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Neuron-specific cellular functions of Interleukin-9 in Alzheimer's Disease

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Abstract

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By Samsara Wynona Upadhya

The deposition of beta-amyloid and tau initiate immune responses in the brain that lead to neuroinflammation in Alzheimer's Disease (AD). Increased rates of pro-inflammatory and anti-inflammatory cytokines such as Interleukin-9 (IL-9) are observed in AD. Previously, our lab found African Americans with AD had a higher increase in Cerebrospinal Fluid (CSF) IL-9 levels than those with Normal Cognition (NC), but this was not true for Caucasians. Using immunohistochemical analysis for brain IL-9, we found greater brain IL-9 levels also associated with AD in African Americans but not Caucasians. The direct mechanism by which IL-9 acts on brain specific neurons remains to be elucidated. Here, we aimed at studying the role of IL-9 in the transmission and modulation of neuronal signals in the brain by investigating differential IL-9 and IL-9 Receptors (IL-9R) expression in cases with and without AD. Using postmortem brain slides from older African Americans and Caucasians, we utilized an immunohistochemical staining method to stain for IL-9R. We also aimed to observe the differences in density of the IL-9 receptor with or without ongoing cell loss. For the first time, we successfully identified the presence of IL-9R on neuronal cells, localizing around the cell body. We also found an upregulation of IL-9R in AD in comparison to control cases, and increased levels of perivascular IL-9 in comparison to parenchymal IL-9 in Caucasian AD cases which was not true for African American AD cases. These results support the finding that IL-9 has AD specific effects on the brain, and may be a key cytokine leading to the observed racial differences in AD.

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Introduction

As of 2019, 5.8 million Americans are living with Alzheimer's Disease (AD) and early onset Alzheimer's. AD is now 6th leading cause of death in the United States. Over the past 10 years while deaths linked to HIV, stroke and heart disease have decreased, Alzheimer's related deaths have increased by 145%[1]. AD is clinically associated with cognitive, verbal and visual decline. These manifest as typical AD symptoms of memory loss, difficulties completing familiar tasks and having conversations, and confusion with place or time [2]. AD is universally fatal.

Pathologically, AD is associated with abnormal protein depositions as well as loss in neuronal mass, synapses and synaptic function [3]. Extracellular amyloid beta-proteins (Aβ), primarily Aβ42 but also Aβ40, form neuritic plaques. Intracellular tau proteins form neurofibrillary tangles, more commonly localizing around the nucleus of the cell. Tau in AD is hyperphosphorylated, and can interfere with microtubular arrangement and functions [4]. Over the better part of the 20th century, post-mortem detection of plaques and tangles was necessary for the diagnosis of AD.

Research has shown that the main AD-related proteins (Aβ and tau) can be measured in Cerebrospinal Fluid (CSF) [5]. In AD, Aβ 42 levels are lower and tau levels are higher. These biomarkers have been shown to have race dependent concentrations [6]. African Americans have lower Cerebrospinal Fluid (CSF) levels of p-tau181, t-tau and Aβ40 than Caucasians with similar Aβ42 levels [6]. African Americans have also been shown to have higher quantities of low-grade inflammation, and higher inflammatory rates in stroke patients [7, 8] but the effects of race on AD-related inflammation are unknown.

The deposition of Aβ42 and tau initiate immune responses in the brain that lead to neuroinflammation [9]. Both Aβ42 proteins and extracellular plaques are targets for immune response[10] mediated by microglia[11]. Perhaps as a result, increased CSF levels of pro-inflammatory and anti-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-6, IL-9, IL-12, IL-18, IL-4, IL-10, IL-13 and transforming growth factor-beta (TGF-β) have all been observed in AD [12] [9].

Data suggests the incidence of AD among African Americans (AA) is twice that of Caucasians. This may be an effect of comorbidity with other conditions such as vascular disease and diabetes. There is a higher diabetes burden in older African Americans compared to Caucasians, which proves as a risk factor for one group more often [13, 14] due to disparities in lifestyle and education quality. Increased AD risks may also be associated with limited or delayed access to healthcare [14]. Varying genetics is also thought to play a role in this racial disparity, as African American populations are consistently found to have a higher proportion of the risk genes such as APOE4, and unique genetic risks such as those associated with ABCA7 [13]. Large scale studies involving the measurement of biomarkers for AD have been deeply rooted in data collected from a majority of Caucasians [15] and may not be generalizable to persons with AD from all racial backgrounds. Furthermore, the mechanism for higher AD prevalence in African Americans is generically hypothesized to be related to diet, genetic and environmental factors with no specific mechanism established [13, 14]. This also highlights the potential for underdiagnosis in African American and other minorities with AD as they might not have the same cut-off values for AD biomarkers and cognitive testing as Caucasians. Therefore, the diagnostic thresholds must be modified from person to person to increase the efficacy of diagnosis.

We are particularly interested in the role of AD-related interleukin 9 (IL-9). IL-9 is a cytokine produced primarily by Th9 cells and has pleiotropic effects in the body and brain. IL-9 is implicated in the growth and survival of mast cells, in lymphocyte production, enhancement of chemokine production in lung endothelial cells, and the regulation of inflammation in a tissue specific manner [16, 17]. In the brain, IL-9 has been shown to protect cortical neurons against developmental apoptosis[18].

Previously, our lab found healthy African Americans to have lower CSF IL-9 levels than healthy Caucasians, with an AD-associated increase in CSF IL-9 only in African Americans ($p=0.01$) but not in Caucasians [17, 19]. The concentration differences in protein biomarkers present in the CSF of African Americans and Caucasians lead us to hypothesize that the mechanisms of targeted immune response may be race based and poses the question: does the concentration of cytokines in persons with AD differ by race?

Using immunohistochemical analysis in brains of people who died with and without pathologically confirmed AD, I made the novel observation that IL-9 was expressed in the brain. IL-9 immunoreactivity showed up in two ways: light staining in parenchymal cells, and dark staining in the perivascular regions. Similar to CSF findings, I found AD-related increase in parenchymal IL-9 immunoreactivity as well as pathologic and gene expression profiles consistent with mast cell activation only in African Americans. Together, these results show that race modified the inflammatory processes in response to AD [17].

The concentration differences in protein biomarkers, and specifically IL-9 present in the CSF and brain of African Americans and Caucasians lead us to hypothesize that the mechanisms of

targeted immune response may be race dependent and to pose the question: what types of cells express IL-9 in the brain at baseline, what types of cells have IL-9 receptors (IL-9R), and what cells are responsible for IL-9 increases in AD? Furthermore, we wondered why IL-9 is necessary in the brain – thought to be an immune-privileged site, whether it may have signaling functions beyond inflammation. Studies have also shown that cytokines IL-1β, TNF-A and IL-6 function as neurotransmitters. IL-1β is shown to have pro-convulsive effects in mice models, TNF-A application increases Calcium currents in neuronal cultures, and IL-6 has been shown to cause electrophysiological alterations in the cerebellum [20].

Our previous experimentation shows the presence of IL-9 in superior middle frontal cortex through immunohistochemical (IHC) staining [17]. We hypothesized that cells present in the region must express IL-9R. Specifically, we predicted IL-9R will localize on synaptic clefts, on the post synaptic membranes we have seen IL-9 localized in and around neuronal cells [17]. Using postmortem brain slides from older African Americans and Caucasians, we utilized an IHC method to stain for IL-9R. We also aimed to observe the differences in density of the IL-9 receptor with or without ongoing cell loss. We hypothesized that cases with cell loss will have a higher density of IL-9R staining per cell as we have observed an increase in IL-9 in conditions of cell loss in comparison to control cases. As our model for cell loss, we utilized superior middle frontal cortical and hippocampal sample slides from Alzheimer's Disease (AD) cases as neuronal loss and tissue composition changes are correlated with AD [21]. In order to shed light on the mechanism by which IL-9 is seen in neuronal cells, we also looked at the differential expression levels of parenchymal and perivascular IL-9 to determine its mechanism of action.

Materials and Methods

1. Sample Selection

To perform immunohistochemcal (IHC) analysis on postmortem brain samples, superior middle frontal gyrus tissue was requested for 34 subjects from the Emory Alzheimer's Disease Research Center Brain Bank. Of this group, 16 were African Americans and 18 were Caucasians. Cases were age and gender matched between the races. Due to a limited sample size, African American cases without major non-AD pathology were selected based on availability (Table 1).

		AD	Control
Race	AA	10	8
	NHW	7	9
Gender	Male	10	8
	Female	7	9
Average age of Death		67.00	61.89

Table 1: Demographic information for dataset used in this study (n = 34). AA = African American, NHW = Non-Hispanic White

2. Immunohistochemical Staining

The Lab-Vision Auto Stainer 480S platform (ThermoFisher, Waltham, MA) was used to stain formalin-fixed superior middle frontal cortical sections in all cases using primary antibodies targeting IL-9 (1:1500, Cat#66144-1, Millipore Sigma, Burlington, MA), and IL-9R (1:250, Cat#LS-C199119, LifeSpan Biosciences, Seattle, WA) and detected via a DAB-based detection method. Initially, to test the optimal dilution for primary antibodies, multiple dilutions were used. The dilutions which best distinguished between positive stain and background were selected.

Slides were digitally scanned using the Aperio Digital Pathology Slide Scanner (Leica Biosystems, Buffalo Grove, IL). Quantitative pathologic analysis was performed using Aperio Image Scope (12.3.3.5048) to generate total area of positive staining.

For parenchymal IL-9 and IL-9R, a random field approach was utilized to sample each slide. A 10 x 10 digital grid was layered on to each slide to create 100 50 μM x 50 μM fields. Using a random number generator, a random row and column were selected to generate a field coordinate. The selected field was analyzed using Aperio ImageScope if 1) more than half of the field was occupied by cortex and/or white matter by visual inspection; 2) there was no large mounting artifact (tear, fold); and 3) there was no significant staining artifact (e.g., DAB debris). This was repeated for 25 fields per case. The mean immunoreactive area across 25 fields was used for group-level statistical analysis. Immunoreactive areas were categorized into 'Strong Positive,' 'Positive,' and 'Weak Positive,' by Aperio software and only 'Positive' pixel counts were used in data analysis (Figure 1).

For perivascular IL-9, 25 random fields were selected for morphological analysis. All blood vessels in that field were manually identified and measured. Blood vessels were identified

based on the appearance of a complete luminal wall with red blood cells in the central lumen. For vessels with perivascular cells expressing IL-9, an irregularly shaped outline containing perivascular and intraluminal areas was traced to capture contiguous perivascular IL-9-immunoreactive cells. If IL-9-immunoreactive cells were only on one side of the vessel, the outer vessel wall was traced to complete the shape. The portion of this area containing strong intensity staining is then calculated as A_{peri}. After the first 5-10 blood vessels containing perivascular IL-9-positive cells were analyzed, an average rectangular area was approximated to analyze blood vessels without any perivascular IL-9-immunoreactivity. For each vessel, the intraluminal space was manually traced, and the portion of intraluminal area with high intensity IL-9-staining was then subtracted from A_{peri}. The perivascular area with high-intensity IL-9-staining of all blood vessels from the 25 random fields was then averaged for each case.

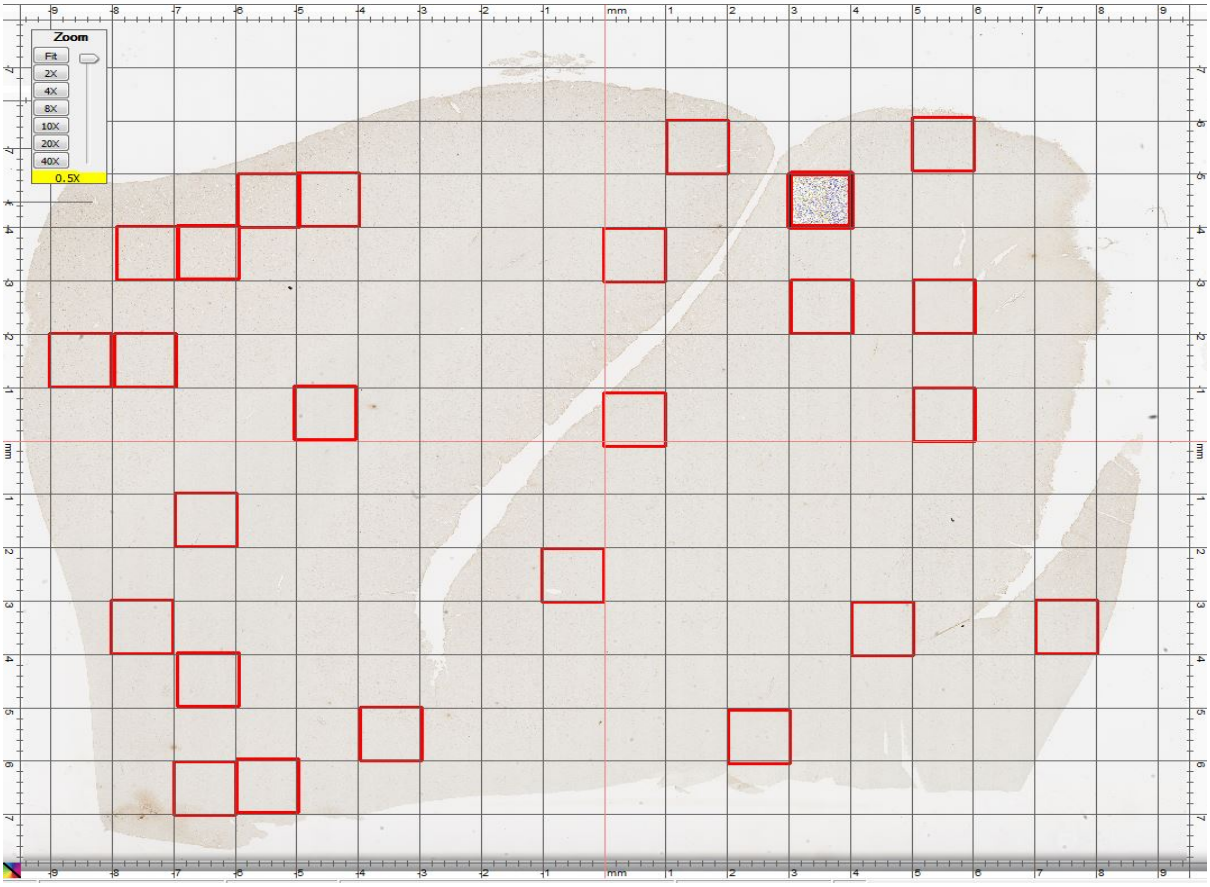

Figure 1: Representative image of random field selection after generation of grid on slide

3. Statistical Analysis

Each case's immunoreactive area was represented as a ratio of the mean intensity between the case and Caucasian control subjects, as pixels are arbitrary units. To minimize bias, the quantitative analyses were performed while blinded to race, gender and diagnosis for all cases.

All statistical analyses were performed on IBM-SPSS 24 (Armonk, NY). In order to investigate the differential ratio of Parenchymal over Perivascular Intensity of Strong Positive (IsP), a nonparametric Mann Whitney U test was used due to the nonrandom distribution of the data. Mean brain IL- 9R -immunoreactive areas were also investigated with a nonparametric Mann Whitney U test to compare differences in diagnosis and race as the data was nonnormally distributed.

Results

1. AD associated with Increased Perivascular IsP:Parenchymal IsP of IL-9

We observed an increase in Perivascular:Parenchymal IL-9 levels in AD (Figure 2B) ($p=0.067$). NHW cases had a higher ratio in comparison to AA cases for AD and. The NHW AD ratio was comparable to the AA control ratio. An increase in perivascular IL-9 was observed in both AA and NHW cases with AD in comparison to Control cases.

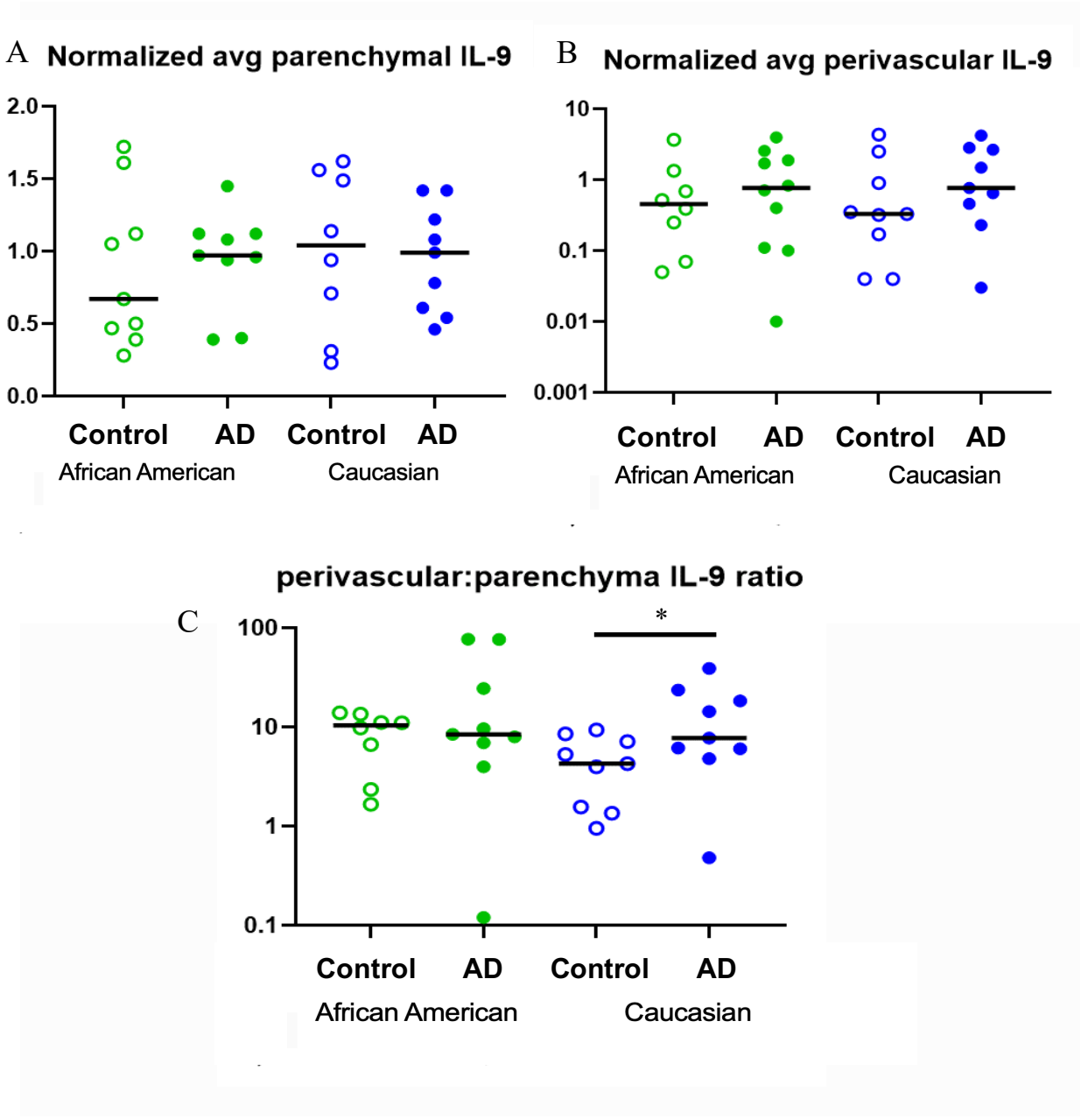

Figure 2: AD is associated with an increase in perivascular IL-9 without associated parenchymal changes in NHW cases. A: In AA cases, there is an upregulation of IL-9 in AD, this is not true for NHW cases. B: Perivascular IL-9 is upregulated in both AA and NHW cases with AD, in comparison to Controls. Data log-transformed due to non-normal distribution C: NHW AD cases have a higher perivascular:parenchymal ratio IL-9 staining ratio than NHW controls ($p=0.094$). This is not true for AA cases. Data log-transformed due to non-normal distribution.

2. IL-9 Receptors are expressed in the brain

By staining for IL-9R, for the first time we were able to confirm the expression of IL-9R in neuronal cells of the cortex. Expression was localized mostly to cell bodies in contrast to the axon terminals. (Figure 3A, B)

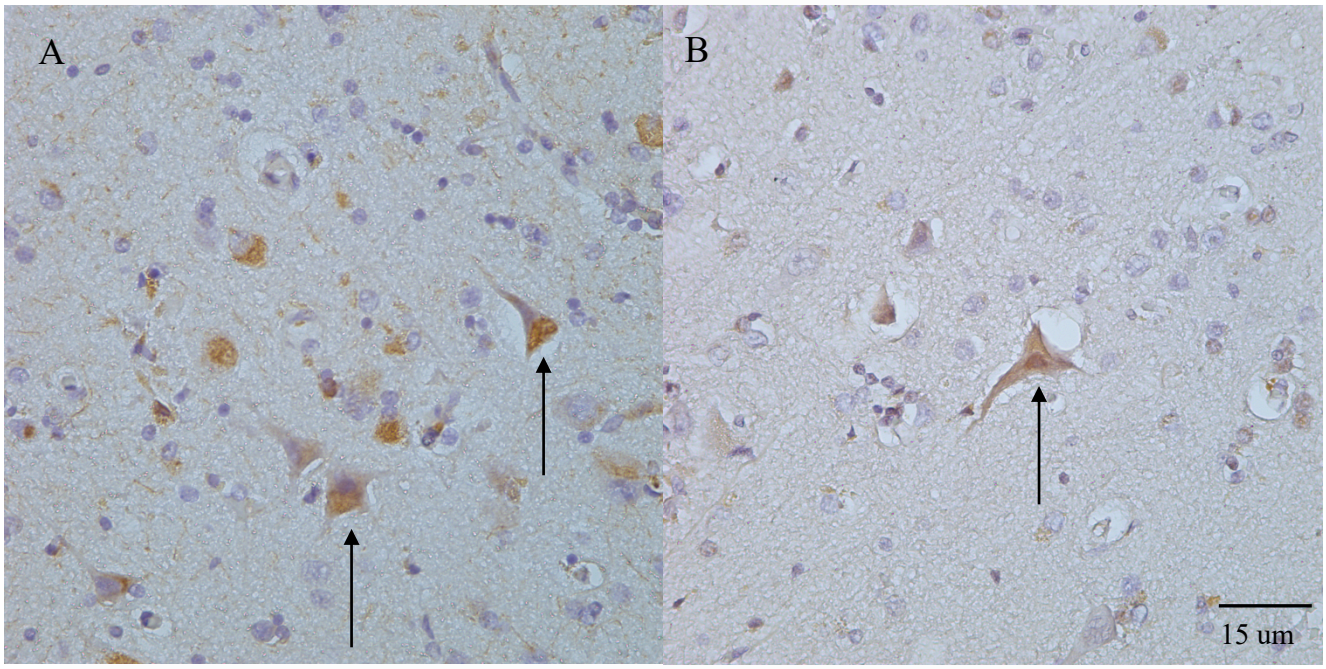

Figure 3A, B: Immunohistochemical analysis reveals IL-9R staining in neuronal cells. Positive stains are brown, marked by arrows.

3. AD associated with higher IL-9 Receptor levels

Immunohistochemical analysis revealed a significant upregulation in positive staining for IL-9 receptors in AD cases compared to control cases (Figure 4A, B) ($p<0.01$) when combining both cohorts. AA cases with AD had a lower average intensity of positive staining than Caucasian (NHW) cases with AD (Figure 4A). NHW cases had a higher intensity of positive staining for both AD and Control in comparison to AA cases (Figure 4A).

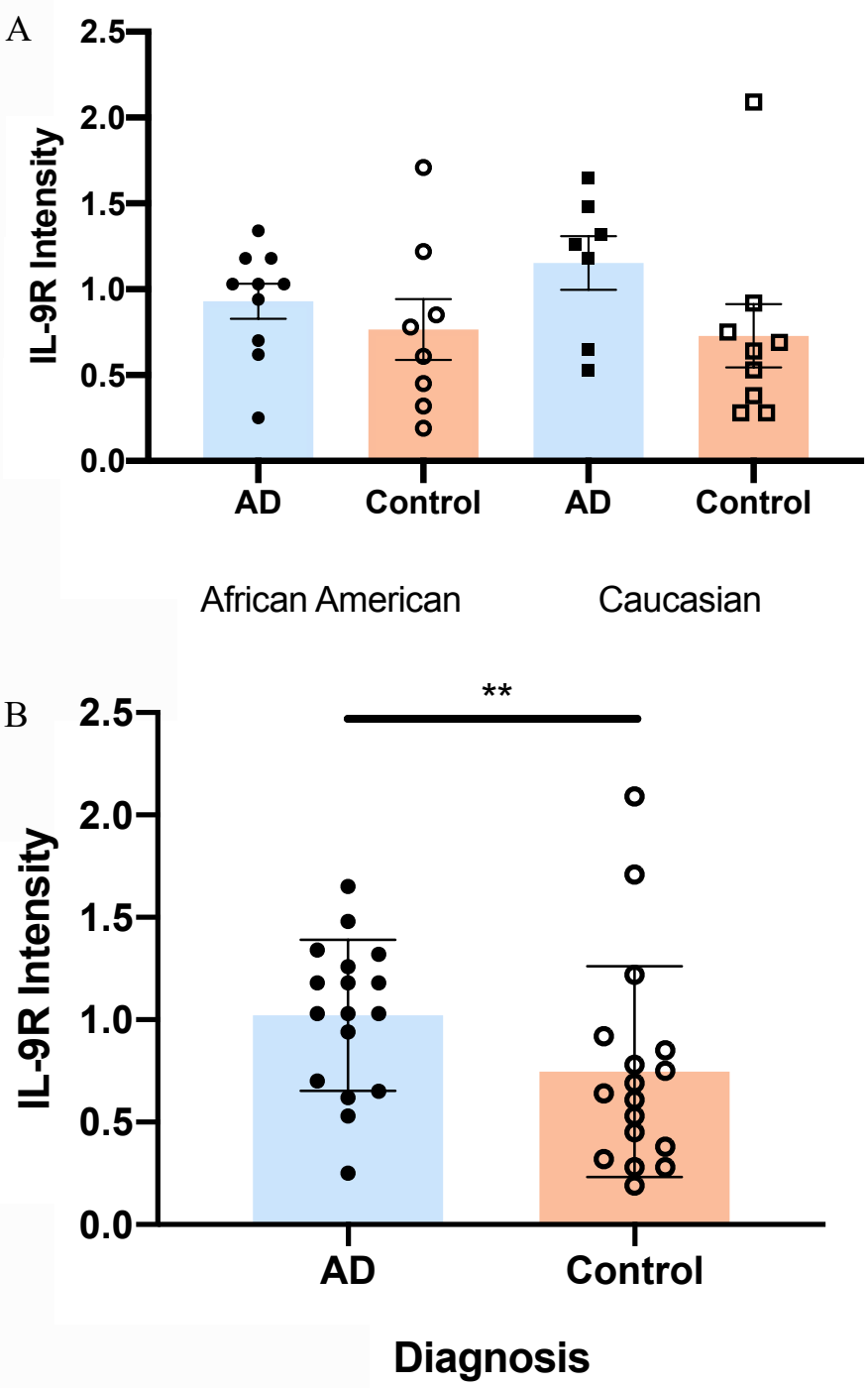

Figure 4: IL-9R levels are upregulated in AD. A: In both AA and NHW cases, an upregulation of IL-9R staining is observed only in AD. B: A significant increase ($p<0.00$) is observed in both AA and NHW cohorts combined.

Discussion

The direct mechanism by which IL-9 acts on brain specific neurons remains to be elucidated. The effects of IL-9 on the transmission and modulation of information in the brain is unknown, as well as its effect at the molecular level and cellular level. Here, we aimed to elucidate the role of IL-9 in the transmission and modulation of neuronal signals in the brain by investigating differential IL-9 and IL-9 Receptors (IL-9R) expression in cases with and without AD.

Our results show for the first time the expression of IL-9R in the brain, and its localization near the neuronal cell body. This finding suggests that IL-9 can theoretically act on neurons. We also show for the first time that IL-9R expression is upregulated in AD, and an attenuated increase in IL-9R for African Americans may mirror their AD-associated increase in brain and CSF IL-9. These complex changes in IL-9 and its receptor in a race- and disease-dependent manner suggests there is a correlation between the cytokine, its receptor, and neuronal function which remains to be explored.

The decreasing ratio of Parenchymal/Perivascular observed only in AD shows an increased level of Perivascular IL-9 compared to Parenchymal IL-9 in AD but not in controls. Controls have more comparable levels of Parenchymal and Perivascular IL-9. The increase seen mostly in Perivascular IL-9 may be showing the movement or diffusion of IL-9 through the arterial walls that is significantly upregulated in AD. This is consistent with our finding that IL-9 is upregulated in AD in AA cases. Overall, NHW cases had a higher ratio than AA cases, implying a higher of Perivascular IL-9 in comparison to Parenchymal IL-9, which may suggest a decreased mechanism of action of IL-9 only in Caucasians.

The involvement of IL-9 in the biological processes of the brain is indicated by the vast array of pathways it is incorporated in throughout the neurodevelopmental spectrum of humans.

Understanding the effect of IL-9 at the molecular and cellular level of neuromodulation and neurotransmission can shed light on other biological processes that implicate the production and secretion of IL-9. The identification of brain-specific cells that express IL-9R provides novel information that will aid the field in future research regarding IL-9 and its effects. As IL-9 levels have shown to be race-dependent, understanding the downstream effects it causes will provide additional information as to how race may modify the susceptibility of varying pathologies and stresses the importance of diverse cohort analysis for future science research to reduce biases. It also highlights the importance of a diverse cohort to make research equitable. Understanding the role of IL-9 in a tissue specific manner will enhance current knowledge of the biological mechanisms by which IL-9 acts.

This study also has its limitations as it is based on a southern US population and may not be applicable through the US or to native African persons. The sample size was limited due to the lower rate of minority participation in brain donation. The high biological variance of IL-9 R in the brain must be taken into account when considering the results and replication with a larger sample size could control for these variations. The observed differences may also be caused by genetic, environmental, cultural and behavioral factors affecting race-based differences that must all be considered when conducting analysis.

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