04$) Table 1.

Table 1: Mean body weight, brain weight and the neuronal density in the experimental and control groups

Variable	control group (n=10)	experimental group (n=10)	p value
Body weight in grams on Day one	129±1.95	128.9±2.6	0.952
Body weight in grams on Day seven	130.5±1.82	124.5±2.1	0.04
Brain weight in grams on Day seven	1.448±0.01	1.443±0.004	0.53
CA 1 Neuron density per 0.11mm^2 area	7.9±0.23	6.5±0.27	0.001

Table 2: Paired sample t-test comparing the mean weights in grams of both groups on day one versus day seven.

	Mean	S.E.M	P- value
Control			
Day 1	129	1.95	0.01
Day 7	130.5	1.82	
Experimental			
Day 1	128.9	2.6	0.002
Day 7	124.5	2.1	

Intra-group comparison of the mean body weight on day one and seven in the experimental group revealed a significant weight reduction. (128.9±2.6gm vs 124.5±2.1gm) $P<0.002$.(Table 2) The control group animals appeared to gain weight significantly between days one and seven (129±1.95gm vs 130.5±1.82gm) $P<0.01$ (Table 2).

The mean brain weight in the control and experimental groups at the end of the experiment did not differ significantly. (1.448 + 0.01 gm vs 1.443 + 0.004gm) $P>0.53$ (Table 1). Histopathological evaluation of the sections revealed homogenous staining in both groups. The anatomic arrangement of the hippocampal layers was preserved. (*Stratum oriens, pyramidale, radiatum, lacunosum, moleculare*).There was hypodensity of the pyramidal layer neurons in the experimental group (Figure 2). The pyramidal layer neuron density in the control group appeared adequate (Figure 1).

There was a statistically significant decrease in the density of the cells in the stratum pyramidale (per 0.11mm^2 area of the CA 1 region of the Hippocampus) in the experimental group compared to the control group (6.5±0.27 vs 7.9±0.23) p=0.001 (Table 1).

Discussion

The vulnerability of the hippocampal CA 1 pyramidale layer neurons to various kinds of physical and chemical injury has attracted previous investigations [13]. The importance of the CA 1 subfield of the hippocampus is further highlighted by the fact that it provides the major cortical output of the hippocampus with its projections to the subiculum and lateral septal nucleus being well characterized [5]. Phenytoin is a routinely utilized anti-epileptic medication [6]. The neurotoxic effect of Phenytoin on the cerebellar Purkinje cells in rodents has been reported [8]. This study was aimed at evaluating the effect of Phenytoin on the CA 1 neurons of the hippocampal region of wistar rats.

Our findings from this work revealed a significant reduction in the mean body weight of the experimental group animals at the end of the experiment. This is in agreement with previous workers who have reported similar reduction in mean body weight of animals that had parenteral phenytoin administered to them at a dose of 35mg/kg body weight [8]. The mechanism by which phenytoin brings about this action is highly contentious but some authors have suggested that it may be connected with the ability of phenytoin to alter serum thyroxin levels [14].

The total brain weight in both groups did not differ significantly at the end of the experiment on day seven. This finding differs from the works

ofprevious workers who reported a significant reduction

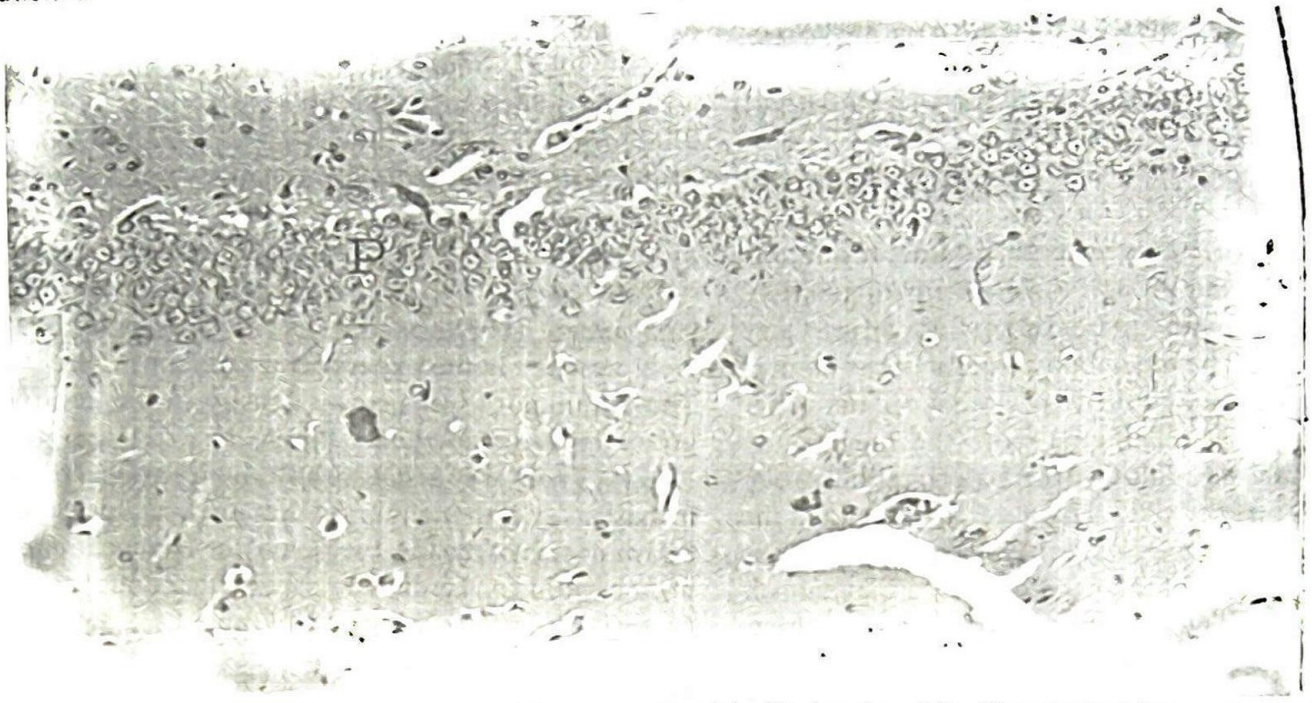

Fig. 1: Photomicrograph sho dale neurons (P) of the CA 1 region of the Hippocampus in t ont roup animals. The neuronal density is adequate

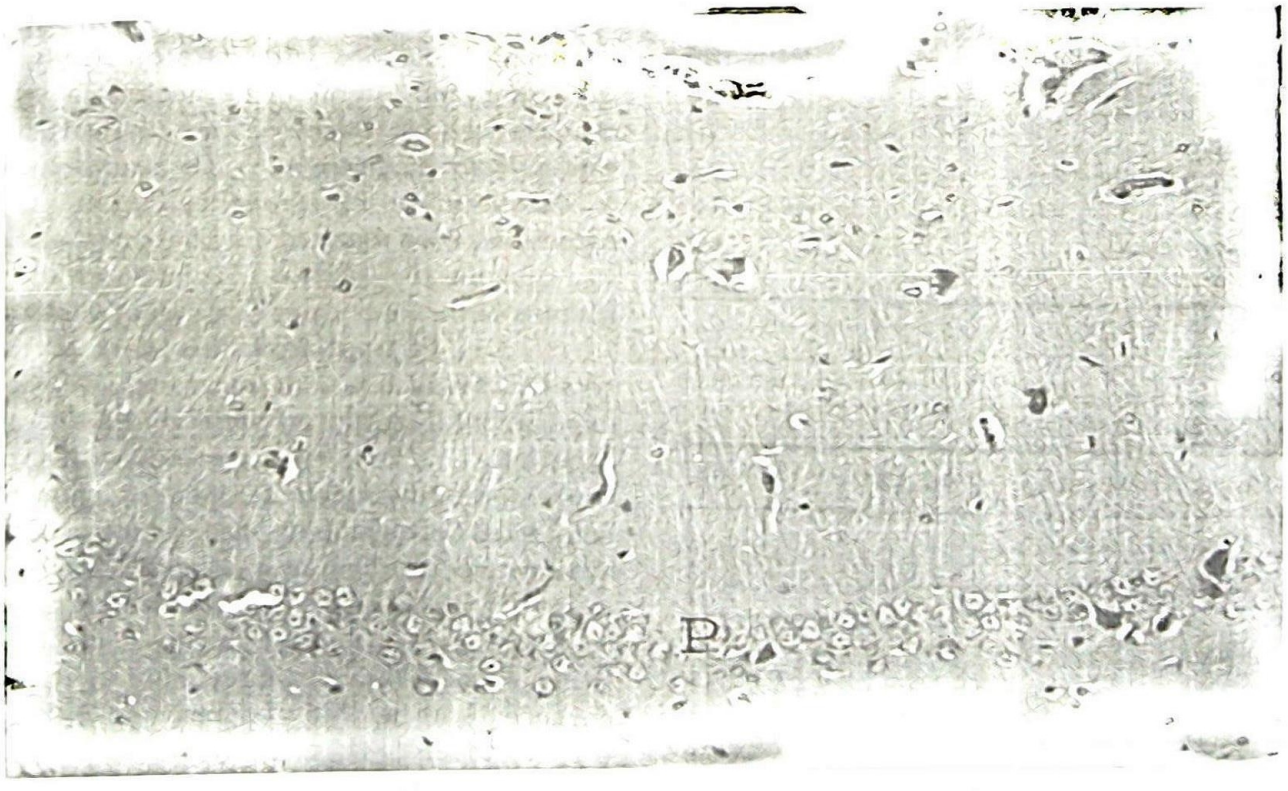

Fig. 2: Photomicrograph showing the pyramidale neurons (P) of the CA 1 region of the Hippocampus in the experimental group animals. There is hypodensity of the neurons.

in the total brain weight of neonatal pups treated with Phenytoin compared to the control group after fifty-six days post treatment [8]. The fact that the animals in our study were adult Wistar rats that had Phenytoin administered to them for just seven days unlike the animals reported above may account for this lack of difference in the total brain weight of the experimental and control animals.

The results of the present study indicate that in the hippocampal formation, CA 1 neurons are depleted in the experimental group when compared to the control group. This finding is in agreement with previous studies showing that the CA 1 neurons are easily vulnerable to insults [15]. CA 1 neurons are the most vulnerable portion of the hippocampus and the phenomenon of selective vulnerability, sometimes designated as 'pathoclisis' have been speculated to be as a result of zonal differences in metabolism [16]. The report by Sommer showing loss of neurons in the CA 1 region of epileptics [17] makes our findings of loss of neurons in CA 1 region of the animals that had phenytoin administered to them more pertinent.

Limitations of this study include the fact that acute administration of phenytoin might not stimulate human conditions where phenytoin treatment is most likely to be chronic. Also, extrapolations from this animal based study to humans may be limited by subtle differences in the neural anatomies of rodents and humans but the preliminary findings presented in this study should form a basis for future studies based on primate brains as a prelude to conducting studies on autopsy specimens of humans who were on phenytoin. We believe this will provide a better understanding of the effects of Phenytoin on the Human Hippocampal CA 1 region.

Conclusion

Our findings suggest that administration of phenytoin at a dose of 25mg/kg body weight to Wistar rats for seven days led to a reduction of the mean body weight and the cell density of the pyramidal cell neurons in the CA 1 region of the hippocampus of Wistar rats and further studies are required to evaluate the functional implications of this findings.

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