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Pyramidal cell morphology and cell death in the hippocampus of adult mice with experimentally induced hydrocephalus

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Abstract: *Background:* Hippocampus is a neural structure in the temporal lobe that plays a crucial role in learning and memory. Cognitive impairment with learning disabilities is a common feature in hydrocephalus and is more prominent in adult-onset hydrocephalus. The aim of this study is to describethe morphological alterations in the pyramidal cells of the hippocampus of adult hydrocephalic mice.
Method: Hydrocephalus was induced in adult albino mice by intra-cisternal injection of kaolin suspension (250 mg/ml in sterile water). They were sacrificed 7, 14 and 21 days post-induction. Morphological analysis was carried out on hematoxylin and eosin-stained coronal sections of the hippocampus: the pyramidal neurons (normal and pyknotic) in the CA1 and CA3 subregions were counted and the pyknotic index (PI) was calculated. The somatic and dendritic features of Golgi-stained pyramidal neurons were examined by light microscopy in both hydrocephalic and control mice.
Result: The PI was significantly greater in the CA1 region of the hippocampus in the hydrocephalic groups compared to the age-matched controls. The dendritic processes of pyramidal neurons in the CA1 region were fewer with shorter terminal branches in the hydrocephalic mice than in controls; this was pronounced at 7 days post-induction. In the CA3 region, there was no difference in dendritic arborization between hydrocephalic and control mice.
Conclusion: Acute adult-onset hydrocephalus was associated with increased pyknosis and reduced dendritic arborization in hippocampal pyramidal cells in the CA1 but not CA3 region.

Keywords: Hippocampal pyramidal cell, Hydrocephalus, Pyknotic index, Golgi stain

Introduction

Hydrocephalus is an abnormal accumulation of cerebrospinal fluid (CSF) in the brain, usually caused by an imbalance between secretion and reabsorption of this fluid.[1] This disorder is a major clinical burden in neurological practice in Africa. Its frequency among other neurological diseases ranges between 13-26%.[2,3] It is mostly a disorder of children with an incidence of 0.48 to 0.81 per 1000 live births and may manifest in the fetal, perinatal and neonatal period.[4] Its occurrence in adults often mimics other neurological diseases in which cognition is impaired, such as Parkinson's disease and Alzheimer's dementia.[5] In hydrocephalus, the severity of the injury to the brain is dependent on the age of onset, the magnitude of the ventricular enlargement and the etiology.[6]

Studies in human and animal models have shown that the hippocampus is vulnerable to injury in hydrocephalus, however the degree is time dependent.[7,8,9] The pathogenesis of brain injury in experimental hydrocephalus has been well studied. The most common method of inducing experimental hydrocephalus is by intracisternal injection of sterile kaolin (aluminum silicate).[10,11,12] Kaolin deposited at the base of the fourth ventricle spreads in the subarachnoid space, where it induces an inflammatory reaction and fibrous scarring in the meninges. This resembles the scarring that develops following meningitis or hemorrhage and leads to an obstruction of the CSF pathways close to the fourth ventricular apertures with ensuing ventricular enlargement.[13] Although this model cannot fully mimic the human condition, it has contributed significantly to our understanding of the pathogenesis of hydrocephalus and potentially to future effective therapies.[14,15,16,17]

Hydrocephalus is most usually characterized by obstruction of the flow of cerebrospinal fluid leading to enlargement of brain ventricles. The pathophysiology of hydrocephalus is multifactorial in nature and consists of primary mechanical injury from stretching of periventricu

periventricular white matter fibers by the dilated ventricles, and secondary injury from ischemic and metabolic epiphenomena.[18,19,20] The hippocampus, especially its CA1 region, is known to be very susceptible to hypoxia and ischemia, and is an important target for the secondary injury originating from hydrocephalic process.[8] Studies have reported neuronal death during the early stage of hydrocephalus which indicate toxic neuronal response to ischemia. Dark pyramidal neurons, reduction in synaptic contacts and hydropic cellular changes in the dendritic processes of neurons have been reported in humans and in animal models.[9,21] However, little is known of the impact of hydrocephalus on the dendritic arbors of the pyramidal neurons in the hippocampus. These are the primary structural specializations for the reception of afferent information which modulate the efferent output of these cells.

In this study, we assessed the injury to the pyramidal cells of the hippocampus in hydrocephalic adult mice us, by examining dendritic morphology and cell death in this population of neurons.

Materials and methods

A total of 70 adult albino mice (49 experimental and 21 control) were used for this study. They were obtained from the central animal house of the Faculty of Basic Medical Sciences, the University of Ibadan. All procedures on animal handling conformed to the guidelines on the ethical use of animals in research. The mice were anaesthetized with intraperitoneal injection of ketamine/ xylazine combination (90/10 mg/kg). With the neck of the mouse in a flexed position, the skin covering the sub -occipital region was shaved, incised and retracted laterally to reveal the rhomboid depression marking the cisterna magna. With a 27- gauge needle, 0.02ml of sterile kaolin suspension (250 mg/ml in sterile water) was slowly injected into the cistern magna. A sham injection was performed for controls in which the cisterna magna was simply punctured. The animals were monitored for about 1hour and returned to their cages. They were housed in groups of six and allowed free access to water and solid pellet feed. They were weighed twice weekly and assessed for signs of development of hydrocephalus such as increased head circumference, affected gait and dull general appearance.

The mice were euthanized in groups on days 7, 14 and 21 post induction of hydrocephalus (designated as E1, E2 and E3 respectively) by transcardial perfusion with 10% formalin. The cranial cavities were opened immediately after perfusion, and the brains removed and stored overnight at 4°c in the same fixative. Subsequently, the brains were sliced coronally at the level of the optic chiasm and blocked in order to obtain tissue samples from hippocampus. Tissue samples were prepared separately for hematoxylin and eosin (H&E) and Golgi staining. The Golgi method was used to demonstrate individual pyramidal cells together with their neural, particularly dendritic processes.

Hematoxylin and eosin staining

The fixed brain specimens were dehydrated in graded series of alcohol, cleared in xylene, infiltrated in molten paraffin wax, embedded. Coronal sections were obtained at the level of optic chiasma and stained with hematoxylin and eosin. Three brain specimens were randomly selected from each of the groups for quantitative analysis. Neuronal counts (of normal and pyknotic neurons) were obtained from the *Cornus ammonis* (CA)1 and three regions of the hippocampus using a digital light microscope (Leica, Germany) at x400 magnification. For each animal, average neuronal counts were obtained by counting four serial coronal sections using a standardized square of 1200mm² with a Motic images plus 2.0 SET UP and pyknotic index as previously used by Taveira et al.[9] and obtained as follows:

$$\text{Pyknotic index (PI)} = \frac{\text{pyknoticneurons}}{\text{Total neurons}} \times 100$$

Golgi Stain

A subset of three brain samples randomly selected from each of the groups were processed for Golgi stain. The fixed tissues were immersed in potassium dichromate solution (3g/100ml of distilled water) for 5 days (with replacement every 24 hours). They were transferred into silver nitrate solution (2g/100ml) for 3 days (with replacement every 24 hours), infiltrated and embedded in paraffin wax. The paraffin blocks were sectioned at 60µm, dehydrated in increasing concentrations of alcohol, cleared in xylene, mounted on adhesive glass slides and cover-slipped with DPX. The sections were viewed under a light microscope at ×400 magnification. Isolated pyramidal cells which had their cell body and processes well delineated were singled out for qualitative/ morphological assessment.

Data analysis

Quantitative data from the tissue sections were statistically analyzed using the Graph Pad Prism version 5.0 for Windows, Graph Pad Software (San Diego, California, USA). Sample means generated after a statistical test to ascertain normal distribution, were compared among the various groups using analysis of variance (ANOVA) and Student t- test with confidence interval calculated at 95% and level of significance fixed at 5%.

Results

Physical observations

The hydrocephalic mice exhibited a general reduction in activity and food intake; developed a dome shaped head usually with varying degrees of unsteady gait and hunched back (Figure 1).

There was an initial reduction in body weights of the hydrocephalic mice two to four days post- induction, then a slow but steady weight gain was recorded over the next three weeks. When compared to their controls, there was a significant reduction in the body weights of

hydrocephalic mice on the 14 and 21 days post-induction ($p < 0.05$) (Figure 2).

Fig 1: Photographs showing the effect of hydrocephalus on the animal (a) hydrocephalic mouse (b) Normal mouse

Fig 1a **Fig 1b**

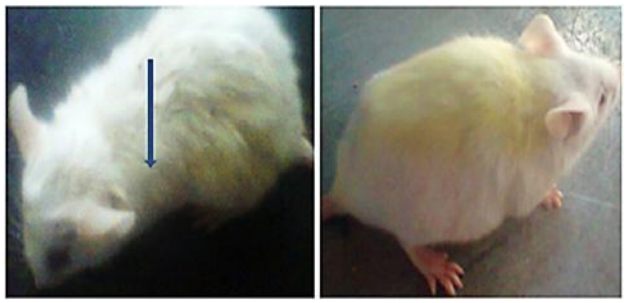
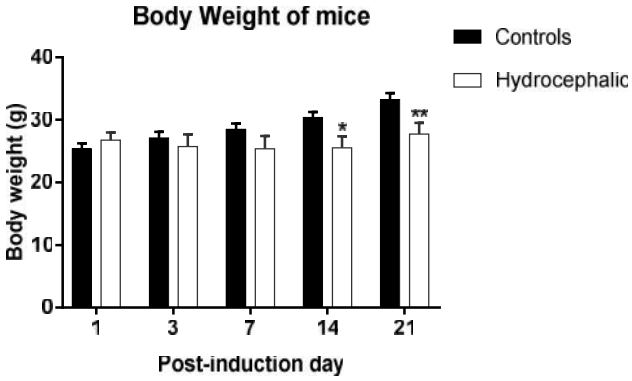


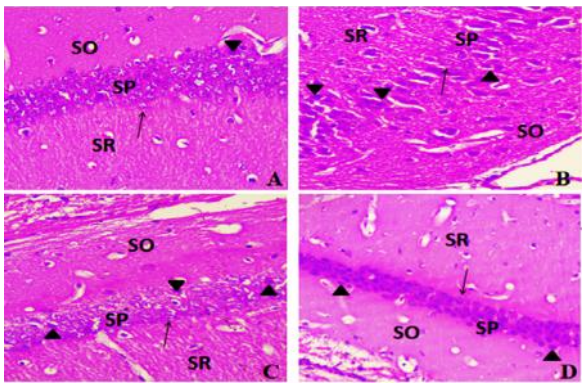
Fig 2: Bar chart showing the weight (g) of control and hydrocephalic mice over a duration of three weeks post induction



Histological analysis

Hematoxylin and Eosin staining of the hippocampus of the control mice brains revealed a compact layering of pyramidal cells and a full neuronal cell population with well-defined nuclei. In the hydrocephalic groups, mild disarray of the pyramidal cells layering, many pyknotic cells with dark shrunken nuclei and abnormal clumping of chromatin were observed (Figure 3). These pyknotic pyramidal cells were particularly evident in the CA1 region of the hippocampus.

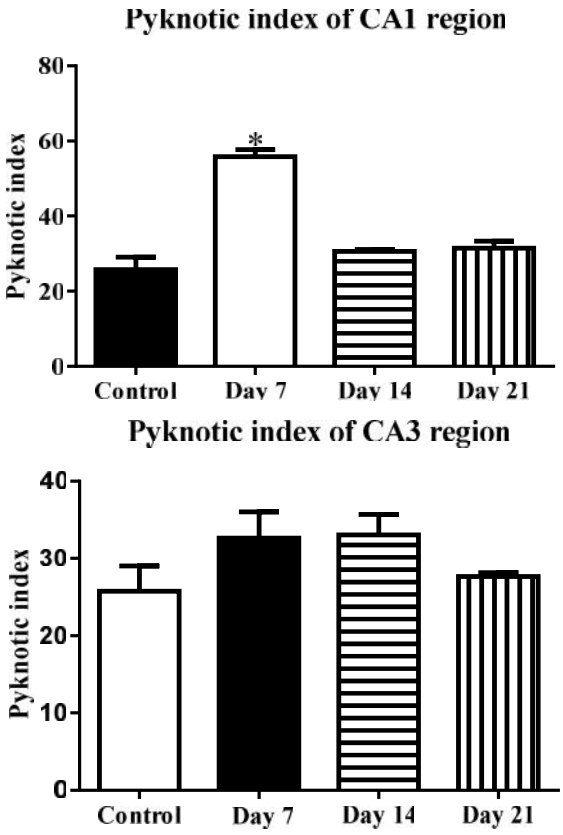
Fig 3: Representative stained sections of *CornusAmmonis* 1 of adult mice hippocampus: (A) Control group (B) E1- 7days post-induction (C) E2-14 days post-induction (D) E3- 21 days post induction. SO, Stratum oriens; SP, Stratum pyramidalis; SR, Stratum radiatum. Dark pyramidal neurons (arrowheads); Normal pyramidal neurons (arrow). H&E.



Neuronal counts

The PI was increased in the CA1 subarea of hippocampus of the hydrocephalic animals. Comparison between the experimental groups revealed a significant increase of the PI in the E1 hydrocephalic group compared to controls, but not in the E2 and E3 groups. The P1 of CA3 subarea did not differ significantly between experimental and control groups at all the time points after induction (Figure 4).

Fig 4: Figure 4: The pyknotic index (PI) of the CA1 and CA3 of the hippocampus of control and hydrocephalic mice / graphical representation of the pyknotic of the CA1 and CA3 of the hippocampus of control and hydrocephalic mice



Pyramidal neuronal morphology

Well-defined pyramidal cells with pear- shaped soma were observed in the control animals. Each soma gave rise to a single apical dendrite which coursed through the surface of the hippocampus and dividing into several terminal branches. The neurons in the hydrocephalic animals demonstrated somatic features similar to that of controls i.e., there were no observable differences in the size and structure of the somata of the pyramidal cells. However, in the hydrocephalic group, neurons possessed apical dendrites with sparse or no terminal branches at all.

Compared to the controls, the dendritic arborizations of the pyramidal neurons in the CA1 region in all the hydrocephalic groups were shortened, but this was most striking one week post induction (group E1). The apical dendrites gave rise to fewer and shorter terminal

branches from the first to the third week post induction (Figure 5). In the CA3 region, no difference in the pattern of dendritic arborization was observed on comparison between the hydrocephalic and control groups (Figure 6).

Fig 5: Representative Golgi stained sections of *CornusAmmonis* 1 region of mice hippocampus: (Aa-Ac) CTRL group; (Ba-Bc) E1 - 1 week post induced hydrocephalus; (Ca-Cc) E2- 2 weeks post induced hydrocephalus; and (Da-Dc) E3- 3 weeks post induced hydrocephalus. Upper panel, Aa-Da represents all the groups at × 100 magnification; middle panel, Ab-Db are at ×400 magnification while the lowermost panel, Ac-Dc are at ×1000 magnification. Apical and basal dendritic arborization appear reduced in all experimental groups but is most pronounced in E1 as shown in Bb and Bc.

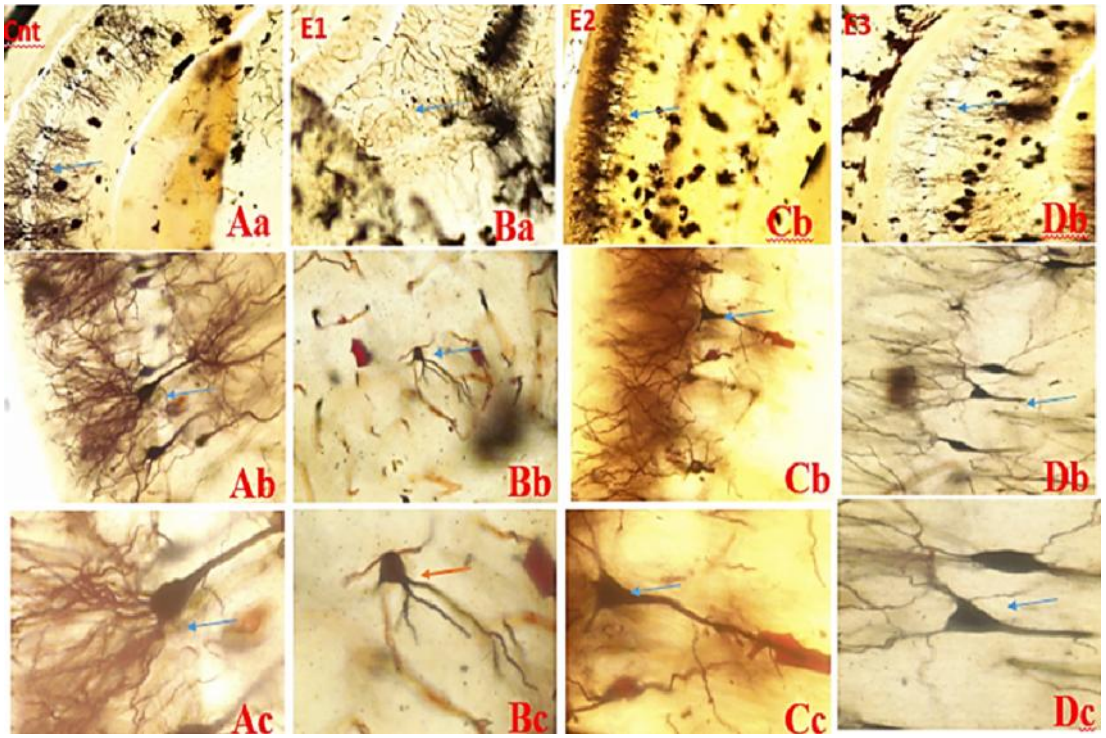
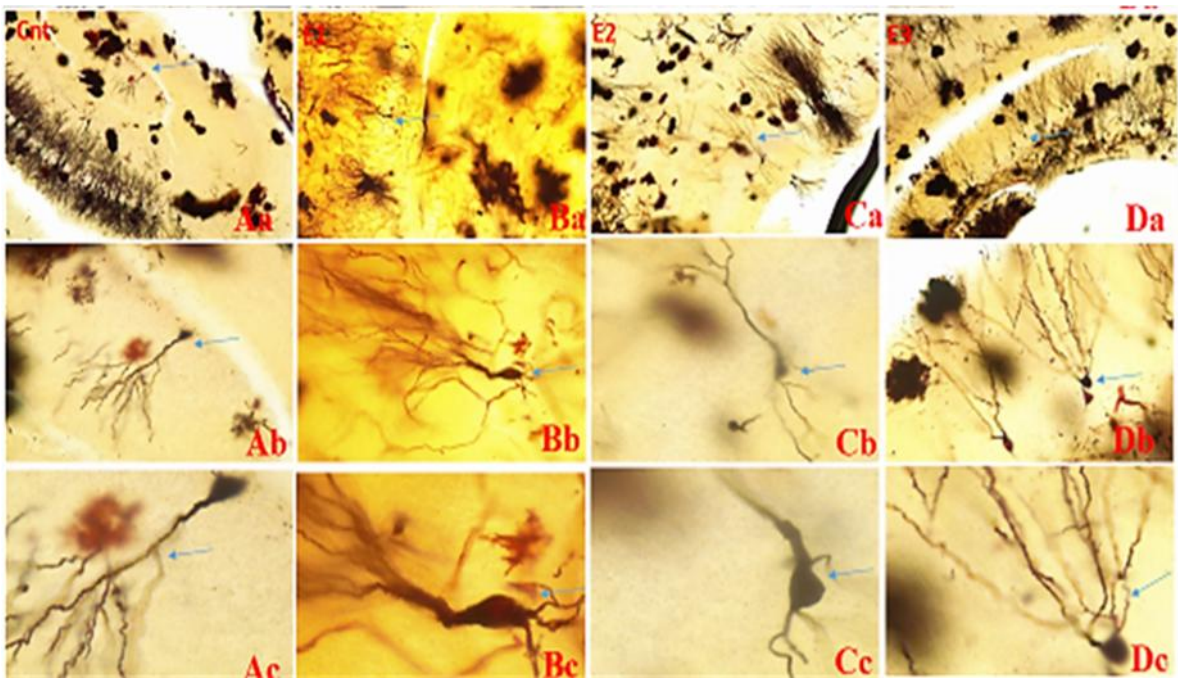


Fig 6: Representative Golgi stained sections of *CornusAmmonis* 3 of mice hippocampus: (Aa-Ac) CTRL group; (Ba-Bc) E1- 1 week post induced hydrocephalus; (Ca-Cc) E2- 2 weeks post induced hydrocephalus; and (Da-Dc) E3- 3 weeks post induced hydrocephalus. Upper panel, Aa-Da represents all the groups at ×100 magnification; middle panel, Ab-Db are at ×400 magnification while the lowermost panel, Ac-Dc are at ×1000 magnification. No observable differencein the pattern of dendritic arborization across the groups.



Discussion

In this study, we report that induction of hydrocephalus in adult mice led to reduced body weight, increased pyknotic index and reduction in the dendritic arborization of the pyramidal cells in the CA1, but not CA3 region of the hippocampus. Intracisternal injection of kaolin in adult albino mice resulted in the development of hydrocephalus. Several studies have successfully induced hydrocephalus in experimental animals with Kaolin.[7,9,22,23] About 75% of the experimental animals developed a hunched back and unsteady gait. However, it was observed that even though an enlarged head was not present in some of the mice, their ventricles were enlarged; this is similar to our previous study.[22] This finding was especially noteworthy in this study because the mice were adults and it is known that cranial sutures except the posterofrontal sutures remain patent throughout life in mice.[24]

The initial loss of body weight observed in the hydrocephalic mice was most likely due to loss of appetite and reduced feeding activity in the early phase of induction of hydrocephalus. Following this, there was steady weight gain in the hydrocephalic mice, but they still weighed significantly less than the controls throughout the duration of the study. Delayed growth is reported to be one of the first signs of hydrocephalus in rodents.[25]
Pyknotic neurons were found to be significantly increased in the hippocampal CA1 region of hydrocephalic groups when compared with their controls. Previous studies reported the vulnerability of CA1 to ischemia.[8,26] Ischemia is a restriction in blood supply to tissues which causes a shortage of oxygen and glucose needed for cellular metabolism.[27] Though we did not examine the blood vessels in this study, others have reported cerebrovascular compression with reduced flow of blood to the hippocampus in hydrocephalus resulting in impairment of protein synthesis and disturbed energy metabolism.[28,29]

The sensitivity of CA1 to hypoxia and ischemia caused by hydrocephalus could likely account for the increased pyknotic index observed in this region. Though a number of dark neurons were also seen in the CA3 region of hydrocephalic mice, this was comparable to what was observed in the controls. This suggests that in hydrocephalus in mature rodents, pyramidal cell injury in the hippocampus is more pronounced in the CA1 region. Kriebel and McAllister[21] who studied the hippocampus in hydrocephalic young cats similarly found pyknotic neurons dispersed in the neuropil (dense network of neuron and glia). Cabuk*et al*.[8] proposed that the hydrocephalic process affects hippocampal pyramidal cells by the mechanism of excitotoxic injury through elevation of extracellular glutamate secondary to the activation of nitric oxide synthase (NOS). NOS is in selective neurons in the brain and plays an important role in mediating excitotoxic injury due to decrease in cerebral blood flow.

Kriebel and McAllister[21] have suggested that the dark neurons observed in the hippocampus in hydrocephalus could be a result of deafferentation arising from stretching of the periventricular axons; although they only examinedthe CA3 subarea. Contrarily, Del Bigio*et al*.[7] proposed that the cell death could be due to the destruction of fimbria-fornix connections, rather than a direct effect on the hippocampus. In this study the pyknotic index was found to be most increased in the subarea CA1, similar to the findings of Chen et al.[30], who reported that increased dark pyramidal neurons in the CA1 region correlated with the reduction in the expressions of presynaptic vesicle marker synaptophysin and glutamatergic postsynaptic density marker PSD95 in the region, thus indicating loss of excitatory connectivity. This supports the assertion that pyramidal cell death in the CA1 subregion may be due to other mechanisms involved in neuronal death other than deafferentation.[9] It also suggests that the pyramidal neurons in the CA1 region are more susceptible to the hypoxic and ischemic effects of hydrocephalus than the pyramidal neurons in the CA3 region.

Silver-staining techniques have been used as a sensitive method to determine neuronal damage in animal brains.[31,32,33] This method is used in observation of individual cells together with their projections particularly dendritic processes. Golgi analysis revealed differences between the hydrocephalic mice and their controls in the somatic and dendritic morphology of pyramidal neurons from the hippocampus. These differences were observed in the neurons within the CA 1 region which had fewer dendritic arborizations and shorter terminal branches.

This is similar to our earlier study of the sensorimotor cortex in neonatal mice, although we reported diminished arborization in the basal but not apical dendrites.[34] Being the major output of the hippocampus, morphological changes in the CA 1 pyramidal neurons provide structural data to explain deficits in learning and memory.[35] Dendrites are the branched projections of a neuron that act to propagate the electrochemical stimulation received from other neural cells to the cell body, or soma, of the neuron from which the dendrites project.[36] Therefore, if this structure is implicated, it may compromise the function of a neuron.

The changes observed in the apical dendrites of the hydrocephalic mice could be as a result of increased pressure or mediated by a secondary mechanism and may account for some of the cognitive impairment observed in hydrocephalic patients.[37]Studies such as those by Kriebel and McAllister[21] have suggested that dark neurons observed in hydrocephalus may contribute to the harmful effect on learning and memory.

However, since the insult which resulted in damage to parts of the neuron (i.e. the dendrites, in this case) could eventually lead to neuronal death, this could actually be part of a continuum.
Qualitative study of the Golgi stained hippocampal pyramidal cells showed that the apical dendrites in the 1-week post induction group were shorter and fewer than

in the 2-weeks and 3-weeks post-induction groups. This is consistent with by the fact that the PI was significantly increased at 1 week in hydrocephalic mice, thus showing that hippocampus was greatly affected early in the hydrocephalus. At 2 weeks post-induction, however, longer apical dendrites with reduced arborization were observed, compared to the control. This suggests that there may be some recovery later in the evolution of the process.

Several studies have reported similar findings at different timelines during hydrocephalus. Since it has been reported that one of the factors that determines the severity of brain injury in hydrocephalus is the age of the onset, it is interesting to note that our study presented the same pattern of damages as reported by other studies carried out at different ages of induction of hydrocephalus.[8,9,28,30] It has been reported that the effect of hydrocephalus on the dendritic arbors of CA1 region in juvenile Wistar rat was more prominent than that of the CA3 subregion.[30] A more detailed study is needed that would look into magnitude of ventriculomegaly and the differential effects of hydrocephalus across the *Cornu Ammonis*1 to 3 regions.

Conclusion

In this study, hydrocephalus resulted in alterations in the morphology of the hippocampal pyramidalcells, specifically attenuation of the dendrites and their arborizations. Such alterations provide a structural link between the disease and the frequently observed deficits in learning and memory. Further studies are needed to clarify and amplify this relationship.

Conflicts of Interest: None
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