Spring 6-15-2024

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Abstract

Polyamines, and their rate-limiting enzyme ornithine decarboxylase (ODC), are crucial for many functions in the central nervous system but levels decrease with age. In neurodegenerative diseases, like Alzheimer's Disease (AD), polyamine levels begin to increase again. Yet, there are still many unanswered questions surrounding polyamine's possible role in AD, especially in those with Down Syndrome (DS), who also have an extra copy of the amyloid precursor protein (APP) and tend to get AD far earlier than the general population. We aim to investigate if there is colocalization between amyloid plaques and Ornithine Decarboxylase (ODC) in patients with AD and AD/DS, see if there is a relationship between polyamines and amyloid plaques, and if this might be a contributor factor underlying AD pathogenesis. This will be done through immunofluorescence staining of paraffin-embedded human hippocampal tissue for ODC, β-amyloid, and collagen as a molecular marker for vascularity. The results indicate limited to moderate colocalization of ODC and amyloid plaques that tend to be located near vascularity, and that this colocalization is present, especially in those with DS and AD. This suggests a relationship between polyamine metabolism and AD pathology supporting that this may be a factor contributing to AD pathogenesis.

Document Type

Undergraduate Thesis

Degree Name

B.S.

First Advisor

Daniel Paredes

Keywords

Colocalization, Polyamines, Alzheimer's Disease (AD), Down Syndrome (DS)

Subject Categories

Biochemistry, Biophysics, and Structural Biology | Diseases | Life Sciences | Molecular Biology | Nervous System Diseases

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Abstract

Polyamines, and their rate-limiting enzyme ornithine decarboxylase (ODC), are crucial for many functions in the central nervous system but levels decrease with age. In neurodegenerative diseases, like Alzheimer's Disease (AD), polyamine levels begin to increase again. Yet, there are still many unanswered questions surrounding polyamine's possible role in AD, especially in those with Down Syndrome (DS), who also have an extra copy of the amyloid precursor protein (APP) and tend to get AD far earlier than the general population. We aim to investigate if there is colocalization between amyloid plaques and Ornithine Decarboxylase (ODC) in patients with AD and AD/DS, see if there is a relationship between polyamines and amyloid plaques, and if this might be a contributor factor underlying AD pathogenesis.

This will be done through immunofluorescence staining of paraffin-embedded human hippocampal tissue for ODC, β-amyloid, and collagen as a molecular marker for vascularity. The results indicate limited to moderate colocalization of ODC and amyloid plaques that tend to be located near vascularity, and that this colocalization is present, especially in those with DS and AD. This suggests a relationship between polyamine metabolism and AD pathology supporting that this may be a factor contributing to AD pathogenesis.

Introduction

Alzheimer's Disease (AD) is the most common neurodegenerative disease pathologically characterized by extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles. The Aβ peptide that these plaques consist of comes from the amyloid precursor protein (APP) being cleaved by the γ-secretase complex at a different location, as opposed to α-secretase, leaving the fragments that are prone to aggregation (Sadigh-Eteghad et al. 2015). In humans, the gene that encodes for APP is located on chromosome 21 (Selkoe and Hardy 2016). In Down Syndrome (DS), chromosome 21 is triplicated, leading to an overexpression of APP. All people with DS virtually have AD pathology by the age of 40, as compared to the general population (Lott and Head 2019). This finding was a key factor in the 'amyloid-cascade' hypothesis of AD (Selkoe and Hardy 2016).

Polyamines have a variety of widespread biological functions important for normal cell function, growth, and differentiation (Morrison and Kish 1995). Polyamine levels decrease with age (Minois et al. 2011) but in AD, neocortical neurons showed a prevalence for L-Ornithine decarboxylase (ODC) (Bernstein and Muller 1995) which is the rate-limiting enzyme in the polyamine biosynthetic pathway (Pegg 2006) and polyamine levels in AD brains are substantially increased (Liu et al. 2014). Yatin et al. (1999) suggested that this increase in polyamine metabolism is a reaction to Aβ-mediated oxidative stress, but further work showed neurotoxic effects of the polyamine spermine and Aβ (more so than Aβ on its own), suggesting that in AD the metabolism of polyamines is dysregulated, resulting in toxic accumulation of polyamines (Yain et al. 2001). Polyamine could aggravate neuronal damage (Singh et al. 2010) and in vitro studies have shown that polyamines may modulate Aβ peptides by inducing accelerated aggregation (Luo et al. 2013). Therefore, polyamines may be a pathway contributing

to the neurotoxicity in AD that underlies its pathogenesis, but few studies have been done on human AD tissue to examine if this relationship between polyamines and AD pathology can be seen in patients.

This study aims to answer the question of whether there is a relationship between polyamines and amyloid plaques in AD and AD/DS patients through colocalization analysis in human hippocampal tissue. AD/DS phenotype will allow for more analysis of amyloid plaques and give a better understanding of this possible relationship. It is important to investigate this as many questions still surround AD pathogenesis, even though it is the most common neurodegenerative disease, and these results may support or refute the growing theory that polyamines are integral to understanding AD (Polis et al. 2021).

Methods

Hippocampal Tissue Collection and Preservation

All brain tissues used were from the Down Syndrome Biobank Consortium at the University of Colorado Anschutz School of Medicine. All donors, 2 without AD or DS, 1 with AD, and 1 with AD and DS gave informed consent to have a brain autopsied and the materials be used for research purposes. Diagnoses were given by a board-certified pathologist post-mortem with specific diagnosis and demographic information being included in Table 1.

Table 1. Demographic and diagnostic information.

Case ID	Age (years)	Gender	Pathology
UCI 2008-38	63	Female	AD & DS
HB93	96	Female	AD (Braak Stage I)
HB86	80	Male	AD (Braak Stage IV-V)
HB76	63	Female	Control

The brains were autopsied and placed in a formalin solution to be fixed. After fixation, the hippocampus was dissected and embedded in paraffin. 5µm thick sections were cut using a microtome from each paraffin-embedded hippocampal section and placed onto glass slides. Each case was represented by one hippocampus.

Immunofluorescence

There was 1 slide stained for HB76, HB86, and HB93 and 2 slides stained for UCI 2008-38. The slides were deparaffinized and rehydrated by being placed in solutions of xylene (Millipore) twice, 100% ethanol (Fisher Chemical) twice, 95% ethanol, 70% ethanol (Fisher Chemical), 50% ethanol, and finally MilliQ water for five minutes each. Heat-induced antigen retrieval was done with citrate buffer [10mM citric acid (Sigma Life Sciences), 0.05% Tween 20 (Sigma Aldrich), pH 6.2] and 0.05% citraconic acid [98% citraconic anhydride (Sigma Aldrich), pH 7.4] at 100°C in a vegetable steamer for 20 minutes each and allowed to cool to room temperature (RT).

A hydrophobic ImmEdge pen (Vector Laboratories) was used around the tissues which were then washed with 1x tris-buffered saline [TBS, 10x TBS (Thermo Scientific)] three times for five minutes each. The tissues were then blocked with Block-Aid (5%, Thermo Fisher Scientific) and Triton X-100 (0.1%, Fisher Bioreagents) in 1x TBS for one hour at RT. 1x TruBlack [20x TruBlack Lipofuscin Autofluorescence Quencher (Biotium), 70% ethanol (Fisher Chemical) was placed on the slide, for 30 seconds each to reduce autofluorescence, and then washed three times in 1x TBS for three minutes. Slides were incubated with the primary antibodies shown in Table 2 (in a 1x TBS and 0.05% Block-Aid solution) overnight.

After the slides had warmed to RT, they were washed three times in 1x TBS for five minutes each. The slides were then incubated with the secondary antibodies shown in Table 2 (in a 1x TBS and 0.05% Block-Aid solution) for one hour at RT. Finally, the slides were washed three times for five minutes in 1x TBS and then cover slipped with Prolong Gold with DAPI (Thermo Fisher Scientific) being used for the mounting media. They were left to dry for five hours at -4°C. All slides were stained at the same time to reduce variability.

Table 2. List of primary and secondary antibodies used.

Primary Antibody	Supplier	Dilution	Secondary Antibody	Supplier	Dilution
Anti-β-Amyloid Peptide Antibody, Chicken Polyclonal	Aves Lab, #AB2313536	1:2000	Goat anti-Chicken, Alexa Fluor 488, Goat Polyclonal	Invitrogen, A32931	1:500
Anti-Collagen IV Antibody, Rabbit Polyclonal	Abcam, #AB6586	1:400	Goat anti-Rabbit, Alexa Fluor 594, Goat Polyclonal	Invitrogen, A11012	1:500
Anti-Ornithine Decarboxylase Antibody, Mouse Monoclonal	Abcam, #AB193338	1:2000	Goat anti-Mouse, Alexa Fluor 647, Goat Polyclonal	Invitrogen, A32728	1:500

Imaging and Analysis

Three 20x magnification images per slide were taken using confocal microscopy performed on an Olympus Fluoview FV3000 confocal/2-photon microscope using Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647, and DAPI channels. These images were in locations where the most amyloid plaques were seen or if none were present, areas of vascularity. Image acquisition settings were kept constant for all slides and were photographed at the same time (roughly 5 hours after completing the immunofluorescence stain).

Images were exported as .oir files and analyzed in ImageJ Fiji, using the plugin JACoP. Pearson's colocalization correlation coefficient (PCC) and Manders' colocalization analysis (M1 and M2) to get a well-rounded view of colocalization. Thresholds were set for each image manually and the whole image was used for the analysis. This was done for the Alexa Fluor 488 channel and Alexa Fluor 647 from β-amyloid and ODC, respectively. A one-sample t-test was used to compare PCC among images within the same case type (AD, AD/DS, or control), and a two-sample t-test with unequal variances between cases and for analyses regarding Manders' colocalization, according to Mcdonald and Dunn (2013). Statistical significance was set at $p < 0.05$.

Results

Immunofluorescence staining of ODC and β-amyloid in AD/DS patients showed limited to moderate colocalization on average (Pearson's $r = 0.393 \pm 0.0416$, one-sample t-test; $t=9.44$, $df=6$, $p<0.0001$). β-amyloid overlapped with ODC an average of 31.0% of the time (Manders' $M1=0.310 \pm 0.0453$) and ODC overlapped with β-amyloid 35.9% of the time (Manders'

M2=0.359±0.0342) in AD/DS patients. Figure 1 demonstrates one such image used in the analysis.

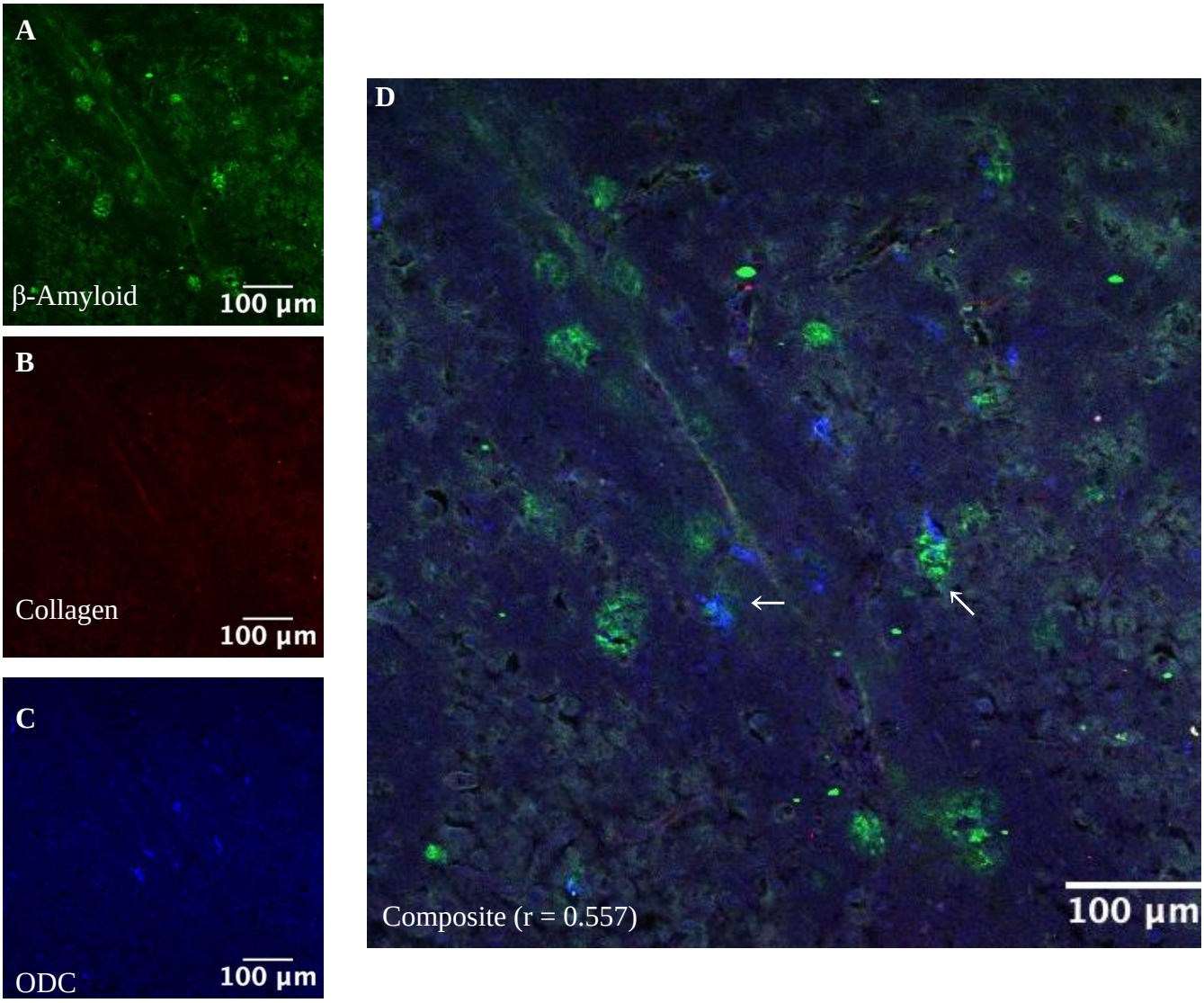

Figure 1. Immunofluorescent stain of an AD/DS hippocampus for β-amyloid, ODC, and collagen. (A) β-Amyloid stained with Alexa Fluor 488. **(B)** Collagen stained with Alexa Fluor 594. **(C)** Ornithine decarboxylase (ODC) stain with Alexa Fluor 647. **(D)** Merged image of the three. ODC and β-amyloid exhibited a Pearson's colocalization correlation coefficient of 0.557 for this specific image. Arrows point to areas of colocalization. This AD/DS patient was a 63-year-old female.

Similarly, ODC and β-amyloid also showed limited to moderate colocalization in AD patients (Pearson's r = 0.382±0.0480, one-sample t-test; t=7.97, df=4, p<0.001). However, β-amyloid overlapped with ODC an average of 14.8% of the time (Manders' M1=0.149±0.0380) and ODC overlapped with β-amyloid 34.6% of the time (Manders' M2=0.346±0.0275) in AD patients. No plaques were found in the control patient so no colocalization analysis could be done. A summary of the colocalization is provided in Table 3.

Table 3. Summary of Colocalization Data

Case, Slide & Image Number	Pearson's Coefficient (r)	Manders' Coefficient, M1 (fraction of β-amyloid overlapping with ODC)	Manders' Coefficient, M2 (fraction of ODC overlapping with β-amyloid)
UCI 2008-38, 1, 1	0.519	0.533	0.429
UCI 2008-38, 1, 2	0.391	0.204	0.461
UCI 2008-38, 1, 3	0.267	0.381	0.347
UCI 2008-38, 1, 4	0.393	0.255	0.377
UCI 2008-38, 2, 1	0.334	0.180	0.189
UCI 2008-38, 2, 2	0.557	0.321	0.402
UCI 2008-38, 2, 3	0.290	0.298	0.308
Average	**0.393**	**0.310**	**0.359**
Standard Error of Mean (SEM)	**0.0416**	**0.0453**	**0.0342**
HB93, 1, 1	0.425	0.212	0.370
HB93, 1, 2	0.546	0.266	0.437
Average	**0.382**	**0.149**	**0.346**
Standard Error of Mean (SEM)	**0.0480**	**0.0380**	**0.0275**
HB86, 1, 1	0.275	0.080	0.326
HB86, 1, 2	0.310	0.085	0.324
HB86, 1, 3	0.356	0.101	0.273
Average	**0.307**	**0.107**	**0.347**
Standard Error of Mean (SEM)	**0.0260**	**0.0560**	**0.1275**

The PCC values for the AD and AD/DS patients were not statistically different (two-sample t-test; $t=0.167$, $df=9$, $p > 0.05$) and neither were M2 values (two-sample t-test; $t=0.296$, $df=10$, $p > 0.05$). However, AD patients did have significantly lower M1 values (two-sample t-test; $t=2.73$, $df=10$, $p < 0.05$). The difference between M1 and M2 values in AD/DS was not significant (two-sample t-test; $t=-0.858$, $df=11$, $p > 0.05$) while it was significant in AD (two-sample t-test; $t=-4.21$, $df=7$, $p < 0.05$).

In addition, qualitatively areas with amyloid plaque and ODC colocalization tend to be near vascularity, as seen in Figure 2, but this was not seen in all images. Figures 3 shows images from the control patient.

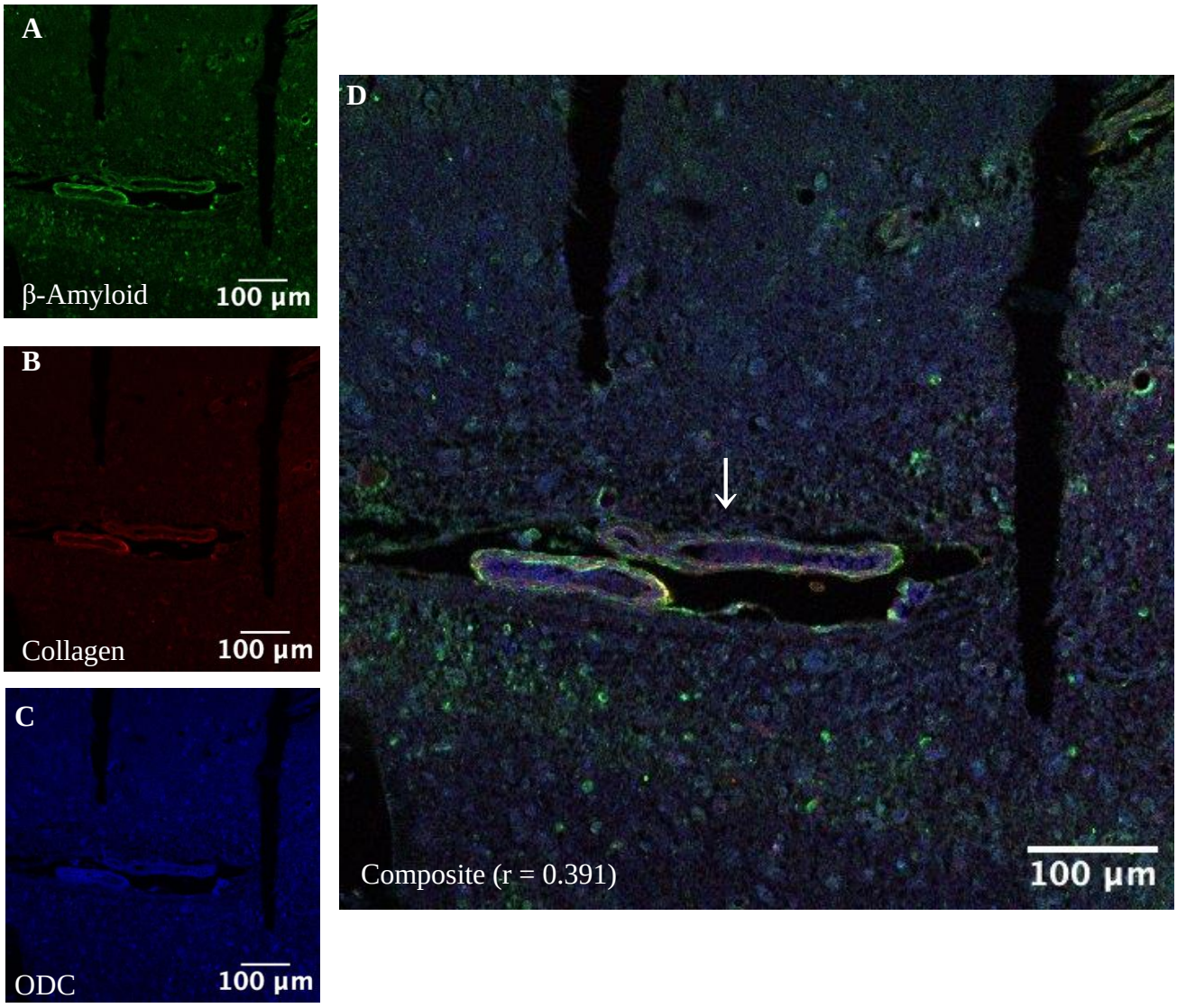

Figure 2. Immunofluorescent stain of an AD/DS hippocampus for β-amyloid, ODC, and collagen near vascularity. (A) β-Amyloid stained with Alexa Fluor 488. **(B)** Collagen stained with Alexa Fluor 594. **(C)** Ornithine decarboxylase (ODC) stain with Alexa Fluor 647. **(D)** Merged image of the three. ODC and β-amyloid exhibited a Pearson's colocalization correlation coefficient of 0.391 for this specific image. The arrow points to a blood vessel. This AD/DS patient was a 63-year-old female.

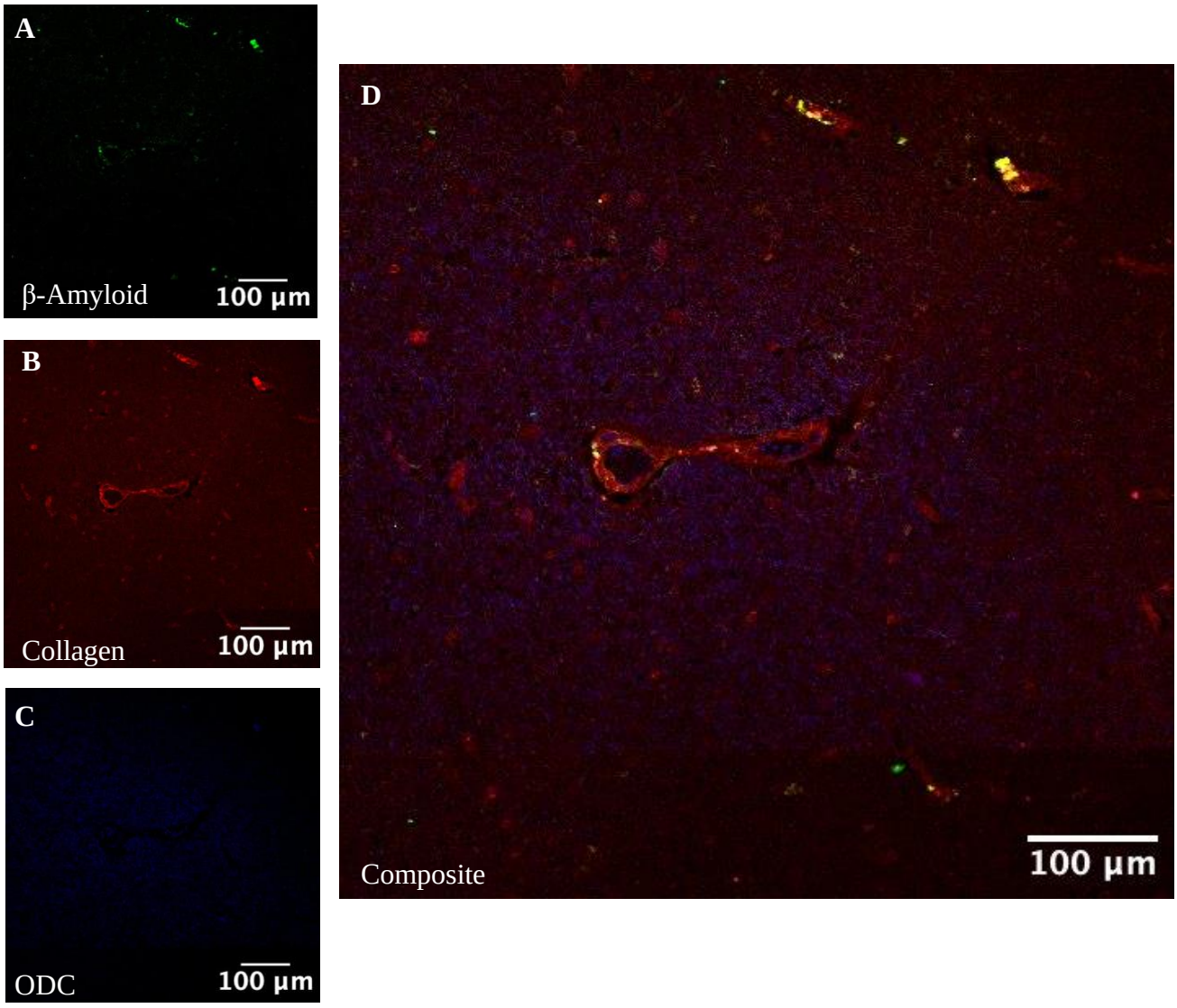

Figure 3. Immunofluorescent stain of a control patient's hippocampus for β-amyloid, ODC, and collagen. (A) β-Amyloid stained with Alexa Fluor 488. **(B)** Collagen stained with Alexa Fluor 594. **(C)** Ornithine decarboxylase (ODC) stain with Alexa Fluor 647. **(D)** Merged image of the three. This control patient was a 63-year-old female.

Discussion

Colocalization of ODC and amyloid plaques seems to be strongest in AD/DS patients. Control and AD patients had some colocalization, but specifically, ODC overlapping with amyloid plaques. All colocalizations were limited or moderate, but even this indicates a relationship between Amyloid plaques and ODC. This colocalization supports the idea of a relationship between amyloid plaques and polyamines which supports prior work conducted by Polis et al. (2021) pointing to the polyamine metabolism being vital in AD.

The difference between M1 values (Manders' colocalization coefficient for the fraction of β-amyloid overlapping with ODC) between AD and AD/DS patients indicates a somewhat stronger colocalization between ODC and amyloid plaques in AD/DS but this could be explained by the higher amyloid plaque density in AD/DS (Head et al. 2016). The difference between M1 and M2 values (Manders' colocalization coefficient for the fraction of ODC overlapping with β-amyloid) may indicate ODC aggregation and colocalization to be a byproduct of amyloid plaque formation but that is difficult to conclude without further experimentation to specifically examine directionality. This difference was only found in AD, not AD/DS, but this could be from different stages being included in AD while there was a single AD/DS case.

The qualitative finding of colocalization of amyloid plaques and ODC near areas of vascularity is unsurprising considering that polyamine metabolism requires vast nutrient exchange and that polyamines have a cardiovascular protective effect (Xuan et al. 2023).

Although these results do point to a relationship between polyamine metabolism and amyloid plaques, there are limitations. Antibody validation was not completed and the ODC antibody used seemed to produce considerable background noise. This was alleviated to some extent by manually setting a threshold but still may impact specifically Pearson's Colocalization

Coefficient values, which explains why these values show a stronger colocalization. Future steps should utilize other ODC antibodies to test the validity of these findings. Additionally, a small sample size was used, so the generalizability of these findings cannot be certain. The next steps should include a large sample size with multiple patients in each tissue type (control, AD, and AD/DS) and a most robust analysis could be done.

These findings of limited to moderate colocalization of ODC and amyloid plaques suggest a relationship between polyamine metabolism and AD pathology, pointing to a possible pathway of AD pathogenesis. Future studies with a large sample size, a different ODC antibody, and antibody validation could further solidify these preliminary findings. But these findings in their own regard warrant more research and a closer look into how polyamines interact with AD.

Acknowledgments

Thank you to Daniel Paredes for supporting and mentoring me in the creation of this experiment and thesis as well as Andres Sola who helped me greatly in this process. Thank you to Ann-Charlotte Granholm-Bentley, Jeremiah Phares, and Anah Gilmore for teaching me the fundamentals of immunohistochemistry which supported this thesis.

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