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Role of CCR3 in aging rhesus monkey brain

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ROLE OF CCR3 IN AGING RHESUS MONKEY BRAIN

by

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ABSTRACT

Each year, aging and age-related deficits in cognitive function affect larger population worldwide. Research on aging has focused on changes in gray matter and white matter with age. A quantitative analysis of magnetic resonance images from healthy subjects of 16-79 years showed a significant negative correlation between gray matter volume and age (Taki et al., 2004). In addition, age-related cognitive decline is reported to be associated with white matter changes such as myelin damage, a result of both the inability of microglia to clear out damaged myelin debris and oligodendrocyte to support remyelination. Eotaxin-1 (CCL11) belongs to a group of eosinophil-specific chemoattractant originally found in peripheral immune system mediating allergic inflammation, asthma and atopic dermatitis (Garcia-Zepeda et al., 1996; Spergel, Mizoguchi, Oettgen, Bhan, & Geha, 1999). Recently it has been reported to have endogenous sources in the CNS and to increase with age in cerebral spinal fluid (CSF) as well as periphery in blood plasma. While CCL11 has been identified to increase with age, injection of CCL11 inhibit neurogenesis in young mice, which is likely to be mediated by C-C chemokine receptor type 3 (CCR3). CCR3 is also the only receptor for CCL11 that is expressed by oligodendrocyte precursor cells (OPCs) and by activated microglia in mice, which means it may participate in the process of microglial phagocytosis and oligodendrocyte myelination. To investigate if CCR3 is an important factor in the normal aging brain and its potential role in these existing findings, immunohistochemistry,

stereology and densitometry were performed in the anterior cingulate cortex and cingulum from brain tissue of 4 young adults and 6 aged rhesus monkeys that were behaviorally tested previously to 1) demonstrate any association between CCR3 expression level and age 2) characterize changes in CCR3 level in relation to cognitive impairment 3) identify cellular localization of CCR3. We found a significant increase in amount of CCR3 cingulate cortex with age, which suggests its pro-disease effect in other pathways such as the interaction between CNS and T cell immune system. Although for aged group increase in CCR3+ cell density in white matter appeared insignificant, we found that CCR3 was expressed exclusively in OPCs but was absent in mature oligodendrocytes. indicating its role in OPC proliferation, oligodendrocyte maturation and myelination.

TABLE OF CONTENTS

TITLE i

COPYRIGHT PAGE ii

READER APPROVAL PAGE iii

ABSTRACT iv

LIST OF TABLES viii

LIST OF FIGURES ix

LIST OF ABBREVIATIONS x

INTRODUCTION 1

METHODS 5

Subjects 5

Fixation 7

Tissue Processing 7

Immunohistochemistry 8

Data Collection and Image Analysis 12

Statistics 13

RESULTS 14

Overview of CCR3 Expression Pattern for Each Subject 14

Effect of Age on CCR3 Expression Level 15

Associations between Age and CCR3 Expression Level 16

Effect of Cognitive Status on CCR3 Expression Level.....18

Identification of Cell Types Expressing CCR3 19

DISCUSSION.....23

Correlation between Gray Matter CCR3 Increase and Aging.....23

Limitations.....25

Conclusion 26

REFERENCES 28

CURRICULUM VITAE.....33

LIST OF TABLES

Table	Title	Page
1	Subject List	6

LIST OF FIGURES

Figure	Title	Page
1	Overview of CCR3 Expression Pattern for Each Subject	14
2	Levels of CCR3 Expression with Age	16
3	Associations between Age and CCR3 expression Level	17
4	CCR3 Expression Level in Subjects with Different Cognitive Status	19
5	Immunofluorescence of CCR3 Receptor and Glial Cells	20

LIST OF ABBREVIATIONS

AD……Alzheimer's Disease

BBB……Blood Brain Barrier

CII……Cognitive Impairment Index

CNS……Central Nervous System

CSF……Cerebral Spinal Fluid

CCR3……Chemokine Receptor Type 3

GM……Gray Matter

NCA……Normal Cognitive Aging

NGS……Normal Goat Serum

NHP……Non-human Primate

OPC……Oligodendrocyte Precursor Cell

ROI……Region of Interest

TBS……Tris-Buffered Saline

WM……White Matter

INTRODUCTION

Aging has become an increasingly significant issue around the world. It's reported that the population aged 60 years or over has tripled since 1950, reaching 600 million in 2000 and surpassed 700 million by 2006. Current projections suggests that the number will triple again in another 50 years (Mba, 2010). While the increasing proportion of the aged puts a higher demand on social and medical services, it also motivates researchers to investigate the effects of the aging process in the structure and function of the brain.

Aging is accompanied by gradual loss of physical strength and dexterity as well as functional decline in the sensory and visceral organs and the immune system, among which age-related cognitive decline is known to occur in both neurodegenerative diseases and in normal aging. Alzheimer's disease (AD) is the most devastating age-related dementia and involves progression of cognitive impairment (Walhovd et al., 2010). In contrast to traditional view that there is neuronal loss with advanced aging, a large number of recent studies are pointing to white matter (WM) pathology. A quantitative analysis of stereology data used autopsied subjects excluding those that died of diseases that could affect CNS directly (e.g. cerebrovascular incidents) or indirectly (e.g. diabetes) and showed that only 10% of neocortical neurons are lost in autopsied humans from 10 to 90 years old but there was a reduction of WM volume by 30% with age (Pakkenberg, 1997). While white matter pathology is poorly understood, it is well known that myelin sheaths provide for isolation between neurons for controlled electric signal conduction as well as rapid, saltatory conduction. Our lab has shown that age-related increases in

myelin pathology play an important role in the pathogenesis of age-related cognitive decline (Bowley, Cabral, Rosene, & Peters, 2010)

Although numerous studies use rodents as an animal model to study normal cognitive aging (NCA) because they are smaller, fast-reproducing and can be easily housed, non-human primates (NHP) are a better model to investigate normal aging in humans in that not only are they phylogenetically closer to human and they do not develop AD in spite of Aβ deposits that are similar to that in human (Heuer, Rosen, Cintron, & Walker, 2012; Jucker, 2010). In this study, rhesus macaques (*Macaca Mulatta*) were used because they share similar maturation progress to humans, their brain anatomy resembles human brain and they naturally demonstrate age-related cognitive decline which can be assessed by cognitive tests similar to those administered on human. Rhesus monkeys age at about three times the rate of human's with a maximum lifespan of ~40 years with puberty occurring at 2.5-4.5 years old (Colman et al., 2009). Therefore, in this study monkeys with age ranging from 6-9 years old were categorized as "young" whereas 26-30 years old grouped as "old".

Chemokines are a family of chemoattractant cytokines. They are small proteins that may be secreted or membrane-bound. Together with their receptors, they play a vital role in cell migration in response to a chemical gradient, a process known as chemotaxis. Originally found in the periphery, their primary functions include regulation of lymph organ development, T-cell differentiation and tumor cell metastasis but fairly recent studies have shown that they function in the central nervous system as neuromodulators. Specifically CCL11, also known as eotaxin-1, although previously found to act in

periphery, has been shown to be produced by choroid plexus epithelial cells in aged mice and is able to undergo bidirectional transport across the blood brain barrier (BBB) by either binding to cellular components in blood or by direct interaction with BBB. They are found in various brain regions including frontal cortex, hypothalamus, hippocampus, occipital cortex and cerebellum, suggesting direct function in the CNS (Erickson, Morofuji, Owen, & Banks, 2014). Furthermore CCL11 was observed to be increased in the brain and cerebral spinal fluid (CSF) in the dorsal lateral frontal cortex(DLFC) in human subjects with chronic traumatic encephalopathy (CTE) compared with Alzheimer's disease patients and healthy controls. In healthy agers CCL11 level was reported to increase in the cerebral spinal fluid and blood plasma. It was also found that young mice exposed in an aged systemic environment have decreased synaptic plasticity, impaired conditional fear conditioning and spatial learning, suggesting CCL11's role in adult neurogenesis during normal aging process (Villeda et al., 2010).

CCR3 is one of the receptors that bind to CCL11 and it is known to be expressed by astrocytes, microglia and neurons(van der Meer, Ulrich, Gonźalez-Scarano, & Lavi, 2000). Specifically microglia are a major target of HIV-1 infection and according to an early in vitro study, CCR3 along with CCR5 expressed by microglia were reported to promote efficient infection of CNS by HIV-1(He et al., 1997). A later study showed that mice lacking Apolipoprotein E(ApoE) demonstrated more neuron damage in CA3 region, greater microglial activation and a greater percentage of activated microglia expressing CCR3(DUAN et al., 2006). Other studies looking at oligodendrocytes in rats suggested a role for CCR3 in myelination process since among all the chemokine receptors only

CCR3 is expressed on oligodendrocyte precursor cells (OPCs) where it appears, in a concentration dependent manner, to mediate a functional increase in OPC proliferation, differentiation, and inhibition of migration when bound by CCL11 (Maysami et al., 2006)

Our laboratory has previously proposed two mechanisms that may underlie myelin damage during normal cognitive aging. First, with age, oligodendrocytes may be compromised and therefore less capable of maintaining myelin or remyelinating after damage. Second, age-related microglial dysfunction may lead to inability of these resident macrophages in the brain to clear debris, damaged myelin byproduct may accumulate (Shobin Eli, 2018). Alternatively, gray matter volume was found to decline gradually with age beginning in early adulthood (Taki et al., 2004). Also taking into account recent findings in other animal models that neuronal, OPC and microglial expression of CCR3 play important roles in either neurodegenerative diseases or normal cognitive aging (NCA), the present study aims to investigate the association between CCR3 expression levels in neurons and glial cells and NCA processes, and tries to introduce CCR3's role in our existing myelin damage proposal in aging rhesus monkey brain.

METHODS

Subjects

Brain tissue from 10 rhesus monkeys (five males and five females) was used in this study. Their age ranges from 6.2 to 29.6 years old. Monkeys were procured from the Emory University Yerkes National Primate Research Center (Atlanta, GA) and were housed individually under 12 hour light/dark cycle at the Laboratory Animal Science Center at the Boston University School of Medicine. Aged animals used for this aging study were brought in at old age after living in an open social colony and remained here until the end of life without experimental intervention. Both the Yerkes National Primate Research Center and the Boston University Laboratory Animal Science Center are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Prior to subject selection, health records were screened to exclude any confound diseases that could impact normal aging process by affecting brain tissue composition and behavior.

Upon entry of this project, all monkeys were behaviorally tested on a battery of tests assessing learning, memory and executive function. The behavioral testing includes: Delayed Non-Matching to Sample (DNMS) testing learning and recognition memory, Delayed Recognition Span Task (DRST) looking at working memory span and the Category Set Shifting Task(CSST) assessing executive function (Herndon et al., 1997; Moore et al., 2007). Based on the principal component analysis (PCA) that includes three test scores – DNMS-acquisition, DNMS-120s delay and DRST-spatial condition, a weighted average of these combined scores is computed and converted into a z-score.

The stack of z-scores then yield a cognitive impairment index (CII) such that animals with CII of 1.0 or less are considered unimpaired, 1.0-2.0 standard deviation above the mean is considered mild impairment and scores above 2.0 reflect serious cognitive impairment (Moore et al., 2007). In this study ten rhesus monkey with various age and cognitive status are included: four young adults, three old cognitively impaired and three old unimpaired rhesus monkeys including both male and female.

Table 1. Subject List. Details on subjects used for this study.

Animal	Gender	Age	CII	Group by CII
AM180	F	29.6	3.513	impaired
AM181	F	27.5	5.89	impaired
AM243	M	24.4	4.402	impaired
AM188	F	6.5	0.364	spared
AM275	F	27.8	1.598	spared
AM284	M	25.4	1.465	spared
AM301	M	28.4	1.14	spared
AM205	M	6.2	0.079	spared
AM227H	M	7.8	0.361	spared
AM255	F	9.5	0.123	spared

At the conclusion of behavioral testing monkeys were given MRI scans and had blood and CSF collected before brain tissue collection.

Fixation

For brain tissue harvest monkeys were sedated with ketamine hydrochloride (10mg/mL, intramuscular) and then deeply anesthetized with sodium pentobarbital (25 mg/kg, intravenous) followed by transcardial perfusion with 4% paraformaldehyde solution in 0.1M phosphate buffer (pH=7.4) at 37˚C. Some subjects were first perfused with 4˚C Krebs-Henseleit buffer (pH=7.4, a mixture of 6.4 mM Na2HPO4, 137Mm NaCl, 5 Mm glucose, 0.3Mm CaCl2, 1 Mm MgCl2; Sigma-Aldrich) before the paraformaldehyde perfusion step. This step not only flushes the brain vasculature but also rapidly cools down the tissue, limiting the extent of potential proteolysis resulting from anoxia. After perfusion, brains were removed intact, weighed and photographed and post-fixed for less than 24 hours.

Tissue Processing

After perfusion, brains were cryoprotected by immersion in 10% glycerol and 2% DMSO in 0.1M phosphate buffer for one to three days until the entire block sank. Then the block was transferred into solution with 20% glycerol and 2% DMSO followed by rapid freezing in -75˚C isopentane. This method has been used in the lab for decades and has been proven to generate the least amount of freezing artifact which is mostly ice crystals formed by the freezing process itself and might disrupt the integrity of tissue by displacing the tissue structure (Rosene, Roy, & Davis, 1986). For brain cutting, the

frozen blocks were removed from the -80 ˚C freezer and fix on the stage of sliding microtome so that they were cut in entirety in the coronal plane. The blocks were maintained frozen by surrounding them with pulverized dry ice that was re-packed every 15 to 20 minutes as needed. Each block was cut into 10 interrupted series of 30 μm frozen sections. 24 vials were prepared for each series and contained 15-20mL of 15% glycerol in phosphate buffer. During cutting, each section was collected and put into the corresponding vial so that each vial contained 6-8 sections that were about 7200μm from their immediately previous section and sections within a series were spaced at 300μm intervals. After cutting, all vials were moved to -80˚C freezer for long-term storage and would be picked out and thawed upon further processing.

Immunohistochemistry

Antibodies labeling CCR3, astrocytes, mature and immature oligodendrocytes and microglia were used to assess tissue.

Oligodendrocytes: CC1 antibody is most commonly used to specifically detect mature oligodendrocyte cell bodies and it is marketed as anti-adenomatous polyposis coli (APC, a tumor suppressor protein) clone CC1. Although APC was originally found to be linked to colon cancer, it has been reported to have a high expression level in the adult brain (Ratan et al., 1994). It has been established that antibodies targeting the N-terminal peptide (like CC1) have light staining for astrocytes and an intense staining for oligodendrocytes, which owes to the cross-reactivity with an unrelated antigen (Brakeman, Gu, Wang, Dolin, & Baraban, 1999). CC1 is primarily used to identify

mature oligodendrocyte cell bodies but its state-specific expression patterns haven't been fully characterized(Ness, Valentino, Mciver, & Goldberg, 2005).

Oligodendrocyte precursor cells: NG2 antibody is directed against the NG2 chondroitin sulfate proteoglycan, therefore identifies early oligodendrocyte progenitors and adult oligodendrocyte progenitors(Ness et al., 2005). Oligodendrocyte precursors are first identified at embryonic day 12-14 (E12-14) by their expression of mRNA for the platelet-derived growth factor alpha receptor (PDGFαR), when they also responded positive for NG2 (Nishiyama, Lin, Giese, Heldin, & Stallcup, 1996). As they mature into oligodendrocytes they gradually lose expression of NG2 antigens and enter an intermediate pro-oligodendrocyte stage recognized by O4 immunoreactivity, before they are identified as mature, fully differentiated oligodendrocyte upon detection of respective markers such as myelin basic protein(MBP) and myelin oligodendrocyte glycoprotein (MOG) (Pfeiffer, Warrington, & Bansal, 1993). Therefore in addition to O4 and PDGFαR, NG2 is also widely accepted as a marker for OPCs.

Astrocytes: GFAP (glial fibrillary acidic protein) is widely used as the best marker for astrocytes. It was first isolated as a protein highly concentrated in old demyelinated plaques from multiple sclerosis patients and was found to be associated with reactive astrocytes in such plaques and other pathological contexts. It was later found to be an intermediate filament protein expressed by astrocytes in the CNS and it's involved in the structure and function of cytoskeleton. In astrocytes, GFAP is thought to help maintain mechanical strength. Studies in transgenic mice indicated that the expression of GFAP is not essential for the normal appearance and function of most astrocytes, but is essential

for the process of reactive astrogliosis and glial scar formation (Pekny & Pekna, 2004; Pekny et al., 1995). However GFAP-negative mice managed to display post-traumatic reactive gliosis, indicating that GFAP regulation, although a hallmark for reactive gliosis, is not a necessity for this process (Pekny et al., 1995).

To eliminate variation in chromagen staining across cases, for any each specific stain, sections from all subjects were removed from -80°C freezer at the same time, rapidly thawed to room temperature and batch processed all together using the same reagents for chromogen staining. Briefly, for each monkey 6-12 sections were pulled out at section intervals of approximately 1200 μm and thawed to room temperature. Prior to primary antibody incubation, tissue sections were rinsed to remove the glycerol and treated with 10% hydrogen peroxide for 30 minutes to eliminate endogenous peroxidase activity. Sections went through blocking solution (10% normal goat serum plus 0.3% triton in TBS buffer) for an hour to reduce non-specific binding and background signal and were then incubated into primary antibody solution with 1:5000 CCR3 antibody (R&D system, USA), 5% NGS and 0.3% triton in TBS buffer overnight at room temperature. The next day tissue sections were rinsed and incubated with secondary antibody for an hour, in a solution consisting of 1:1000 biotinylated secondary antibody, 2.5% NGS and 0.3% triton in TBS buffer. Sections were then rinsed and incubated in 0.45% A/B reagent. Specifically, biotin was conjugated to the secondary antibody and avidin, a large glycoprotein that binds to biotin, can be labeled with peroxidase. DAB solution was activated with 0.0083% hydrogen peroxide right before reacting with tissue sections and then peroxidase was developed by DAB for seven minutes to produce colorimetric end

products. Sections were then mounted on gel-subbed slides, dried for 24 hours before coverslipping with permount.

Immunofluorescence was used for double-labeling and was performed on tissue sections from another young animal for a qualitative analysis on CCR3-targeted cell types. Briefly, sections went through quenching for two hours with 0.075g/mL glycine together with 10% NGS and 0.3% triton, eliminating any free aldehyde groups that could non-specifically bind antibodies. Then for oligo fluorescence, sections were incubated with primary antibody solution containing 1:250 rat CCR3, 1:500 mouse CC1 and 1:250 rabbit NG2 plus 5% NGS and 0.3% triton and microwaved twice at 250 watt, 30 ˚C for five minutes and continued overnight at room temperature. For astrocyte or microglia fluorescence procedure, in addition to CCR3, 1:500 GFAP from rabbit or 1:500 mouse LN3 and 1:500 rabbit IBA1 were used as primary antibodies. The next day sections were incubated for two hours with 1:1000 goat anti rat, 1:600 goat anti mouse and 1:600 goat anti rabbit secondary antibodies with excitation spectrum at 488 μm, 647 μm and 568 μm respectively, and mounted immediately after rinse. Next, autofluorescent eliminator was added to the slides to eliminate lipofuscin-like autofluorescence that could potentially accumulate with age and affect actual fluorescent signals. The slides were then coverslipped in DABCo.

All rinses consisted of five-minute incubation with TBS (pH=7.39) for three times and all steps took place on a rocker unless stated otherwise.

Data collection and image analysis

After coverslipping, slides were dried for at least 24 hours and cleaned right before analysis. Slides were blind coded to prevent experimenter bias. Tissue sections were selected from the point where corpus callosum appears anteriorly to the end of posterior hippocampus. For each subject five to twelve sections were processed within this range. For cell counting. Stereoinvestigator (SI) software from Microbrightfield Inc was used to map out cingulum, a collection of white matter fibers projecting from the cingulate gyrus. For each section, optical fractionator program on the SI program was used that is connected to a Nikon Eclipse E600 microscope, and the contour of ROI was outlined under 2x objectives. The percentage of area for counting was set at 2% and 40x objectives was used to make sure that there were 5-10 cells in each counting frame. Then a grid was generated with the overall layout of all counting frames. Cells were counted based on their staining intensity (cells with certain color intensity was chosen as a standard and any cells deeper than that get counted. The standard remained consistent throughout counting all subjects). Then for each section, total number of cells was estimated by dividing the number counted with area percentage. Finally, cell density was estimated based on total number of each section, area of each section and section intervals.

Tissue imaging was done for densitometry analysis. In order to measure CCR3+ cell density in cingulate cortex, gray matter was mapped out for each section using the same set of slides from chromagen staining. Images were taken from anterior to posterior. Specifically, SRS acquired workflow on SI software was used to obtain images. Contour

was outlined at 2x objective on a Nikon Eclipse E600 microscope and 7% of the contour was randomly selected for taking image under 20x objective. Z plane for imaging was set where the most cells were in focus. Images where white matter occupies over 50% of the area were ruled out. Images were then imported into FIJI and run with a macro looking at percent area in the binary form indicating CCR3+ cell density, and mean density in the grayscale photo indicating intensity of staining in gray matter.

Statistics

R Studio was used for all statistical analysis. CCR3-positive cell density in cingulum, percent area, mean density and integrated density for CCR3-positive cells in cingulate cortex were calculated and run through one-way ANOVA with between group factor "age" (young, old) and "cognitive status" (impaired, spared), respectively. Data also went through two-way ANOVA with factors "gender" and "age" or "gender" and "cognitive status" to look at potential interaction.

For correlation and regression, since there was only one independent variable for each analysis, single linear regression was run through on the same program.

RESULTS

Overview of CCR3 Expression Pattern for Each Subject

Scatterplots show CCR3+ cell density in cingulum, percent area of staining mean staining intensity in cingulate cortex. Through preliminary observation there might be an increase in WM cell density and GM percent area staining in aged group. Effect of cognitive impairment on CCR3 expression level might be insignificant. Detailed analyses are revealed as followed.

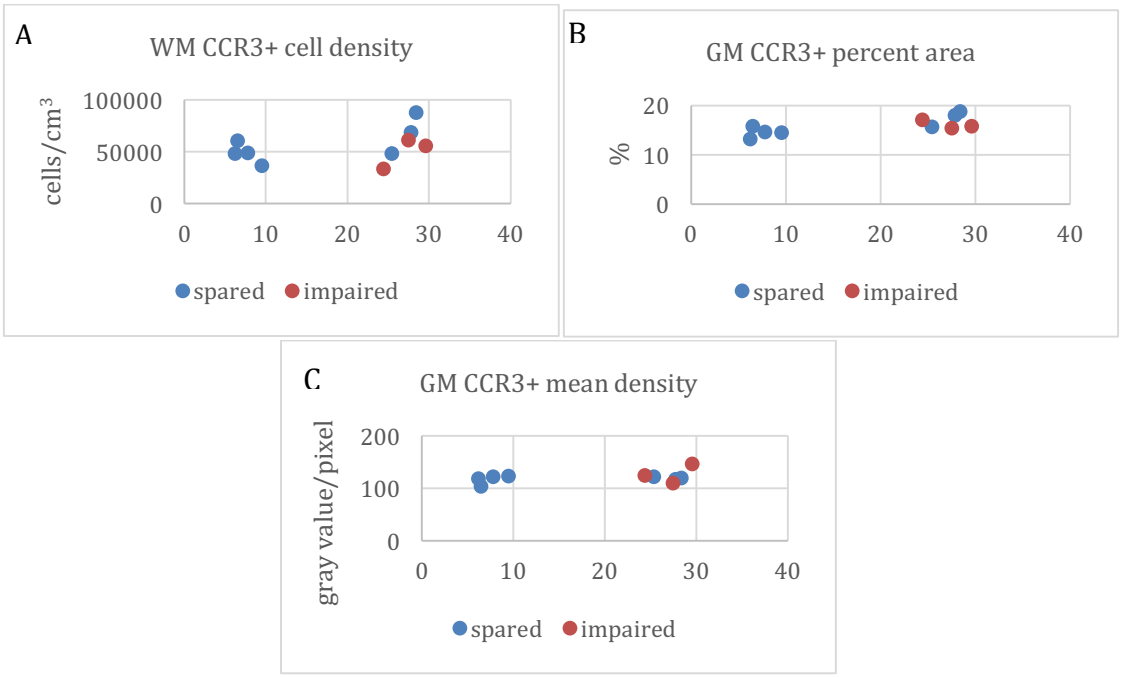


Figure 1: Overview of CCR3 Expression Pattern for Each Subject. This figure shows age-related changes of CCR3+ cell density in cingulum(1A), percent area(1B) and mean staining intensity(1C) in cingulate cortex.

Effect of Age on CCR3 Expression Level

A two way ANOVA comparing CCR3+ cell density in cingulum (Figure 2A) with between-group factor "age" and within-group factor "gender" showed no significant difference between groups ($F(8,1)=0.815$, $p=0.401$), no significant difference between male and female ($F(8,1)=0.073$, $p=0.796$) and in interaction between age and gender ($F(8,1)=0.060$, $p=0.815$). Although there was not a significant difference with age, there was a trend that for aged group there were slightly more cells expressing CCR3 in cingulum. A two-way ANOVA comparing percent area of CCR3+ staining in cingulate cortex (Figure 2B) showed a significant difference between group "young" and "old" ($F(8,1)=6.531$, $p=0.043$), no significant difference between gender ($F(8,1)=0.001$. $p=0.9752$) and no interaction between age and gender ($F(8,1)=1.219$, $p=0.3118$). A two-way ANOVA comparing CCR3+ mean staining intensity in cingulate cortex (Figure 2C) showed no significant difference with age ($F(8,1)=0.589$, $p=0.472$), no significant difference between males and females ($F(8,1)=0.040$, $p=0.848$) and no interaction between age and gender ($F(8,1)=0.299$, $p=0.604$).

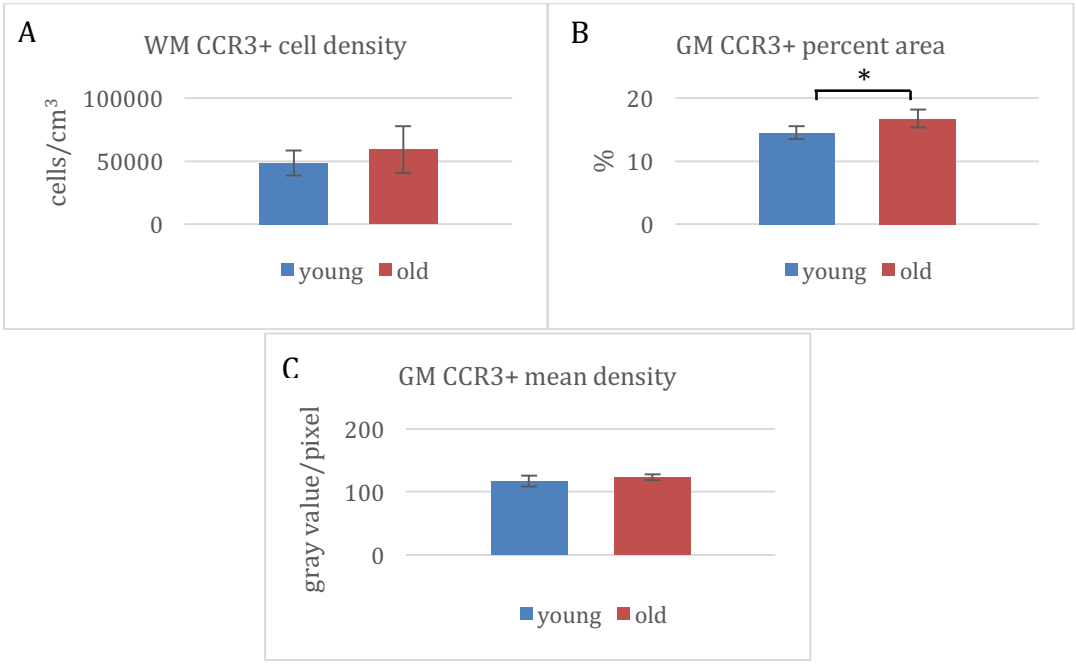


Figure 2: Levels of CCR3 Expression with Age. Figure 2A shows stereology data of the number of CCR3+ cells in cingulum of young compared to old. Figure 2B and 2C were from densitometry and Fiji for image processing. Figure 2B showed the proportion of area that was stained with CCR3 antibody. Figure 2C represented sum of grayscale value of each pixel of the image divided by number of pixels, the result of which showed mean staining intensity of different age groups. ANOVA showed that in aged group there is a significantly larger area where CCR3 is present ($F(8,1)=6.531$, $p=0.043$). This suggests that aged animals have more CCR3 expressed in cingulate cortex.

Associations between Age and CCR3 Expression Level

A simple linear regression model was used to correlate white matter CCR3 expression level with age (Figure 3A) and showed no significant correlation (R2=0.05345, p=0.2543). A simple linear regression model was used to correlate gray matter CCR3 expression level with age (Figure 3B, 3C). As showed by Figure 3B, significant correlation was found between age and percent area of staining (R2=0.4153, p=0.0263) but no significant correlation was found between age and mean staining intensity (Figure 3C) (R2=0.01335, p=0.32).

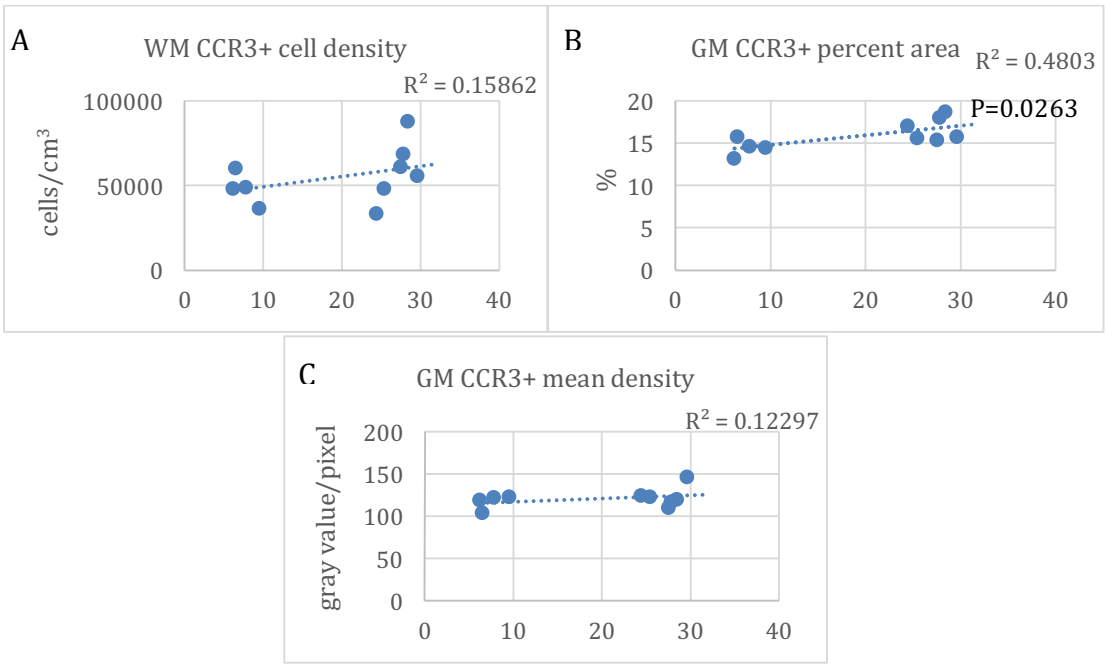


Figure 3: Associations between Age and CCR3 Expression Level. Single linear models reveal that CCR3 percent area staining in gray matter is associated with age (R2=0.4153, p=0.0263) (B).

We showed that more advanced age of a subject is correlated with larger proportion of cells in cingulate cortex that express CCR3.

Effect of Cognitive Status on CCR3 Expression Level

A two-way ANOVA comparing CCR3 expression level in both cingulum and cingulate cortex with between-group factor cognitively "impaired" versus "unimpaired" and within-group factor "gender" showed no significant effect of cognitive status on cell density in cingulum (Figure 4A, $F(4,1)=1.235$, $p=0.382$), staining percent area (Figure 4B, $F(4,1)=1.207$, $p=0.387$) or mean staining intensity(Figure 4C, $F(4,1)=0.224$, $p=0.831$) in cingulate cortex. Only data from the six aged monkeys were used in this analysis to only look at effect of cognitive impairment. No significant effect of gender was found on WM cell density ($F(4,1)=0.548$, $p=0.536$), GM percent area of staining ($F(4,1)=0.049$, $p=0.845$) or on GM mean staining intensity ($F(4.1)=0.001$, $p=0.98$). No interaction was found between gender and different cognitive status regarding WM cell density ($F(4,1)=0.485$, $p=0.558$), GM percent area of staining ($F(4,1)=0.716$, $p=0.486$) and GM mean staining intensity ($F(4,1)=0.059$, $p=0.831$).

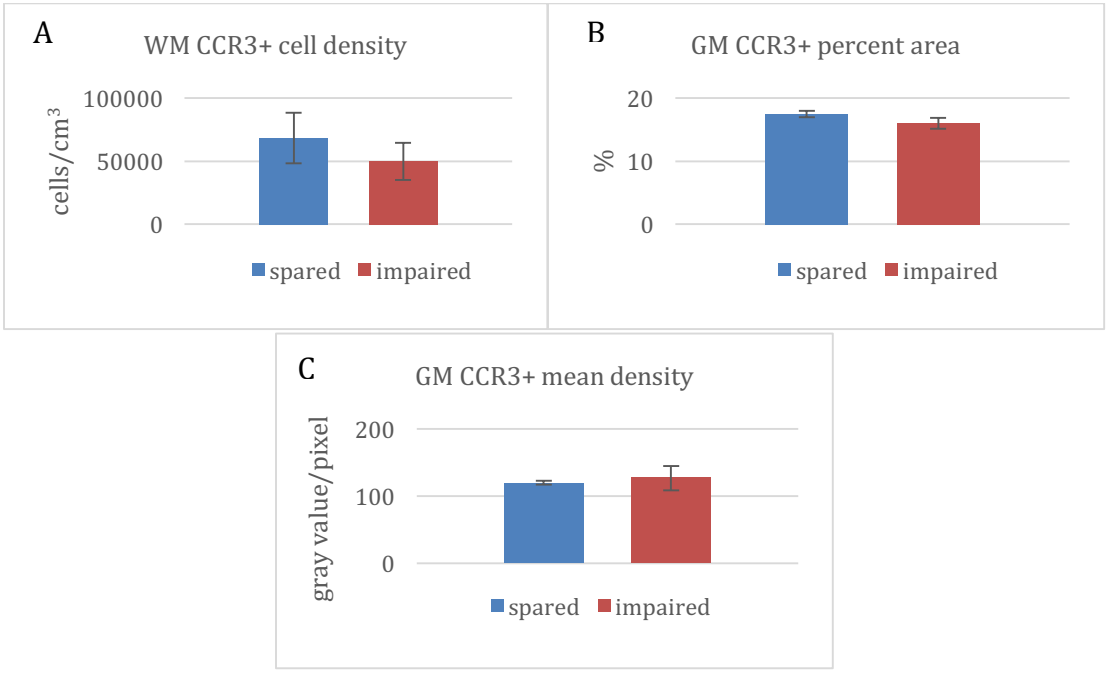
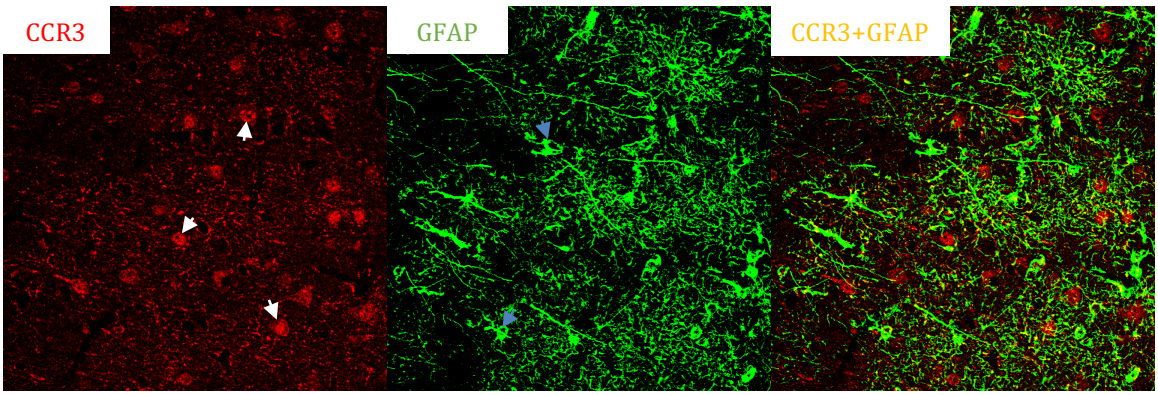


Figure 4: CCR3 Expression Level in Subjects with Different Cognitive Status. Graphs showed number of cells expressing CCR3 in cingulum (A), CCR3 percent area staining (B) and mean staining intensity in cingulate cortex (C) of six aged monkeys that were grouped by extent of their cognitive impairment based on their CII scores. Two-way ANOVA shows that there is no significant difference between cognitively spared and impaired subjects regarding CCR3 expression level in either white matter or gray matter.

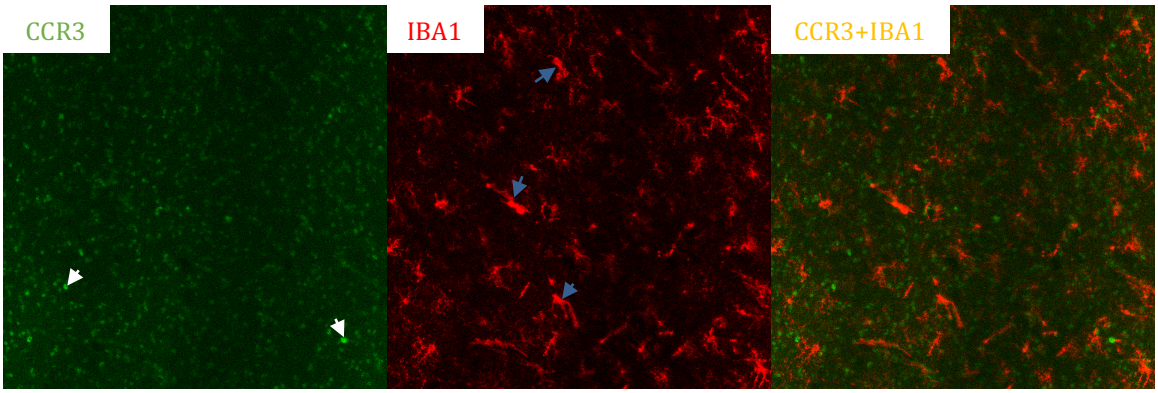
Although non-significant, Figure 4A showed a tendency that subjects that were more cognitively impaired tend to have less CCR3 expression in cingulum.

Identification of Cell Types Expressing CCR3

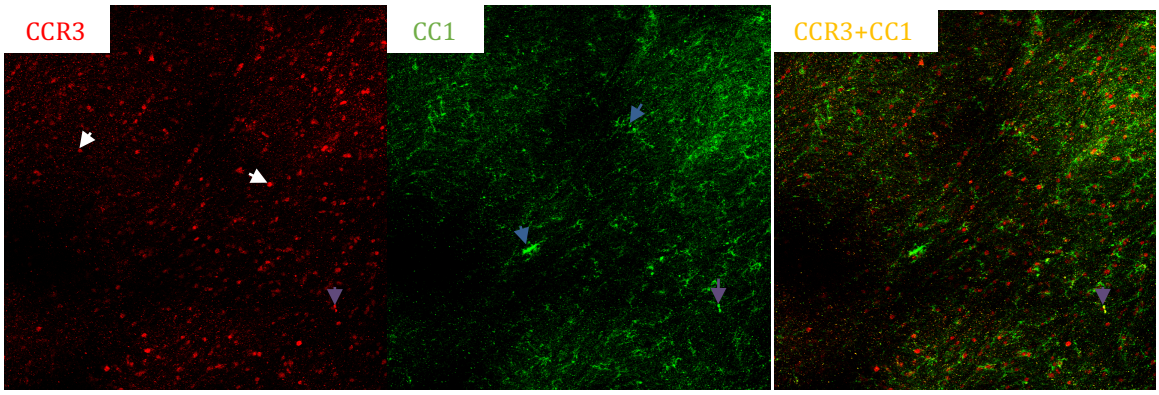
To further identify cells that were stained positive for CCR3, immunofluorescence was performed on tissue sections from another young, unimpaired animal to double-label cells with CCR3 antibody and various cell markers in both gray matter and white matter. Figure 5A-C show labeling in gray matter under 40x with color red showing cells with CCR3, green for GFAP but no overlapping signals were present in the merge image. This means that CCR3 in gray matter may not come from astrocytes. Figure 5D-E show staining for CCR3 and microglia, respectively. The merged image (Figure 5F) shows no overlapping signals, indicating that microglia in the white matter doesn't express CCR3. Figure 5G-I and Figure 5J-L show co-localization of CCR3 and mature oligodendrocytes or immature oligodendrocytes, respectively. Figure 5I shows very few but some overlapping signals while Figure 5L has much more overlapping signals, indicating while some mature oligodendrocytes express CCR3, the main resource of this receptor is from immature oligodendrocytes, or OPCs.



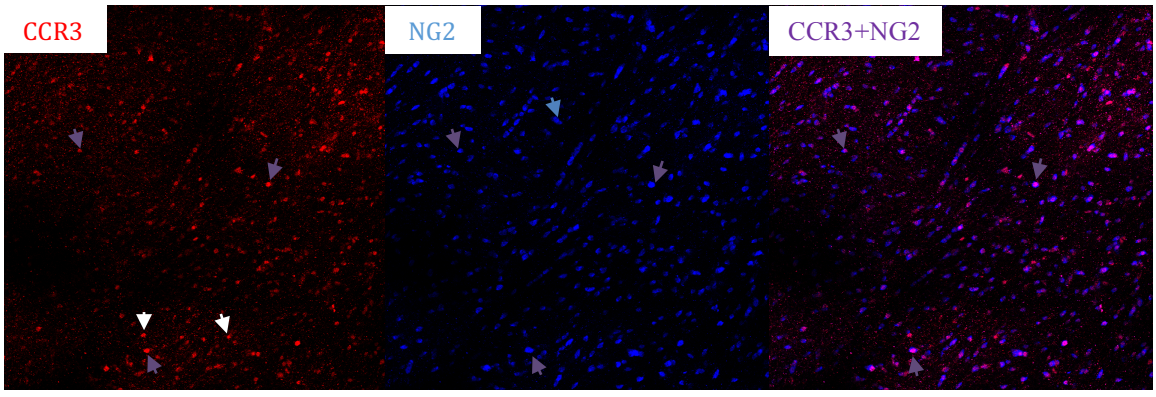
A B C



D E F



G H I



J K L

Figure 5: Immunofluorescence of CCR3 Receptor and Glial Cells. Images demonstrate double-labeling of CCR3 and four different markers for various type of glial cells. some true signals were pointed with arrowheads as examples for them to stand out from background signals. Each image shows the entire range of Z from top to bottom of the section (approximately 30μm), therefore although colors appear to be overlapping in Figure A-C and D-F, they are in fact present in different Z planes. Real co-localization was showed in Figure I and L where co-localized signals in merged images were represented with a different color. Purple arrowheads point to several cells as an example where they were stained positive for two different antibodies.

Here we demonstrate that presence of CCR3 in gray matter is mainly from neurons not astrocytes, and that CCR3 in white matter is mainly expressed by immature oligodendrocytes.

DISCUSSION

Chemokines were previously shown to be elevated in the serum and CSF during aging and produced by activated glial cells in process of neuroinflammation in AD brain (Choi et al., 2008; Leung et al., 2013; Soares et al., 2012). In this preliminary study we investigated the general role of C-C chemokine receptor type 3 (CCR3) in normal aging rhesus monkey brains by measuring CCR3 expression level in the white matter of the cingulum and the gray matter of cingulate cortex. Our previous findings demonstrate that clearance of damaged myelin may be impaired in aging and cognitive decline as a result of microglia becoming dysfunctional with age and OPCs being halted in their normal function (Shobin Eli, 2018). In addition, in healthy aging myelin debris are effectively removed whereas in cognitively impaired agers myelin debris remains in the brain, causing further damage (Shobin Eli, 2018). In the present study, histological examination of CCR3 level in young adult and aged rhesus monkeys reveals that (1) in aged subjects there is a significant increase in number of cells in cingulate cortex that express CCR3. Additionally, the increase followed a linear regression model and correlated with age. (2) Although not significant, there was a trend for aged subjects to have more cells in white matter that express CCR3 and a trend that cognitively impaired agers tend to have smaller number of CCR3+ cells. (3) In young adult monkey brain, oligodendrocyte precursor cells are the primary source of CCR3 in white matter.

Correlation between Gray Matter CCR3 Increase and Aging

In the present study we showed an age-related increase in number of cells expressing CCR3 in cingulate cortex. While the amount of CCR3 per cell remains not significantly different, we could postulate an overall increasing amount of CCR3 in cingulate cortex during aging. Although simply based on present data it remains unknown whether the increasing amount of CCR3 facilitates aging process or it is part of aging pathology, another in vitro study has found the interaction between CCR3 and CCL11 to be involved in the production of Aβ and dendritic spine loss in AD pathogenesis in that these effects could be blocked by CCR3 specific antagonist (Zhu, Xu, Sun, Zhu, & Sui, 2017) suggesting a causative effect of CCR3 on neuroinflammation.

Two more recent studies have pointed to a pro-disease effect of CCR3. In 2016 Zhang et al used mouse cortical culture and found that inhibition of CCR3 provides protection from cell death caused by ischemia and glucose deprivation, therefore identified CCR3 as a mediator for neuronal death (Zhang et al., 2016); Ahmad et al treated BTBR mice with CGS, A2A receptor agonist, and found a decrease in C-C chemokine receptor signaling. A2A is known to have neuroprotective, immunomodulatory and analgesic properties and its down-regulation of chemokine receptor signaling pathways indicate receptors including CCR3 might facilitate progression of neuroimmunological diseases (Ahmad et al., 2018). Indeed substantial evidence pointed to the interaction and crosstalk between T cell immune system and CNS. The role of T cells on CNS could be both beneficial and pathologic (Anderson et al., 2014; Olson & Gendelman, 2016; Yshii, Gebauer, Bernard-Valnet, & Liblau, 2015), and CNS itself can also induce changes in the immune system via neuro-endocrine-immune network (Hirokawa, Utsuyama, & Kobayashi, 1998;

Procaccini, Pucino, De Rosa, Marone, & Matarese, 2014; Tanriverdi, Silveira, MacColl, & Bouloux, 2003). Thymus, central organ of the T cell immune system, also undergoes natural aging process characterized by involution resulting in autoreactive T-cells altering permeability of BBB and attacking choroid plexus (Kunis et al., 2013; Schwartz & Baruch, 2014; Shechter, London, & Schwartz, 2013), the two major paths by which CCL11 enters the brain. Our current result that aged monkeys have an increase in amount of CCR3 is in accordance with these above-mentioned findings, meanwhile specifically underlines the significance of CCR3 and puts forward the potential role of CCR3 in immune system – CNS interaction.

Limitations

Although there has been substantial evidence from our previous study that myelin damage and other shite matter pathologies are key to cognitive impairment along with normal aging progression, current study failed to identify any significant changes in CCR3 level in white matter either with different age groups or various extent of cognitive impairment. It is possible that (1) amount of CCR3 simply is not altered or (2) its role failed to be demonstrated due to small sample size, relatively large within-group variation, difference in tissue processing and complicated background of individual subjects. For example, one old impaired subject (AM180) and two young unimpaired subject (AM188, AM205) was perfused only with 4% paraformaldehyde while the rest went through Kreb-Henseleit solution first for ten minutes in which case cells are exposed to prolonged anoxia and potentially more prone to cell stress. While AM180 showed a relatively lower level of CCR3 in white matter compared to other aged

subjects, CCR3 level of the two young subjects remain within the range of other young ones. If CCR3 were to be involved in white matter pathology during aging, AM180, being more sensitive to cell stress, could possess a smaller amount of CCR3 compared to reality where it was perfused with paraformaldehyde ten minutes later. In addition, another old subject (AM301) was tested cognitively spared (CII: 1.14) yet had the highest number in WM CCR3+ cell density. He had a history of diabetes and had been treated with resveratrol, which is known to have effective antioxidant and anti-inflammatory function. This could help with his cognition to the extent that balances out the adverse impact CCR3 possibly has on cognitive function. Considering the limited sample size, although these variations are individual, they could still have a specific but profound influence on some of the results.

In summary, although current study failed to demonstrate significant changes in white matter CCR3 expression level, considering the aforementioned limitations, we couldn't deny the potential role of CCR3 in white matter pathology involved in normal cognitive aging.

Conclusion

Aging is accompanied with volume loss of both gray matter and white matter in the CNS. Ever since Villeda et al found injection of CCL11 into (Villeda et al., 2010) young mouse brain resulted in memory impairment and inhibition of neurogenesis, numerous studies have identified it to be pro-inflammatory. However, through which pathway does CCL11 "talk" to resident cells in brain and to invoke such changes remained unknown. As our previous findings attribute age-related cognitive impairment to white matter pathology,

this study focuses on CCR3 whose expression was found to be active but highly regulated in mouse brain, uses a more translational animal model and tries to illustrate CCR3's involvement in our existing aging model and other hypotheses. Specifically, we found in this study that there is an increasing amount of CCR3 in gray matter with age. Although such increase was not observed in white matter, nor did we find significant changes in CCR3 with cognitive impairment, we did identify OPCs as a crucial resource of CCR3 After OPCs develop into mature oligodendrocyte, CCR3 expression wasn't observable. Due to the limited sample size, background variability of our subjects and the unique expression pattern by OPCs, it's possible that CCR3 is involved in white matter aging pathology. Therefore it is necessary for future research to include larger sample size, to further assess changes in types of cells that express CCR3 along with age, and to investigate level of CCL11 in gray matter and the change of CCL11-CCR3 interaction during aging.

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CURRICULUM VITAE