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**Relative Contribution of Plaques, Tangles, and PKAN Neurons in Patients with Cognitive
Impairment**

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**An Undergraduate Honors Thesis Submitted in Partial Fulfillment of the Requirement for
the Degree of Bachelor of Science
in University Honors
and
Biology**

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Abstract

Dementia is cognitive impairment often associated with old age diseases namely, Alzheimer's Disease. However, contrary to popular beliefs, dementia is divided into multiple subgroups according to age and disease progression. Interestingly, patients in the older subgroups often do not display characteristic pathologies, such as beta-amyloid plaques and neurofibrillary tangles, in high abundance as observed in patients with Alzheimer's Disease. Instead, cerebrovascular disease in the form of large or many smaller strokes is more commonly present. A notable novel observation made in the older group has been nonviable but preserved "mummified" neurons which resemble those found in an inherited, childhood disease known as pantothenate kinase associated neurodegeneration (PKAN); hence the name "PKAN neurons" for these lesions. For this project, we focused on quantifying the presence of PKAN neurons, plaques, and tangles in patients with Alzheimer's Disease (AD), mild cognitive impairment (MCI), an older subgroup of cognitive impairment designated based on their clinical and demographic features as "Disease of the Oldest Old" (DOOO), and cognitively control subjects. The PKAN neuron density was calculated to be highest in patients with DOOO (86.39 ± 17.90 N=7) when compared to the age-matched control group (4.488 ± 1.274 N=6) with a p-value of 0.0015. We also noted the inverse relationship between PKAN neurons and plaques and tangles across all age-related dementia groups. Our results confirm a dichotomy of dementia in patients with clinical AD, with younger patients having a large burden of AD-associated lesions and older patients with a combination of AD lesions and inversely related PKAN neurons. The results introduce PKAN neurons recognized as a novel feature of dementia, which complement classic AD-associated lesions as correlates of cognitive impairment in more elderly patients.

Keywords

Mixed dementia; Disease of the Oldest Old; PKAN neurons; cognitive impairment; globus pallidus, Alzheimer's Disease

Introduction

As the population ages, dementia is often attributed to known pathologies that underlie the decline of cognitive function. The most prominent old age-related pathologies, such as amyloid-beta plaques and neurofibrillary tangles, found in Alzheimer's Disease (AD) are frequently identified in patients exhibiting cognitive decline. However, it has been increasingly observed that pure AD is rarely the only drive in the manifestation of dementia, but rather it is a combination of multiple pathologies that constitutes the deterioration of the brain (Power et al., 2018; Tanskanen et al., 2017; Langka et al., 2004; Schneider, 2007). The prevalence of multiple pathologies is highlighted in the older subset of demented group as these characteristics significantly increase dementia risk (Tanskanen et al., 2017; Rahimi and Kovacs, 2014).

Dementia of the Oldest Old (DOOO), also known as mixed dementia, also features β -amyloid plaques and neurofibrillary tangles, which are the hallmarks of many dementia-related diseases and are characteristics of old age, but at relatively low levels of these lesions (Nelson et al., 2005, Rahimi and Kovacs, 2014). DOOO is found most often in patients older than 80 years of age and the relative lack of accumulation of β -amyloid plaques and neurofibrillary tangles suggests that other disease processes must drive cognitive losses (Nelson et al., 2005; Crystal et al., 1993). Commonly, it is noted that the degree of neurodegeneration might be correlated to cerebrovascular disease as multiple infarctions are found to be the second most common neuropathologic feature in older patients (Schneider, 2007; Langka et al., 2004; Tanskanen et al., 2017; Rahimi and Kovacs, 2014). Interestingly, when observed under the microscope, we have found that patients with DOOO also have the hallmark lesions of PKAN, namely ubiquitinated ApoE-positive neurons around infarcted area of the globus pallidus, where ischemia or oxidative stress occurred (Woltjer et al., 2015).

Patients with pantothenate kinase-associated neurodegeneration (PKAN), a rare childhood hereditary subtype of neurodegeneration with brain iron accumulation (NBIA), often exhibit movement difficulties characterized as parkinsonism and also other motor symptoms namely dystonia, choreoathetosis, corticospinal tract involvement, optic atrophy, pigmentary retinopathy, and cognitive impairment (Kruer et al., 2011; Thomas et al., 2004). Histological findings indicate that PKAN leads to a significant neurodegeneration in the Central Nervous System (CNS) particularly in the globus pallidus characterized by iron deposition and neuroaxonal spheroids, a non-specific form of axonal degeneration (Kruer et al., 2011). Other than the prominent accumulation of ubiquitin, apolipoprotein E (apoE)-enriched lesions have also been identified. While the role of apoE in the propagation of PKAN remains unclear, recent studies suggest the upregulation of this protein is present at sites with cellular ischemia and oxidative stress (Aoki et al., 2003; Woltjer et al., 2015). This recent discovery allows us to infer its potential connection with cognitive impairment specifically in the older population of patients.

There were two initial observations made about PKAN neurons in patients with DOOO that prompted the closer investigations described in this study: 1. those with a modest burden of plaques and tangles but with the absence of PKAN neurons tended to stay cognitively intact when alive and 2. those with presence of PKAN neurons and absence of plaques/tangles also stayed cognitively intact. Patients with both PKAN neurons and even modest plaques and tangles, on the other hand, tend to develop cognitive impairment. These interesting observations

together raise the question: What is the relative contribution of plaques, tangles, and PKAN neurons to dementia in patients who were seen at the Oregon Health and Science University (OHSU) Alzheimer's Center? In this project, we sought to perform a quantitative analysis on the presence of PKAN neurons, tangles, and plaques in three groups of cognitive impairment: Alzheimer's Disease (AD), Mild Cognitive Impairment (MCI), and Disease of the Oldest Old/Mixed Dementia (DOOO) to determine the relationship between different hallmarks of neurodegenerative diseases in an attempt to redefine dementia and confirm DOOO as a valid subgroup that is often mistaken as AD.

Materials and Methods

Human subjects

We used postmortem tissues of individuals from the Oregon Brain Bank (OBB) who had autopsy-confirmed diagnoses of AD, VCI, MCI, DOOO as well as of those with age-matched cognitively intact diagnosis. Subjects were enrolled in pre- or post-mortem study after consent was obtained from next of kin through an Oregon Health & Science University Institutional Review Board approved consent process. Patients information and histories were obtained through direct interview with family and/or review of available medical records (Woltjer et al., 2015).

Postmortem processing

The tissues were fixed in 10% neutral buffered formalin for at least 10 days followed by dissection of frontal cortex, basal ganglia, and hippocampus. The regions were processed using standard tissue processing methods, embedded in paraffin, and cut as 7- μ m paraffin sections (Kruer et al., 2011).

Immunohistochemistry and histological evaluations

Basal ganglia paraffin sections were stained with hematoxylin and eosin (H&E) and then luxol fast blue (LFB) myelin stain to locate and evaluate the globus pallidus.

Immunohistochemical stains were applied to sections after deparaffinization and antigen retrieval (5 min after treatment at room temperature with 95% formic acid, followed by 30 min incubation in citrate buffer [pH 6.0] at 85°C). Tissue sections were blocked with 5% nonfat dry milk in phosphate-buffered saline and stained with antibodies to 4G8 (anti-B-amyloid, mouse monoclonal from BioLegend, San Diego, CA) (1:5000); Tau (PHF-1, mouse monoclonal, a kind gift from Peter Davies, Albert Einstein College of Medicine) (1:5000); apoE (goat polyclonal from Academy Bio-Medical Co., Houston, TX) (1:5000). Results were visualized using the appropriate application of secondary antibodies and diaminobenzidine (brown) or Vector Red (Vector Laboratories) as chromagens. All slides were processed using diluted hematoxylin counterstain (Kruer et al., 2011; Woltjer et al., 2015).

Quantification of PKAN neuron density

The globus pallidus of each case was identified and traced using H&E/LFB stained slides. The region was also marked on slide with apoE stain where PKAN neurons were located. Slide grids were adhered to each apoE slide. The biochemical characterization of PKAN neurons were based

on previous studies and the density of PKAN neurons in each globus pallidus was calculated per centimeter squared (Woltjer et al, 2015).

Quantitative assessment of tau and amyloid in frontal cortex

After the slides were stained with anti-tau and anti-amyloid antibodies, 5 representative regions of the frontal cortex of each case were photographed to be analyzed. Image analysis was done using Image J (NIH image). Due to brown color developed from the DAB chromagen, brown was set as default for signal sensitivity detection to locate plaques and tangles. Raw data was plotted and analyzed using GraphPad software.

Results

Table 1: Characterization of study subjects

Diagnosis	Subjects	Age range (years)
Old Control	14	84-96
Young AD	7	60-79
Old AD	14	84-104
Old MCI	13	86-103
DOOO	10	86-105

Table 2: Description of pathologic findings of each group

Cognitive Impaired Group	Braak Stage (Tangles)	Neuritic Plaques
Control	0-4	0-1
Young AD	5-6	2-3
Old AD	5-6	1-2
MCI	1-5	0-2
DOOO	2-4	0-1

Figure 1: The contrast between healthy neurons and PKAN neurons in globus pallidus (intermediate magnification view).

(A) Hematoxylin and eosin (H&E) stain of normal globus pallidus neurons. Dense and uniformly pink background is indicative of full neural connections and axonal function. Nuclei and nucleolus are visible and present.

(B) H&E stain of PKAN neurons in globus pallidus. Note the lacy texture and uneven distribution of pink pigmentation in the background, which implies the sparse synapses and neural connections present. The absence of nuclei and nucleolus is also notable. Intracellular structure is replaced by granular substance, which is composed of remnants of disintegrated neuronal cytoplasm.

(C) Immunohistochemical stain of globus pallidus containing PKAN neurons. Vector Red was used as chromagen to distinguish the dark, pink apoE heavy region from commonly found brown iron deposits found in the globus pallidus due to blood leakage. Lacy degeneration and lack of nucleus/nucleolus are also evident, showing the depletion of neural synapses

Figure 2: Plaque density based on CERAD score assessment and IHC in different diagnoses.

(A) The neocortical plaque density using the Consortium to Establish a Registry for Alzheimer's Disease ("CERAD") plaque score assessment outlines the range of scores assigned for five subgroups: young AD, old AD, DOOO, old MCI, and control. The range of CERAD scores for young AD is 2-3, old AD is 1-2, DOOO is 0-3, old MCI is 0-2, and control is 0-1. The group scored the highest on the plaque assessment is the AD groups in which is consistent with the observation that plaques are one of the two hallmarks of the disease.

(B) The presence of immunohistochemical (IHC) signal detected by 4G8 anti-amyloid antibody applied to sections of the frontal cortex varies greatly by diagnosis. There is a statistical significance between old AD and DOOO in plaques abundance (p-value = 0.0261). There is also a statistical difference between DOOO and old control groups (p-value <0.0011). This presents that even with a small presence of plaque lesions in every old age group, these lesions are identified at a higher level in the AD groups regardless of age compared to the DOOO group.

(C) The graph depicts a linear relationship between plaque scores and stain results obtained from IHC. There is a consistent relationship between the scored assigned and the staining found (p-value < 0.0001) and that lower CERAD scores is equivalent to lower plaque lesions and higher CERAD scores is equivalent to higher plaque lesions.

Figure 4: Quantitation of PKAN neurons in globus pallidus of multiple age-related diagnoses. PKAN neurons were counted per centimeter squared and compared between four subgroups (young and old AD, MCI, VCI, and DOOO) against the control group. Control individuals demonstrate the lowest number of PKAN neurons per cm^2 as expected. AD group regardless of age features a significantly lower density of PKAN neurons than DOOO (DOOO vs old AD p-value = 0.0261; DOOO vs young AD p-value = 0.0005). DOOO group not only reveals a linear spread of PKAN neurons per cm^2 but also contains the most abundant number of PKAN neurons per cm^2 compared to any other groups.

Figure 5: Quantitative comparison between Tau detection and PKAN neuron density of different diagnosis groups. PKAN neuron density is compared to Tau signal in this graph. Control group (green dots) is clustered near the origin, indicative of little to no presence of PKAN neurons or tau signal in the group. Similarly, MCI group also features some concentration of tau and PKAN neurons but not as significantly when compared to other groups. Both AD

groups (black and red dots) are heavily concentrated along the y-axis, revealing variably abundant tau signals identified with relatively low PKAN neuron density. DOOO group (purple dots) appears to be closer to the x-axis, indicative of a higher PKAN neuron count with the addition of some tangle detection. There is an inverse relationship between PKAN neurons and tangles in the older subset of demented group. The more PKAN neurons, the lower tangles detected. The lower PKAN neurons, the higher tangles detected.

Figure 6: PKAN neuron density and tau signal in older demented groups.

(A) The comparison between the two older subsets of demented patients, DOOO and old AD, demonstrates the inversely proportional relationship between PKAN neuron density and tau detection. The AD group is evenly distributed on the y-axis, which is equivalent of detection of variably increased tau signal. The DOOO group is scattered along the x-axis, indicating a higher density of PKAN neurons found.

(B) There is a statistically significant relationship between the two variables, PKAN neurons and tau/tangles (p -value = 0.0210).

Figure 7: PKAN neuron density in relation to 4G8 signal in older demented

(A) The relationship between PKAN neuron density and 4G8 signal is also inversely proportional, similarly to that of PKAN neuron density and tau signal. The old AD group continues to scatter along the y-axis revealing a high concentration of plaques in the brain in

relative of the DOOO group. While some AD also features PKAN neurons, DOOO significantly presents a higher density.

(B) This relationship is also statistically significant ($p\text{-value} = 0.0036$). The higher PKAN neuron density found, the lower 4G8 detection observed and vice versa, the lower PKAN neuron density found, the higher 4G8 detection observed.

Discussion

While individuals with dementia also feature plaques and tangles, they express these lesions histologically at a lower level compared to their AD counterparts (Haroutunian et al., 2008; James et al., 2012; Middleton et al., 2011; Savva et al., 2009;). This suggests that perhaps the presence of the pathological markers of AD are not universally applicable to all patients with dementia, particularly in the oldest old group, in which this uncategorized subset has already surpassed the age group that commonly diagnosed AD patients would fall under (Haroutunian et al., 2008; Savva et al., 2009). The decline in these lesions with increasing age confirms the significance of age as a contributing factor in separating the two subgroups of dementia and furthermore detaches oldest old group as its own existing pathological diagnosis from AD (Middleton et al., 2011; Haroutunian et al., 2008; Savva et al., 2009).

Interestingly, the new subset of dementia is commonly found to have cerebrovascular diseases and infarctions (Schneider et al., 2007; James et al., 2012). This notable observation directs research to an entirely different route in which foretells how the manifestation of dementia might differentiate itself from AD, specifically with the recent observation of a new feature known as PKAN neurons.

The identification of PKAN neurons, in the past, has been noted in a childhood disease where these lesions were also found near areas with infarctions (Woltjer et al., 2015). The quantitative analysis on PKAN neurons in DOOO confirms yet another feature of dementia that is beyond the realm of plaques and tangles. The correlation between PKAN neurons and cognitive impairment distinguishes DOOO from AD in which the previously identified structural changes, plaques and tangles, in cognitive impairment is insufficient to be accounted for the deterioration of the brain (James et al., 2012). The significance of PKAN neurons further highlights that the hallmarks of dementia are not found exclusively in the frontal cortex, but it now involves the basal ganglia, namely globus pallidus, in which motor movement might be considered as a factor in diagnosing patients.

Further research is necessary to fully understand the manifestation of PKAN neurons and its association with cognitive decline. With PKAN neurons recognized as a feature of dementia, genetics information obtained from the studies of the childhood disease, PKAN, can enhance the understanding of these unique features in the DOOO subset. The recent observation about these pathologic features in globus pallidus might also lead to how the control of voluntary movement in the aging population can be studied to initiate more efforts in early detection of dementia.

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Abbreviations

DOOO	disease of the oldest old
MCI	mild cognitive impairment
AD	Alzheimer's disease
PKAN	pantothenate kinase associated neurodegeneration
H&E	hematoxylin & eosin
LFB	luxol fast blue
IHC	immunohistochemistry

Glossary

Infarction: clusters of dead neurons due to lack of oxygen/blood flow (webMD)

Plaques: one of two key hallmarks of Alzheimer's Disease and other age-related cognitive impairments. While there are many types of plaques, these features are generally a breakdown and accumulation of a larger protein known as amyloid precursor protein (National Institute of Aging).

Tangles: one of two key hallmarks of Alzheimer's Disease and other age-related cognitive impairments; they contain hyperphosphorylated and misfolded tau, a microtubule associated protein normally located in axon to facilitate axonal transport (Serrano-Pozo et al. 2011).

Hematoxylin & eosin: a staining technique used to identify various tissue types; hematoxylin stains nucleic acids blue-purple color and in the brain, nuclei of neurons are stained. Eosin stains proteins pink; in the brain, the cytoplasm and extracellular matrix are stained (Fischer, et al. 2008).

Immunohistochemistry: protein identification technique used in pathology to locate hallmarks of certain diseases; uses the unique immunal relationship between antibodies and antigens of the organism(s).

Ischemia: insufficient supply of blood or oxygen.

Globus pallidus: one of three regions of deep gray matter known as the basal ganglia; one of its primary purposes is motor control.

Luxol Fast Blue: a staining technique used to identify extremely fatty substances; in the brain, myelinated axons are stained in the blue color.

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