

## HIPPOCAMPAL NEURONAL LOSS IN THE CA1 AND CA3 AREAS OF ALZHEIMER'S DISEASE PATIENTS

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### SUMMARY

**Background:** It is believed that in Alzheimer's disease (AD) some areas of the brain are particularly vulnerable to specific degenerative processes and that they could exhibit neuronal dysfunction in the earliest stage of the disease. The implications of the hippocampus in memory processes are very well known and it is likely that the hippocampus would be among the first areas of the brain affected by the pathogenic mechanisms occurring in AD. However, the distinction between the neurodegenerative changes that accompany normal ageing and those that characterize AD is not clear. Also, the distribution of the hippocampal cell loss in both normal aging and AD is not very well understood.

**Subjects and methods:** In this context, we focused on the quantification of the neuronal density in the four specific areas of the hippocampus (CA1-CA4) of AD brains, as compared to an age-matched control group, by using the Nissl staining technique.

**Results:** We found a significant reduction of neuronal density especially in the CA1 and CA3 hippocampal areas. The most prominent decrease was found at the CA1 area level, as compared to all other 3 areas which were analyzed.

**Conclusions:** In the present study we managed to demonstrate and confirm a significant neuronal loss of hippocampus in AD, as compared to an age-matched control group. Moreover, it seems that this decrease of hippocampal neuronal density is more prominent especially at the CA1 and also in the CA3 hippocampal areas. This could have important implications in the design of therapeutic and investigative strategies of AD. However, larger samples are necessary in order to provide the basis for firmer conclusions in this area of research.

**Key words:** Alzheimer's disease – hippocampus - CA1 – CA4 areas - Nissl staining

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### INTRODUCTION

Alzheimer's disease (AD) is one of the most frequent causes of dementia, representing 50 to 60% of all dementia cases and affecting 10 to 20% of people older than 65 years (Talmelli et al. 2010). This progressive neurodegenerative disease, characterized by a global cognitive decline, behavioral and functional changes has a great impact on the ability of individuals to perform basic activities of daily living (Inouye et al. 2010, Ciobica et al. 2012). Although many intellectual functions are impaired (attention, orientation, language, judgment) the most prominent symptom of AD is represented by a progressive memory loss.

The various neuropathological alterations within the AD brains include the accumulation of extracellular neuritic deposits and intracellular neurofibrillary tangles, diffuse cerebral atrophy, enlargement of the ventricles associated with a decrease in neuronal synapses, loss of dendritic spines and furthermore a neuronal reduction (Zhang et al. 2011). The neuronal loss is a common pathway for a large number of degenerative processes in AD (van de Pol et al. 2006, Spires et al. 2006) and can be triggered by various factors, such as  $\beta$  amyloid plaques, perturbed calcium

regulation, glutamate, ischemia, inflammatory processes or oxidative stress (Baloyannis 2006, Padurariu et al. 2010, Ciobica et al. 2011).

It is believed that some areas of the brain are particularly vulnerable to the aforementioned specific degenerative processes and that they could exhibit neuronal dysfunction in the earliest stage of the disease (Desikan et al. 2010, Kelley et al. 2011). Hence, the cortex and the hippocampus demonstrate some of the earliest damages in AD (Henke et al. 1999, Herholz and Zanzonico 2010).

The implications of the hippocampal region in the mechanisms of spatial, semantic and episodic memory are very well known (Mavrogiorgou et al. 2011). Therefore, it is likely that the hippocampus would be among the first areas of the brain to be affected by the pathogenic mechanisms occurring in AD. However, the distinction between the neurodegenerative changes that accompany normal ageing and those that characterize AD is not clear (Korbo et al. 2004). Of course, this aspect has important implications for the design of therapeutic and investigative strategies. Additionally, most of the studies focused extensively on  $\beta$  amyloid deposits and neurofibrillary pathology in AD, whereas neuronal loss has been more difficult to evaluate (Simic

et al. 1997). Also, the distribution of the hippocampal loss in both normal aging and AD is not very well understood (West et al. 1994). It may be that this regional selectivity of neuron loss could be relevant for understanding the mechanism involved in AD.

In this context, the present study will focus on the quantification of the neuronal density in the four specific areas of the hippocampus (CA1-CA4) of AD brains, as compared to an age-matched control group.

## SUBJECTS AND METHODS

The present study was established on the morphological analysis of four human brains: 2 from patients who suffered from AD (2 males; 79.1 years  $\pm$  4.9) (and 2 healthy individuals who died accidentally (1 female and 1 male; age: 74.9 years  $\pm$  9.2) obtained from the laboratory of Forensic Medicine and Toxicology of the Aristotelian University of Thessaloniki, Greece.

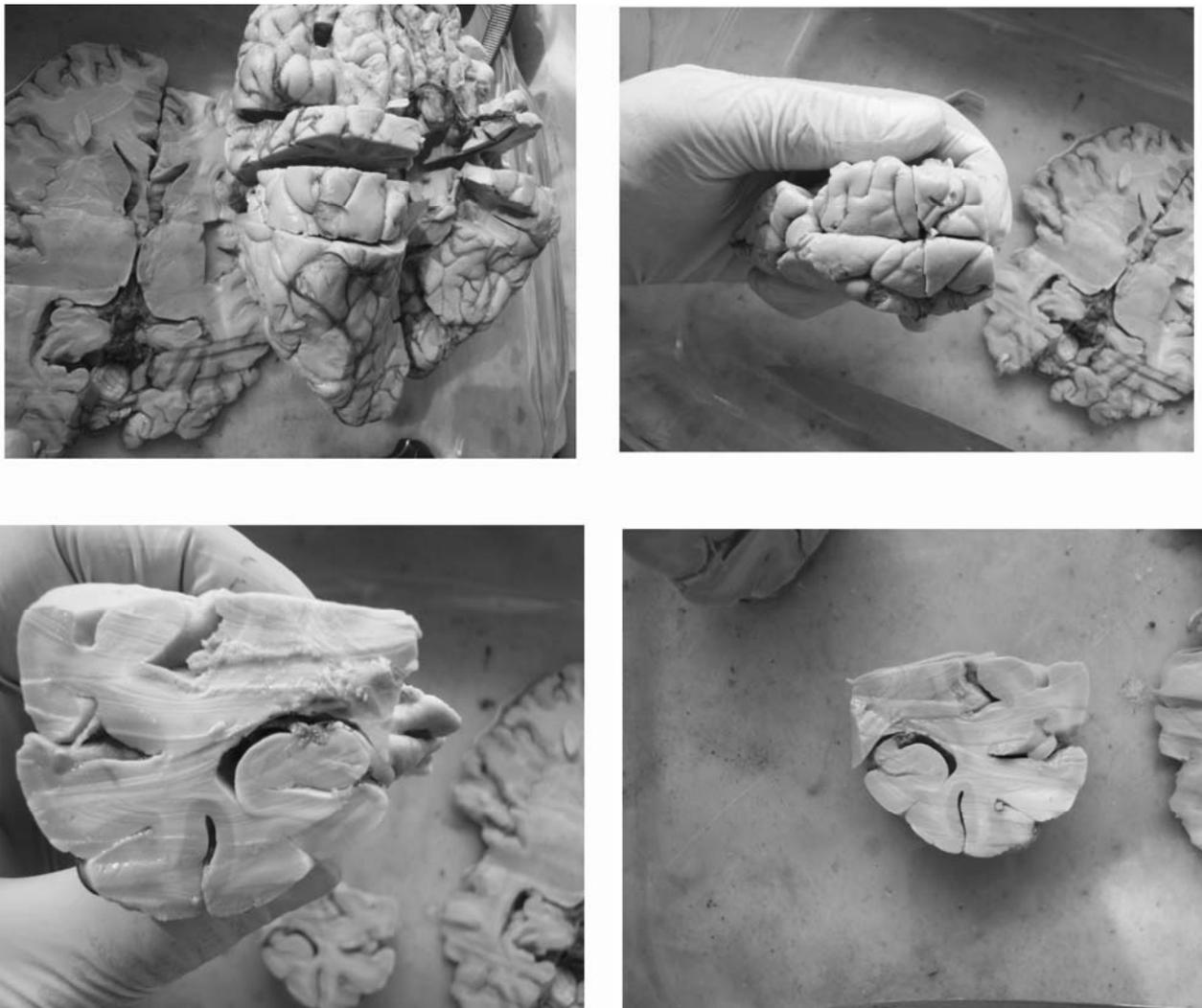
Histological criteria for the diagnosis of AD were those outlined by the National Institutes of Health/American Association of Retired Persons (NIH/AARP) Research Workshop on the Diagnosis of Alzheimer's

Disease (Khachaturian, 1986). They also fulfilled the histological criteria for AD.

The brains were immersed immediately after the autopsy in a fixing solution of 10% of formaldehyde at room temperature.

We collected parts of the hippocampus (Figure 1) which were dehydrated in a graded series of alcohol solutions for 3 days, put in xylene for 24 more hours and then embedded in low melting point paraffin cubes. Afterwards we cut sections with a microtome at a range of 8 $\mu$ m, which were put on microscope slides and after a rapid rehydration were placed in 1% Methylene Blue for 30 minutes. Then the slides were dehydrated, put in xylene for 5 minutes, covered with entellan, cover-slipped and then studied with a Nikon Eclipse 200 photomicroscope.

This technique allowed us to visualize all neuronal somata and was used to provide an accurate idea of the neuronal densities and neuron diameters in the aforementioned hippocampus areas. The density and the size of the neurons were measured on 40 pictures at standard magnifications of x40, using the cell counter plug-in of Image J (by using the masks of our initial pictures - Figure 2).



**Figure 1.** Several steps in collecting the hippocampus and specific areas

For each of the brains, the density of neurons has been counted on blocks of 150 x 150  $\mu\text{m}$  (Mavroudis et al. 2010). The diameter of the neuronal soma was also measured in photomicrographs of the Image J application, after a calibration of the program for the specific types of microscope and microscope camera that have been used.

For the statistical analysis we used the Student's t test (values for  $P < 0.05$  were regarded as statistically significant). All results are expressed as mean  $\pm$  SD.

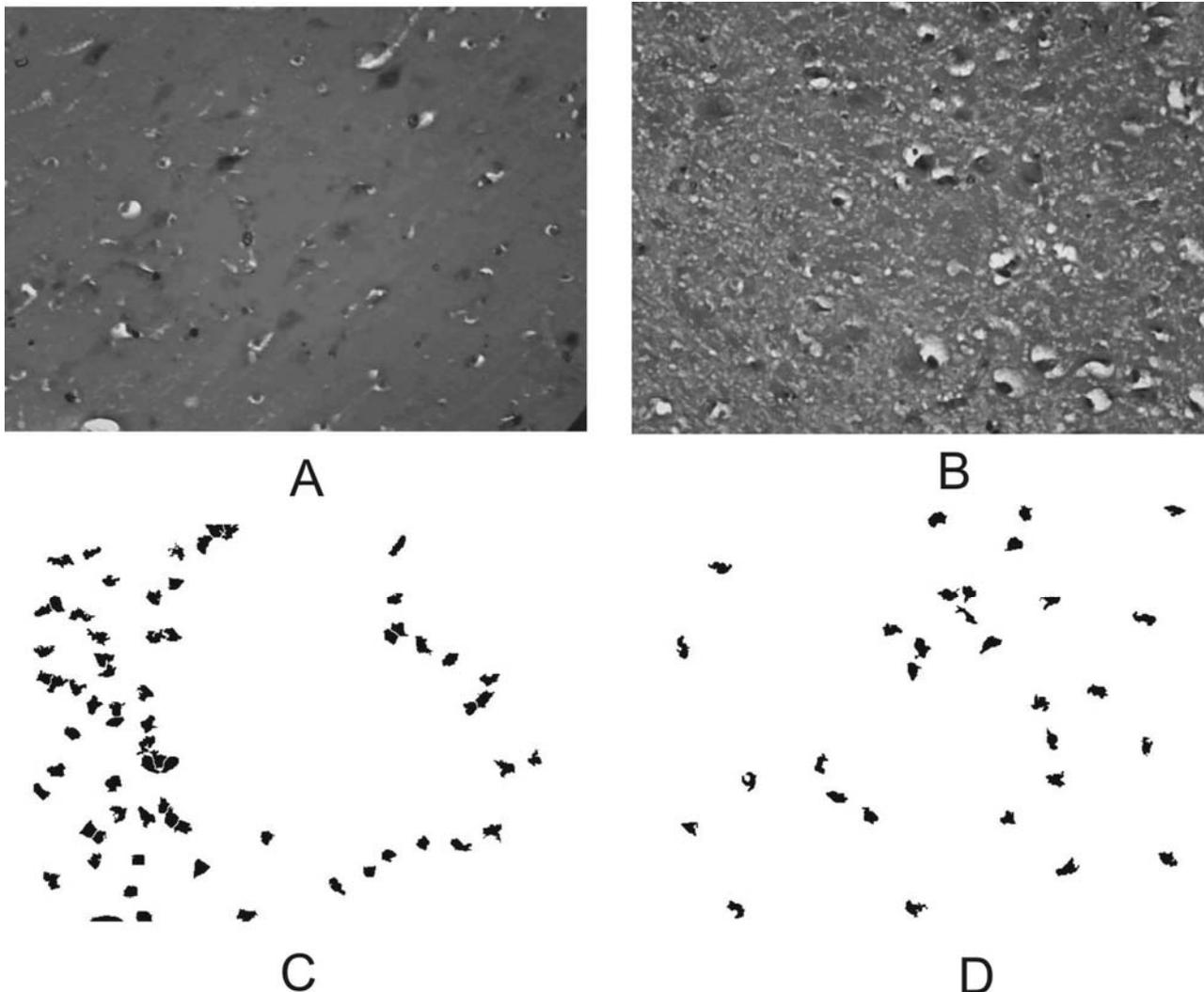
## RESULTS

Regarding the population of neurons counted in the aforementioned areas, we observed a significant decrease ( $p=0.038$ ) of the number of neurons especially in the CA1 area of AD patients, as compared to the control group (Figure 3). This significant decrease ( $p=0.044$ ) in

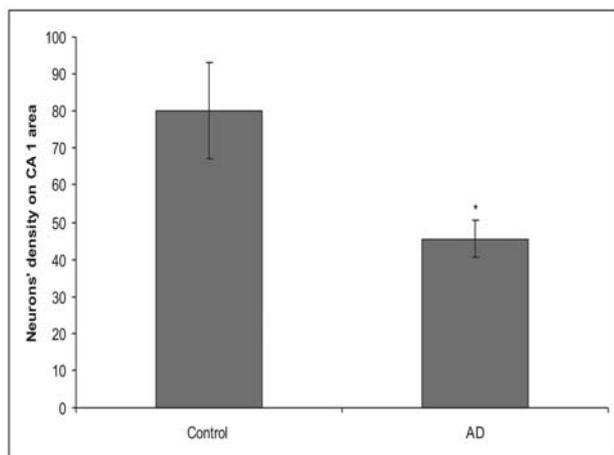
neuronal density of AD patients was observed also in the case of CA3 area, in comparison with the control group, as can be observed in the Figure 4. Additionally, a decrease of the neuronal density was also observed in the case of CA2 and CA4 areas of AD group, but this was not statistically significant ( $p=0.09$  and  $p=0.072$ , respectively) (Figure 5).

Also, when we compared the number of counted neurons for all the 4 areas, in AD patients, we noted a significant decrease in the neuronal density of CA 1 area, as compared to all other 3 areas which were analyzed ( $p=0.034$  vs. CA2,  $p=0.041$  vs. CA3 and  $p=0.039$  vs. CA4, respectively) (Figure 6).

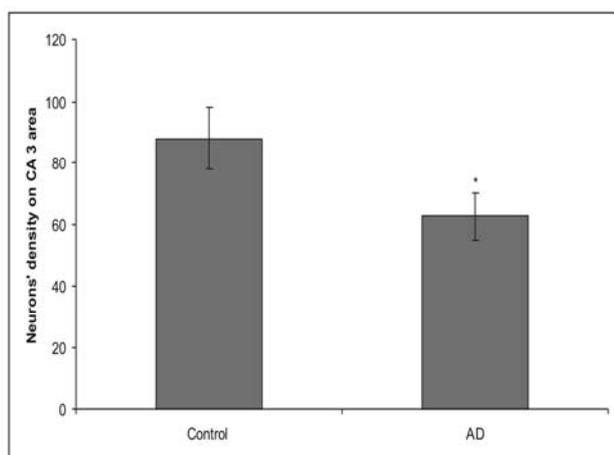
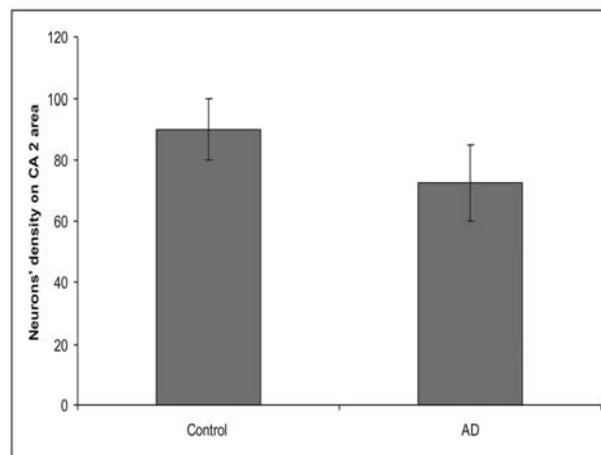
Concerning the diameter of the neurons, we could not find any significant differences between the AD group and the controls, in all 4 areas which were studied ( $p=0.82$  for CA1,  $p=0.9$  for CA2,  $p=0.76$  for CA3,  $p=0.81$  for CA4) (Figure 7).



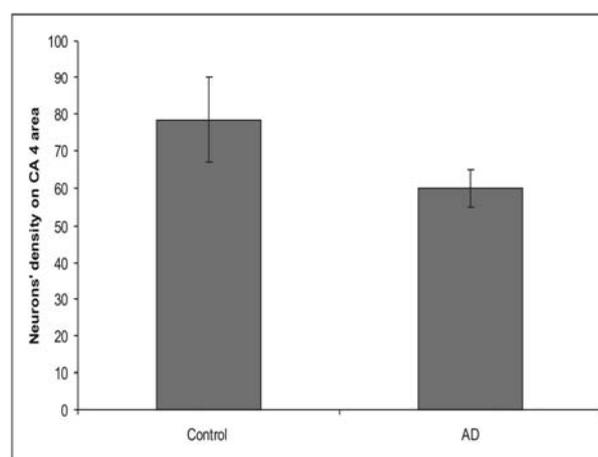
**Figure 2.** Obtaining the masks of the sections and neurons somata in Image J. A. CA1 area in the controls and its mask (C). B. CA1 area in Alzheimer's disease and its mask (D)



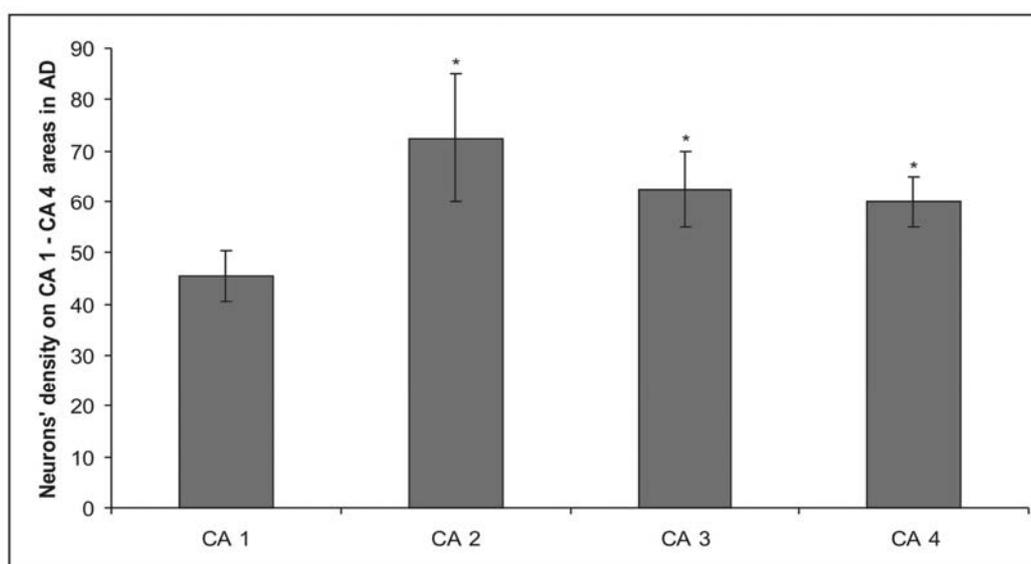
**Figure 3.** Neurons' density in the CA1 area of the hippocampus in Alzheimer's disease brains and controls \*p=0.038



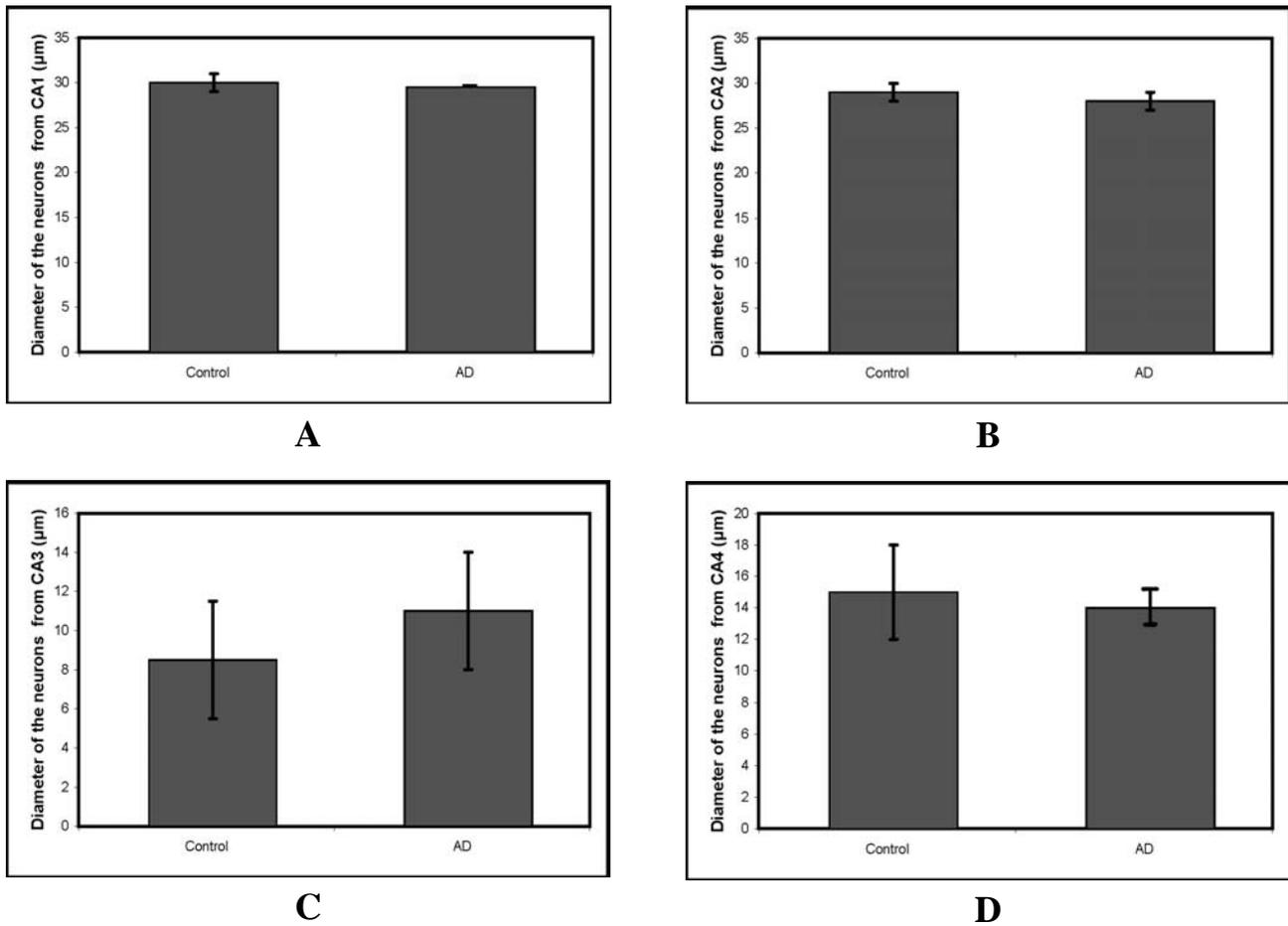
**Figure 4.** Neurons' density in the CA3 area of the hippocampus in Alzheimer's disease brains and controls \*p=0.044



**Figure 5.** Neurons' density in the CA2 and CA4 areas of the hippocampus in Alzheimer's disease brains and controls



**Figure 6.** Neurons' density in the CA1-CA4 areas of the hippocampus in Alzheimer's disease brains \*p<0.05



**Figure 7.** The diameter of the neurons (in micrometers) from CA1-CA4 areas of the hippocampus in Alzheimer's disease brains and controls

## DISCUSSION

Our results provide additional evidence regarding the neuronal loss in the hippocampus in AD, as compared to an age-matched control group. This was mainly expressed by a significant reduction of neuronal density especially in the CA1 and CA3 hippocampal areas. However the most prominent decrease in the neuronal density was found at the CA1 area level, as compared to all other 3 areas which were analyzed.

In particular, the hippocampus is considered to be very vulnerable, many central disorders being associated with hippocampal neuronal loss, such as: epilepsy (Zhang et al. 2009), dementia (Korbo et al. 2004), schizophrenia (Gattaz et al. 2011), major depression and chronic stress (Printha et al. 2009) or Huntington chorea (Walker et al. 2011).

Generally, the number of neurons determines the functional capacity of the brain or any particular neural structure. Previous studies have suggested that the loss of neurons seen in the AD hippocampus could explain the memory disturbances which are clinical signs in AD, even in the preclinical stages (Korbo et al. 2004). Also, in advanced AD, the hippocampus is one of the most profoundly affected regions of the brain and the consistency of hippocampal histopathology has led

some researchers to even use the term ‘‘hippocampal dementia’’ (Simic et al. 1997).

However, the relation of AD to the aging process of the normal human brain has remained unclear. This is mainly due to the fact that the brains of most non-demented elderly individuals contain both senile plaques and neurofibrillary tangles, which led to the idea that the difference in neuron loss between aging and AD might only be quantitative (Simic et al. 1997, Korbo et al. 2004).

In this way, previous reports stated a loss of 25–70% of the neurons in different regions of hippocampus in AD (West et al. 1994) or a similar loss in AD, but with a different distribution (Simic et al. 1997). Also, Kril et al. (2002) have reported a neuronal loss in the hippocampus in microvascular dementia patients.

In all these studies, data from AD patients were compared with cases of normal aging and the general conclusion was that AD is a quantitatively different process from normal aging. In the present paper we also demonstrated a neuronal loss involving the areas of the hippocampus from the AD brains when comparing to normal brains. Also, we report here a significant decrease of the neuronal density in the case of the CA1 area, as compared to all the other 3 areas which were analyzed. In a study by West and colleagues a distinctive

AD-related neuron loss was also seen in the CA1 region of the hippocampus. The same aforementioned paper also reported important qualitative differences in the pattern of hippocampal neuronal loss in normal ageing vs. AD. Additionally, there are studies which have found a main decrease in neuronal density in the CA1 area of a mouse model of tauopathy (Spire et al. 2006).

We also report here a significant decrease in neuronal density of AD patients in the case of the CA3 hippocampal area, as compared with the control group. This can be explained by the important role of area CA3 in modulating the entire hippocampus (Engel 2008).

Of course, the regional selectivity of neuron loss could be relevant for the understanding of various mechanism involved in AD. However, the loss of neurons is not the only mechanism by which neuronal function may be impaired. A reduction in the dendritic branching or the loss of synapses may interfere with central functions, despite the surviving cell bodies (Simic et al. 1997). That is why our future projects in this area will include the evaluation of Golgi stained material, in order to clearly stain and get a full idea about the dendritic arborization (Mavroudis et al. 2010).

However, in the present study we could not find any significant differences between the AD group and the controls concerning the diameter of the neurons in all 4 areas which were studied. This could be explained by the fact that the minimal diameter for the neurons recorded was initially set and established in Image J. In this way, smaller neurons could also be present, but may not have been counted.

There are several limitations to our study. First of all, the sample size is very small (only two patients) and the groups are not very homogeneous, which will lead of course to a certain degree of difficulty in coming to very strong conclusions. Also, the program and method which we have used here present some limitations, including the fact that it can not distinguish between the specific types of neurons, neuroglial cells and some artifacts or vascular formations, which could affect the final results. Thus, in order to reduce these limitations we excluded from the measurements formations (neuronal or not) the elements of which were below or above a specific size and we set the circularity of the desirable formations between specific limits which express the pyramidal neurons of the hippocampus. In this way, the limits that have been used were quite enough to include the majority of neurons, even if significant changes in the cell soma diameter have taken place. Furthermore, the limits were not arbitrary, but extracted after multiple measurements of the circularity and the cell size of hippocampal neurons. In this way, neurons with a soma diameter outside of the limits set do not belong to the neuronal type that we tried to measure in the present study. The measurement of the cell soma diameter was a distinct function of the Image J application and did not take account of the exclusion of smaller neurons.

## CONCLUSIONS

In the present study we managed to demonstrate and confirm a significant decrease in neuron density of hippocampus in AD, as compared to an age-matched control group. Moreover, it seems that this decrease of hippocampal neuronal density is more prominent especially at the CA1 and also in the CA3 hippocampal areas. This could have important implications in the design of therapeutic and investigative strategies in AD. However, bigger samples are necessary in order to provide the basis for stronger conclusions in this area of research.

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**Conflict of interest:** None to declare.

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