EFFECTS OF AGE ON THE DENSITY OF PERINEURONAL NETS AND PARVALBUMIN-EXPRESSING INTERNEURONS IN THE RETROSPLENIAL CORTEX OF BEHAVIORALLY CHARACTERIZED MACAQUES

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Abstract:

Aging takes a toll on all aspects of one's body, including the brain. Deficits in cognitive function, which are associated with normal brain aging, have been seen in both human and macaque models. While several brain aging models are used, Macaca mulattas are useful in that they do not develop neurodegenerative diseases like humans do while also having many anatomical similarities to humans, allowing for better understanding of the normal brain aging process. In previous literature, it has been suggested that extracellular buffering structures, known as perineuronal nets, play a role in the aging brain, specifically with regards to neuronal protection and plasticity. In order to better understand the effect that these perineuronal nets have on brain aging, the density of these nets, along with the density of the parvalbumin interneurons that the nets preferentially surround, was recorded. To further unpack the impact of these nets on cognition, their density was compared to performance on three common behavioral tasks that test object recognition memory, reward-associated recognition memory, and spatial short-term memory. We observed a greater proportion of parvalbumin (PV) neurons that expressed perineuronal nets (PNNs) in adult monkeys compared to aged monkeys. There were, however, no age differences seen in the density of perineuronal nets, or parvalbumin interneurons within the retrosplenial cortex. With regard to the behavioral data, better object recognition performance was significantly associated with a higher proportion of parvalbumin-expressing interneurons surrounded by perineuronal nets. No significant effects were observed in the other behavioral tasks.

Introduction:

Cognition is an important aspect of human health and well-being. While cognition varies on an individual basis, it is known that cognition tends to decline with age (Gazzaley, et al., 2005). An important misconception is that while some individuals will develop disorders related to aging, the majority of individuals do not (Plassman, et al., 2007). This fact makes it critical to dedicate significant efforts towards studying the normal aging process as this is what the majority of people will experience in their lifetime. Furthermore, the average human lifespan has also increased over the past few decades. Specifically, the average lifespan of adults in the United States was 78.6 years old in 2017 and has been increasing by an average of 0.7 years since 2005 (Arias, et al., 2019). This increase of physical health makes it essential to ensure that cognitive health is also preserved in older age. In addition, one major goal of brain aging research is to determine the differences between individuals who will develop age-related diseases and those who will not (Cole, et al., 2019). By learning about the normal aging process of the brain, we can also learn more about the diseased brain. This knowledge will allow for more specialized diagnosis and treatment of age-related neurodegenerative disease to allow individuals to stay active in society longer and fully engage with their families and future generations. It is important to address these issues in order to maintain cognition in healthy individuals and restore cognition in those who are affected by these devastating problems (Cole, et al., 2019). While the majority of cognitive aging research has utilized older human participants, the macaque monkey has also been a useful animal model for studying the effect of age on cognition since they also experience natural age-related declines in brain function (Comrie, et al., 2018).

Macaque monkeys are especially useful in modeling cognitive aging, as they share many of the same cognitive abilities as humans (Aizawa, et al., 2011). Another benefit of macaques in

brain aging research is that they do not develop neurodegenerative diseases, such as Alzheimer's disease (Squire and Zola-Morgan, 1988). This allows for neurobiological changes due to normative brain aging to be unambiguously separated from those due to neurodegenerative disease, which is a confound that is often encountered in human brain aging research. Finally, monkeys have more similar brain anatomy and cognitive abilities to humans compared to other animal models of brain aging, such as rodents (Petrides and Pandya, 1999).

Numerous tasks have been developed to probe distinct aspects of cognition in macaques, and older animals often show impairments on these tests relative to adults (for review see Hara, et al., 2012) The delayed response (DR) test, for example, is a spatial working memory task that requires participants to remember the spatial location of an object over a delay period. Older monkeys show significantly lower correct responses and require more trails to perform the task correctly, indicating that spatial short-term memory function becomes less efficient across the macaque lifespan. This task activates the dorsolateral prefrontal cortex (dlPFC) in functional brain imaging studies, and furthermore, removal of this brain region with localized lesions significantly impairs a monkey's performance on this task. Among other functions, the dlPFC, specifically area 46, is important in playing a role in working memory and executive function (Leubke, et.al, 2010). The delayed non-matching-to-sample (DNMS) test is another test commonly used to assess cognition in aged monkeys. This task tests a subject's ability to recognize visual stimuli. In these experiments, a monkey is presented with an object, then given a delay period. After the delay the animal is presented with a novel object alongside the familiar object. The monkey then learns through trial and error that choosing the novel object leads to food reward. It has been shown that lesions to the hippocampus and perirhinal cortex produce significant impairments to DNMS performance (Zola-Morgan, et al., 1989, 1993, 1994).

Therefore, it is postulated that the medial temporal lobe is involved in this sort of memory function (Corkin, 1984). The last task that is commonly used to evaluate reward-associated memory is the object discrimination (OD) task. It has been proposed that reward associated memory involves the VTA (Lob, et al., 2016). By using these tasks in the same group of animals, we are able to test the function of distinct frontal and medial temporal lobe brain circuits necessary for cognition. Numerous tasks similar to the three described above exist and are commonly used to assess cognition in aging monkeys. One major goal of brain aging research is to uncover neuroanatomical and electrophysiological correlates of age-associated behavioral changes such as those described above.

Perineuronal nets (PNNs) are extracellular structures that surround neurons and have been hypothesized to play a role in the cognitive decline observed in aging by regulating synapse stability and synaptic plasticity (Reichelt, et al., 2019). Perineuronal nets are extracellular matrix structures composed of chondroitin sulfate proteoglycan (CSPGs) and linking proteins that preferentially accumulate around parvalbumin-expressing inhibitory interneurons in certain areas of the cerebral cortex (Wen, et al., 2018). Intriguingly, net degradation with net-specific enzymes has been shown to negatively impact multiple cognitive processes, including working memory and visual stimuli recognition (Paylor, et al, 2018). While it is relatively well understood the purpose these nets serve for neuron function, it not clear how these nets change over time in the normal aging process, or how such alterations in PNN function might impact cognition in older individuals.

The density of PNNs is not equivalent across the brain, and one region in which the density of PNNs is relatively high is the retrosplenial cortex (RSC). This is an area of the brain located at the posterior end of the cingulate cortex, and contains Broadman areas 29 and 30. In

recent years, it has been discovered that the RSC plays a large role in a variety of cognitive functions, such as reasoning, planning, episodic memory, and even navigation (Vann, et al., 2009). The focus of this study is to assess how the density of PNNs that surround inhibitory PV neurons changes during the process of normal aging in the RSC cortex of rhesus macaques. It has been shown that PNN density changes over time in a rat model, and that this change affects cognition. A loss of perineuronal nets in the prefrontal cortex of rats has also been associated with decreased cognition (Paylor, et al., 2018). Therefore, gaining information about how PNN density changes in the aging brain may give us insight into the effect PNNs have on cognition.

Materials and Methods Section:

Subjects:

The subjects that were used for this experiment were 30 male and female rhesus macaques (*macaca mulatta*) ranging in age from 7 to 32 years at the time of data collection (16 aged, mean 24.6 years; 14 middle-aged, mean 13.9 years). Human-equivalent lifespans are estimated to be 3 times that of monkeys (Ershler, et al., 1988; Tigges et al, 1988; Hakeem et al., 1996). Using this estimation, the macaques used here were equivalent to humans in the age range of 21 – 96 years. All subjects were living in the California National Primate Research Center in Davis, California, United States. During this time, long-term behavioral and electrophysiological experiments were carried out with the macaques. The procedures elicited in the behavioral testing procedures below were approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Behavioral testing procedure:

Behavioral tests were carried out in a modified Wisconsin General Test Apparatus (WGTA). This apparatus consists of three wells, a central well and lateral wells on each side of it. Objects, both familiar and novel depending on the specifics of the behavioral test, are placed on top of rewards that are within the wells. Depending on the order or presentation of objects and the rules imposed on the animals, this set up is able to test different aspects of learning and memory behavior in animals. The three behavioral tests conducted within this apparatus were a delayed response (DR) test, a delayed nonmatching-to-sample (DNMS) test, and an object discrimination (OD) task.

The first behavioral test used was the delayed response (DR) task, which examines an animal's spatial short-term memory. In this task, one of the two lateral wells from the WGTA task had a food reward placed in it, with the reward being randomly placed in each well relatively equally. The macaque watched the experimenter place the food reward into the well. Then, the experimenter covered both of the lateral wells with an identical plate, forcing the macaque to remember the location of the food reward. A delay period was then induced by blocking the macaques from viewing the two wells. Afterwards, the block was removed, and the macaque was able to pick one plate to move in order to receive the food reward. If they picked the correct plate, they would be rewarded with the food found in the well under it. Once the monkey had correctly picked the plate with the food reward under it $\geq 90\%$ of the time across 90 consecutive trials at both 0 and 1 second delay conditions, delays of 5, 10, 150, 30, and 60 seconds were applied. Each delay was tested 30 times/day for 3 days with 20 second inter-trial intervals during each delay.

The second behavioral test used was the delayed nonmatching-to-sample (DNMS) task, which examines the animal's ability to differentiate familiar from novel objects, a form of

memory known as object recognition memory. An object was first placed over the central well, which was baited with food reward. After the animal obtained the reward, displacing the object in the process, the reward was moved out of view for 10 seconds using the trap door of the WGTA. After the 10 second delay period, the object that was initially covering the reward was placed on top of one lateral well and a novel object was placed on top of the other lateral well. In this phase of the trial, the animals were only rewarded if they removed the novel object, as the food reward was always placed in its well. A learning criterion of > 90% over 5 consecutive sessions at the 10 second delay condition was used before delays of 15, 30, 60, 120, and 600 seconds were tested. Twenty trials per day were administered for all of the delay conditions with the exception of the 600 second condition in which only 5 were given per day. A 30 second interval being implemented between trials.

The third behavioral test was an object discrimination (OD) task, which examines object-reward association memory. In this training, the macaques were shown pairs of objects that were visually very different and placed over the lateral wells of the WGTA. One of the objects always had the food stimulus below it, whereas the other always did not. Each macaque was allowed 2, 30 trial sessions of training, then given a 2-day rest period. The test was repeated for 4 visually different object pairs, with intervals of 15 seconds in between each trial. A state-space model (Smith, et al, 2004) allowed for estimation of the speed of learning.

Perfusion:

After the conclusion of the behavioral experiments, the animals were anesthetized using sodium pentobarbital (60 mg/kg, i.v.) and trans-cardinally perfused using a solution made up of 4% paraformaldehyde (PFA) in a saline phosphate buffer (0.01M). The purpose of this solution

was to fix the brain to prevent autolysis and putrefaction and to preserve the tissue and its components. After the first solution was administered, a second solution of 10% sucrose and 4% PFA was used to continue fixation to prepare the tissue for freezing. After extraction, the brains were placed in a 30% sucrose and 4% PFA solution at 4 degrees C until they were saturated to ensure that the tissue was fully cryogenically preserved. After saturation was attained, the brains were sectioned coronally at 30 microns and placed in long-term storage at -80 degrees C.

Immunohistochemistry:

The retrosplenial cortex (RSC) in the posterior cingulate gyrus of the brain was the region of interest in this study. This region was studied because it receives sensory information and is thought to be involved in learning and memory (Murray & Bussey, 1999).

The immunohistochemistry began with the removal of the brain slices from the -80 degrees C freezer to allow for thawing. After thawing had completed, the tissue was hemisected into the right and left cortices and the brainstem. The tissue was then rinsed in Tris buffer saline (TBS) three times, for 5 minutes each time. After rinsing, the tissue underwent an antigen retrieval protocol where the tissue was submerged in a sodium citrate buffer solution in a water-bath heated to 86 degrees C for 30 minutes. The tissue was then placed in a room-temperature water bath and allowed to cool down. After, the tissue was washed again in TBS, 3 times for 5 minutes each time. Following the set of three washes, non-specific antibody binding was counteracted using a Tris buffer solution, herein referred to as the “block” solution. The tissue was placed in this block solution of 5.0% normal donkey serum (NDS; Sigma-Aldrich, D9663-10ML) along with 0.3% Triton X-100 (TX; Sigma-Aldrich, x100) and Tris buffer for 1 hour. After the 1-hour block incubation, the tissue was then incubated using two different primary

antibody combinations, one for each hemisphere that was previously hemisected. The first type of primary incubation was composed of parvalbumin (polyclonal antiserum in Guinea Pig, [1.5:1000], ab195004), Aggrecan (polyclonal anti-Aggrecan in Mouse, [5:1000], ab1031), and Wisteria Floribunda Lectin (WFA) ([1.5:100], B-1355) in the block solution mentioned above. The second type of primary incubation was composed of parvalbumin (polyclonal antiserum in Guinea Pig, [1.5:1000]; af195004), WFA ([1.5:100], B-1355), and either HAPLN (polyclonal anti-HAPLN in Goat, [1:100]; af2608) or IBA1 (Synaptic systems, [1:1000], 234003) in the block solution. Note, however, the hemisected tissue stained for IBA1 and HAPLN were not analyzed for this thesis. After the primary, antibodies were added to the tissue, it incubated overnight (approximately 21 hours). The next day, the tissue was washed in TBS four times, 10 minutes each time. Once the washes were complete, the secondary antibodies were added to the well plates containing the tissue and incubated for 2 hours. The tissue that was incubated overnight in the solution containing Aggrecan had three secondary antibodies added to it: Dylight™ 405 (anti-mouse in Donkey, [1:200]; ab141856), Cy™ 2 (anti-Rabbit in Donkey, [1:200]; ab142845), and Cy5® (Streptavidin, [1:200], ab142571). The tissue that had been incubated overnight in the solution containing HAPLN was incubated with Alexa Fluor™ 488 (anti-rabbit in Donkey, [4:2000]; Abcam, ab150073), Dylight™ 405 (anti-mouse in Donkey, [1:200]; ab141856) and Cy5® (Streptavidin, [1:200], ab142571). Following the secondary antibody incubation, the tissue was washed in TBS four times, 10 minutes each time. Once all washes were completed, the sections were mounted on 1.0 mm thick slides (Brain Research Laboratories, #5075-PLUS) and were cover slipped using an 80% glycerol and PBS solution and a #1.5 thickness coverslip (Brain Research Laboratories, #4860-11/2, 0.16 – 0.19 mm thick), sealed along the corners with nail polish.

Spectral Imaging and Data Processing:

"Lambda" Collection:

Slides were left to dry overnight and then were imaged using the "Lambda" collection method on a ZEISS LSM 800 inverted confocal microscope with a field of view through a Plan-Apochromat 20x/0.8 air objective. The lambda collection consisted of taking Z-stacks in the area of the retrosplenial cortex using the software ZEN Black 2.1. These images were collected in tile-scan mode in 3x5, 4x4, and 5x3 fields of view. The Z-stack took images every 1 micrometer deep until it had encompassed the entirety of the tissue containing fluorescent perineuronal nets. These Z-stacks were measured and quantified at 8-bit units and were taken at a resolution of 512 x 512 pixels. Aggrecan sections were visualized using 405, 488, and 633 lasers nm and HAPLN sections were visualized using 405, 488, 561, and 633 nm lasers. The 405 nm laser was used to help with characterization of the autofluorescence spectra. One image was taken from each piece of tissue, and a total of 86 images were further analyzed to determine the density of parvalbumin (PV)-containing interneurons and perineuronal nets (PNNs).

Characterization of Spectral Fluorescence:

Before linear unmixing took place, control data was collected for the 405 and 561 spectra. The reference spectra found auto fluorescence and was compared with the control to make sure that the program running the spectral fluorescence had properly accounted for the autofluorescence (see Pyon et al., 2019 for more details). This was repeated for every image to counteract differences in autofluorescence emissions.

Data Processing and Linear Unmixing:

Data Processing and Linear Unmixing was done using the program ZEN Blue. Unmixing is the process of classifying the spectra of fluorescent emissions contained within each pixel of the image. The program uses a least-squares fit algorithm to discriminate different channels for each pixel. For the images stained with Aggrecan, four reference spectra were used: 405, Cy2 (488), Cy5 (WFA), and the autofluorescence spectra. For the slides stained with HAPLN, five reference spectra were used: 405, 488, Cy3 (HAPLN), Cy5 (WFA), and autofluorescence spectra. Note, again, that the data from the slides stained with HAPLN was not quantified for this thesis.

Results:

Results of Cognitive Assessment/Battery:

Delayed nonmatching-to-sample

In the delayed to nonmatching-to-sample (DNMS) task, aged macaques required more trials than did middle-aged macaques to reach learning criterion (LMM, $\beta = 71.02$, CIs [43.8, -98.2], $t(36) = -5.3$, $p < 0.0001$; Figure 1A). In addition, aged macaques had lower percent correct responses across delays compared to the middle-aged monkeys (LMM, $\beta = -0.00807$, CIs = [-0.0135, -0.00261], $t(227) = -2.91$, $p = 0.00393$; Figure 1B).

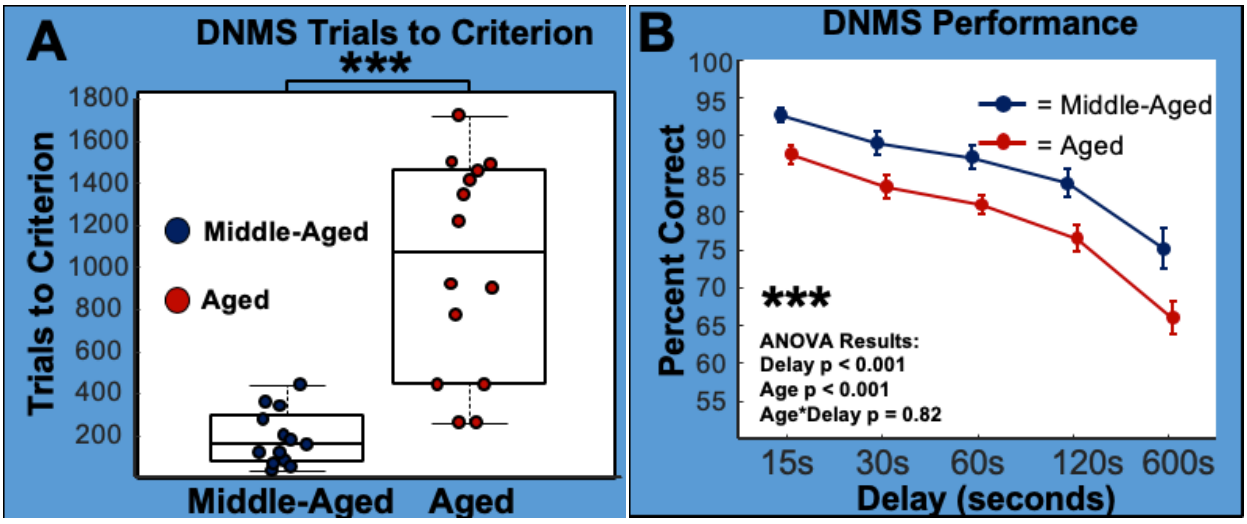

Figure 1: Results from delayed nonmatching-to-sample test of object recognition. **A)** Trials to reach the criterion of ≥90% correct responses over five consecutive sessions for middle-aged and aged monkeys. Note that older animals required more trials than did younger animals to reach the learning criterion. **B)** Average performance across delay conditions in the DNMS task. In both **A** and **B,** blue indicates middle-aged animals and red indicates aged animals. Middle-aged animals performed better on the DNMS task than did aged animals as seen from percent correct responses.

Delayed response test of spatial short-term memory

In the Delayed Response (DR) task, no significant difference was found between aged and middle-aged macaques with regards to trials to learning criterion (LMM, β = -1.45, CIs = [-4.48, 1.58], t(222) = -0.944, p =0.346; Figure A). Similarly, the adult and aged monkeys performed equivalently across all delay conditions (LMM, β = -1.28, CIs = [-4.3, 1.75], t(222) = -0.831, p = 0.407; Figure B).

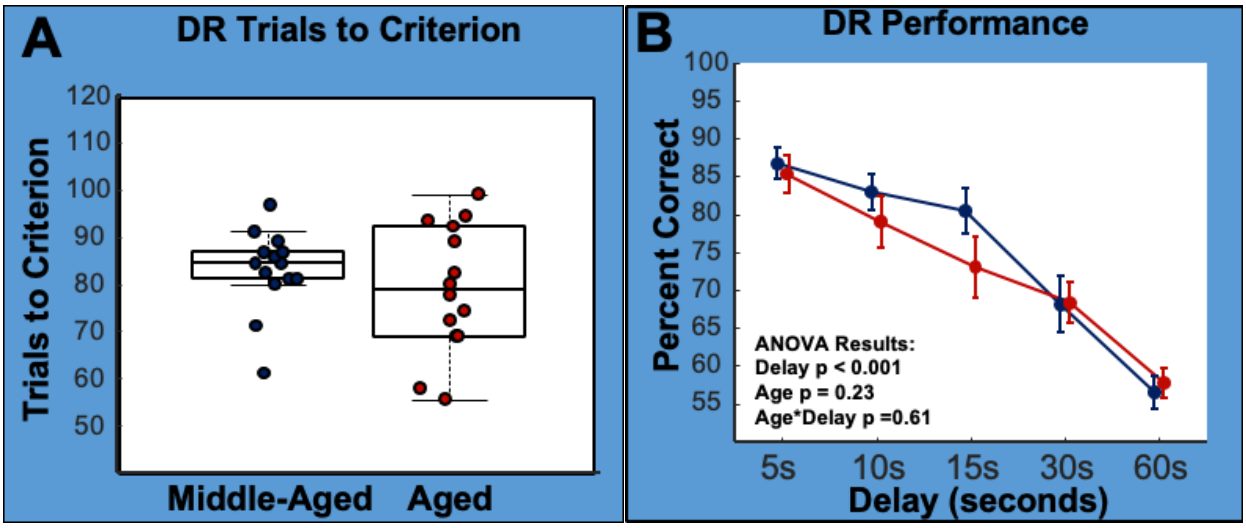

Figure 2: Results from delayed response (DR) test of spatial short-term memory. **A)** DR trials to criterion for middle-aged and aged monkeys. **B)** DR performance across delays for middle-aged and aged monkeys. Blue indicates middle-aged animals and red indicates aged animals. Middle-aged and aged monkeys were not different in the number of trials that they required to learn the DR task, or on their performance across delays.

Object discrimination test of stimulus-reward association learning

The Object Discrimination (OD) task showed a significant difference in learning trials between aged and middle-aged macaques, with aged macaques requiring more learning trials than middle-aged individuals (LMM, Age: $\beta = 1.69$, CIs = [0.568, 2.82], $t(33) = 3.06$, $p = 0.0043$; Figure 3A). Furthermore, middle-aged macaques performed significantly better than

aged macaques in the percent correct for responses in the object discrimination task (LMM, β = -2.60, CIs = [-3.53, -1.66], t(142) = -5.46, p < 0.0001; Figure 3B).

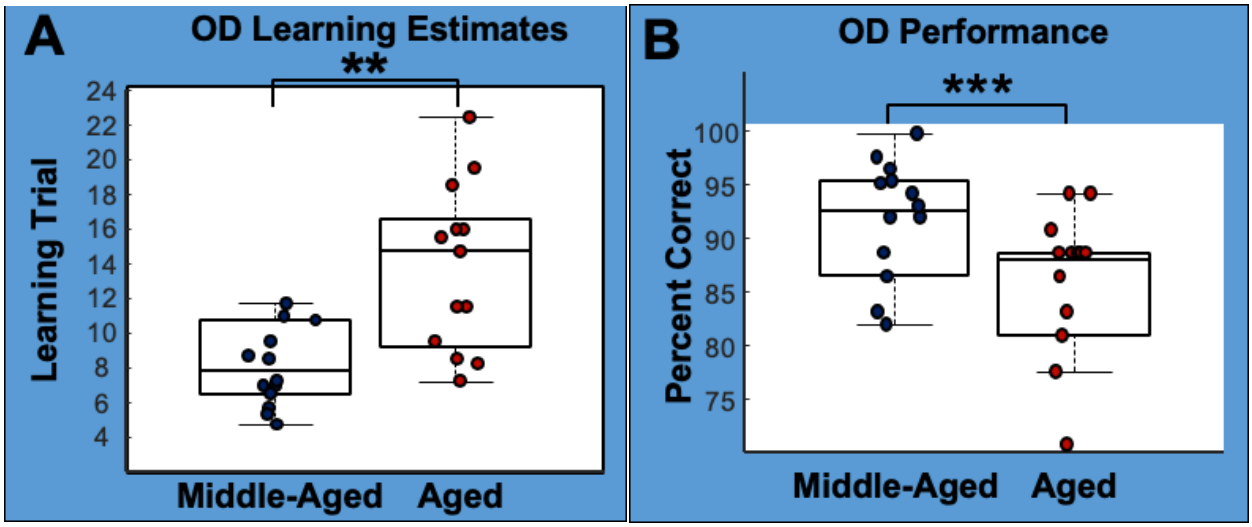

Figure 3: Object discrimination (OD) task acquisition of learning estimates and performance. **A)** Aged animals required more trials to learn the OD task to criterion than did middle-aged animals. **B)** In addition, middle-aged animals out-performed aged animals in the percent correct responses.

Results of Neuron Density:

Parvalbumin-containing neuron density

The density of parvalbumin (PV) neurons within the retrosplenial cortex was not different between adult and aged monkeys (p-value: 0.92016, Tstat: -0.1013, df: 23, sd: 2.7839E+3; Figure 4A). Regression of the density of PV neurons with respect to age was not statistically significant (r: 0.020929, p-value: 0.31998; Figure 4B).

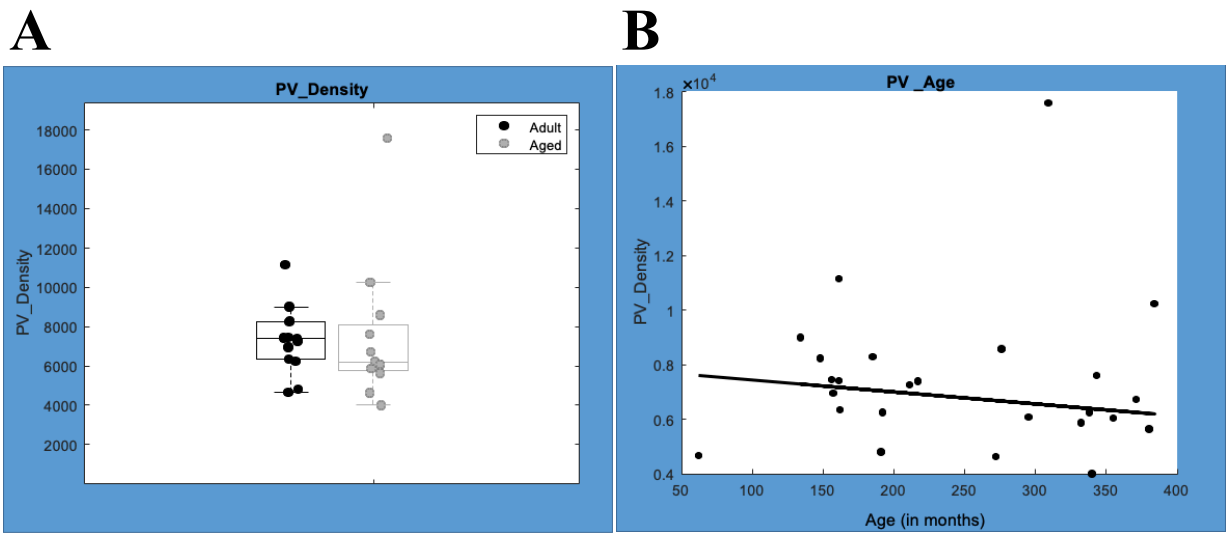

Figure 4: Parvalbumin neuron density**. A)** Adult monkeys tended to have a greater density of PV neurons compared to aged monkeys, although this trend is not statistically significant (p-value: 0.92016). **B)** The relationship between PV neuron density and age was not significant (r: 0.02093, p-value: 0.31998).

Perineuronal net density

Adult monkeys were not shown to have a higher denisty of perineuronal nets (PNNs) compared to aged monkeys (p-value: 0.36455, Tstat: 0.925, dt: 23, sd: 3.1481E+3, Figure 5A; r: -0.1209, p-value: 0.2611, Figure 5B).

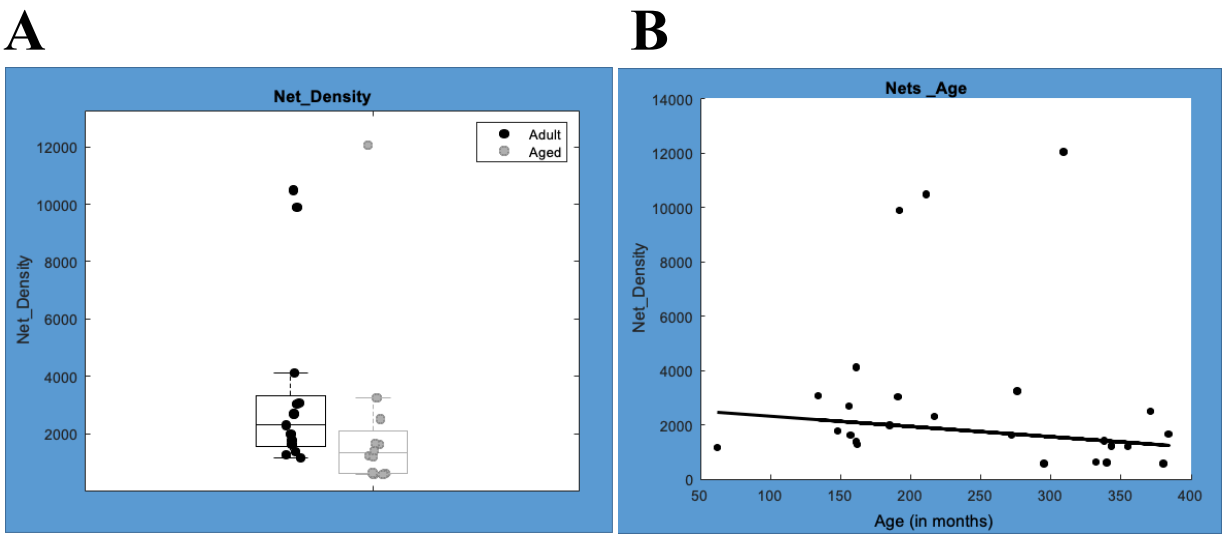

Figure 5: Perineuronal net density in adult and aged monkeys. **A)** The average density of PNNs was not significantly difference between adult and aged monkeys (p-value: 0.36455). **B)** No relationship between PNN density and age was observed (r: -0.1209, p-value: 0.2611).

Proportion of Parvalbumin Cells Expressing Perineuronal Nets

The proportion of parvalbumin (PV) neurons expressing perineuronal nets (PNNs) was greater in adult monkeys compared to aged monkeys (p-value: 0.018917, Tstat: 2.5251, df: 23, sd: 0.0680, Figure 6A, B). However, the regression in Figure 6B was not statistically significant.

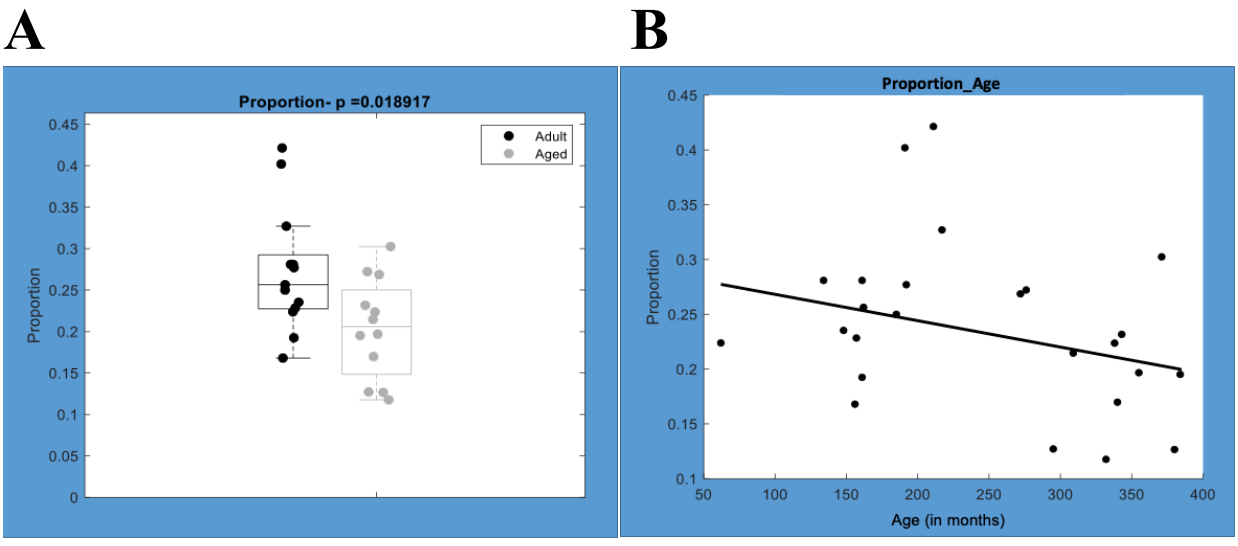

Figure 6: Proportion of PV neurons expressing perineuronal nets (PNNs) in adult versus aged monkeys. **A)** The proportion of PV neurons expressing PNNs in adult monkeys was significantly greater than in aged monkeys (p-value: 0.018917). **B)** The statistical relationship between the proportion of PNN expressing PV neurons and age was not significant (r: -0.34195, p-value: 0.1413).

Results of Relationships to Behavior:

DNMS, DR, OD tasks

The relationship between the proportion of parvalbumin (PV)-containing neurons expressing perineuronal nets (PNNs) and performance on the three behavioral tasks was assessed. There was a significant relationship between proportion of PV neurons surrounded by PNNs and percent correct responses for the DNMS task, as seen in figure 7A (r: 0.43472, p-value: 0.022417). Figure 7B shows that the relationship between PV neurons surrounded by PNNs and the percent correct responses for the DR task was not significant (r: 0.25318, p-value: 0.84928). Similarly, figure 7C shows the relationship between PV neurons surrounded by PNNs and percent correct responses for the OD task was also not statistically significant (r: -0.1596, p-value: 0.27203).

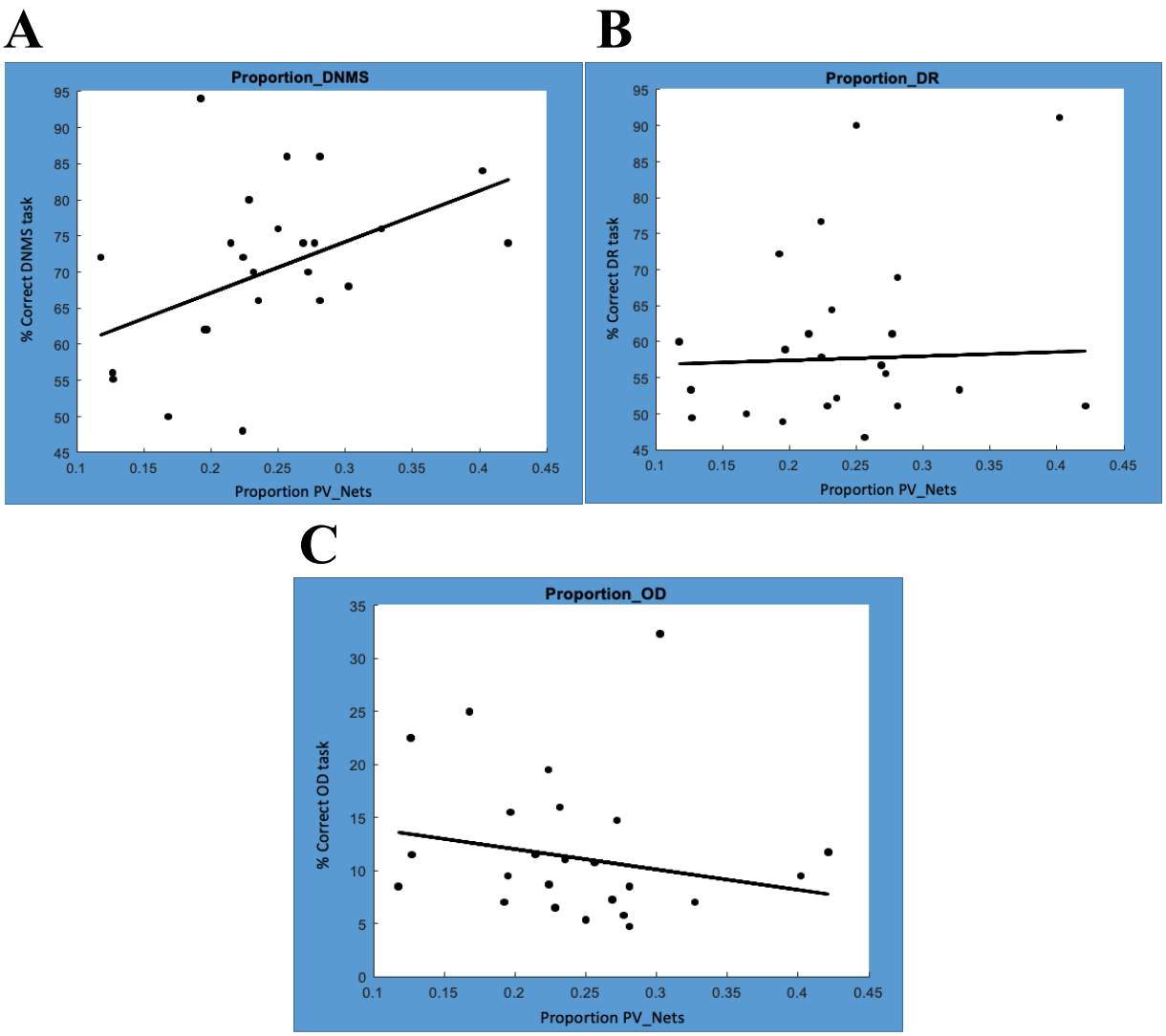

Figure 7: Relationships between the proportion of parvalbumin (PV) neurons expressing perineuronal nets (PNNs) and the percent correct responses on the delayed non-matching to sample (DNMS), delayed response (DR), and object discrimination (OD) behavioral tasks. **A)** The proportion of PV neurons expressing PNNs compared to the percentage of correct responses in the DNMS task showed a statistically significant relationship (r: 0.43472, p-value: 0.022417). **B)** The proportion of PV neurons expressing PNNs compared to the percentage of correct responses in the DR task was not statistically significant (r: 0.25318, p-value: 0.84928). **C)** The

proportion of PV neurons expression PNNs compared to the percentage of correct responses in the OD task was also not statistically significant (r: -0.1596, p-value: 0.27203).

Discussion:

Results of Cognitive Assessment/Battery:

Human brain aging has been modelled using a variety of non-human animals, including rodents and nonhuman primates (Bizon, et al, 2012). Rhesus macaques (Macaca mulatta), in particular, are the most commonly used nonhuman primate model of brain aging as they are the phylogenetically closest model to humans of the common laboratory animals. For our research purposes, another benefit to macaques is that they do not develop neurodegenerative diseases, so we can learn about the normal brain aging process through studying them. While the age at which macaque are considered “aged” is somewhat arbitrary, researchers have generally agreed that 21 years, which equates to a human equivalent of 63 years, is deemed “aged” in these animals (Hara et al., 2012; Tigges et al., 1998). It has also been shown that cognitive decline begins in late teen years for these macaques (Bachevalier, et al., 1991).

The macaque monkey brain is an anatomically comparable model to the human brain, and consequently the processing necessary for many aspects of cognition, such as working memory and matching rules, are similar (Neubert, et al., 2014; Passingham, et al., 2009). This conclusion comes from lesion and structural and functional imaging studies that compare cognitive functioning in non-human primates to humans. Importantly, however, there are also differences between the brains of the two species, one of the most prominent being in the functional organization in higher visual areas (Orban, et al., 2004). Even with these differences in mind, the macaque brain is still a commonly used non-human model for studying human brain

aging. One of the most detrimental aspects of brain aging in humans is the accompanying cognitive decline. Not only does this aging hinder our ability to live fulfilling lives, but it also deteriorates the lives of those around us. One strategy that has been employed to study cognitive aging in macaques is to administer cognitive assessments that are designed to probe distinct aspects of cognition, and consequently the function of different higher order associated brain circuits across the frontal and temporal lobes. The present study employed three commonly used tests: a delayed nonmatching-to-sample test, an object discrimination test, and a spatial delayed response test.

The delayed non-matching to sample (DNMS) task is used to assess recognition memory of non-spatial stimuli (Rapp and Amaral, 1991). Aged macaques were shown to require more trials than middle-aged macaques to learn the DNMS task to a previously defined criterion, and additionally, older monkeys performed more poorly relative to middle-aged animals across the increasing delay conditions (see figures 1A and B). Both of these observations are in agreement with previous reports that older monkeys are impaired on the DNMS task (Bachevalier, et al., 1991). Through lesion studies it has been shown that the perirhinal cortex and the hippocampus significantly impact DNMS performance (Zola-Morgan, et al., 1989, 1993, 1994), which is in agreement with the understanding that aspects of non-spatial recognition memory are mediated, in part, by the medial temporal lobe (Corkin, 1984). Other literature indicates that medial temporal lobe lesions specifically impair the ability to recognize older objects compared to recently presented objects (DeVito, et al., 2010), indicating that stimulus novelty impacts the brain networks involved in completing the DNMS task. The present results that older monkeys are impaired relative to middle-aged monkeys indicates that the perirhinal cortex and hippocampus may not function as well in aged monkeys compared to younger monkeys.

Object discrimination (OD) tasks test an animal's object-reward association memory. Aged macaques required more trials to learn the task compared to middle-aged animals and performed worse with regards to overall percentage of correct responses. Reward-related processing and memory has been shown to involve dopaminergic signaling in the substantia nigra and ventral tegmental (VTA) areas of the brain (Loh, et al., 2016). The substantia nigra is a midbrain structure that heavily innervates the basal ganglia and neocortex. Therefore, one possibility to explain the age-associated OD deficits observed in this study is that damage to the nigrostriatal pathway could lead to impairments in reward-related processes, such as object-reward association memory (Hubble, 1998). Importantly, the present data cannot determine the validity of this hypothesis, and one way to test this idea is to lesion the nigrostriatal pathway in a group of animals and test reward-related processes using a novel associative memory task. Previous lesion studies have also shown reduced object discrimination in monkeys and humans after damage to the perirhinal cortex (PRC) of the medial temporal lobe (MTL). This is an important finding, as it provides support for the hypothesis that the MTL is involved in object discrimination, and that these structures function more poorly in the aged monkey.

The final task used in the present behavioral battery was the delayed response (DR) task of spatial short-term memory. Functional magnetic resonance imaging and localized lesion studies have shown that the dlPFC plays a role in successfully completing the DR task. Within the dlPFC, area 46 in particular regulates much of spatial working memory and executive function (Leubke, et.al, 2010). It has also been shown that executive function depends more on the prefrontal cortex than the medial temporal lobe (Fuster, 2001). In our study, there were no significant age-related differences seen in the DR task, either in the acquisition of the task or in performing it across the increasing delay conditions. While this finding is consistent with some

previous reports (i.e., Dumitriu, et al., 2010), it also comes as a surprise since many other studies have shown that aged macaques are impaired on this task (Darusman, et al., 2014).

One explanation for the non-significant difference in accuracy and task acquisition time for the DR task may be due to environmental enrichment of the macaques under study since they were raised in a primate center with a greater diversity of social enrichment, rather than in a laboratory setting. It is known that one's environment impacts both development and brain function across the lifespan, so this may very well be a possible explanation for the observed differences in response to the DR task (Gluckman, et al., 2005; Gottlieb, et al., 2015). It is possible that this enrichment led to a restoration of executive functioning and working memory within this cohort of macaques. It is interesting to consider that, despite this potential enrichment, older animals are still impaired on the temporal lobe dependent object recognition task, but not on the frontal cortex dependent DR task. Hence, the enrichment might have had a more substantial effect on frontal cortex dependent tasks than temporal lobe dependent tasks. It is also interesting to note that this possible enrichment didn't improve the performance of aged monkeys on the OD task, where aged macaques required more trials to learn the task to criterion.

The finding that aged macaques are impaired in the objection recognition and object discrimination tasks, but not on the delayed response task, is particularly important because it indicates that distinct aspects of cognition undergo different trajectories, which is similar to what is observed in older humans. For example, Nagel showed that older humans tend to become more impaired on tasks involving executive functioning and working memory as they age, but not on tasks that involve tests of fluid ability (Nagel, et al., 2008).

Results of Neuron Density:

Parvalbumin-containing interneurons are important inhibitory cells in the central nervous system and are thought to be critical for many of the behavioral processes discussed above. Nevertheless, there was no significant difference found in the density of PV neurons in the adult macaques compared to aged macaques. Previous literature has also shown a non-significant relationship between PV neuron density and age in a mouse model (Ueno, et al. 2018). However, in a mutant hamster model, the density of PV interneurons increased with age in the striatum (Hamann, et al., 2007). The difference between results from this study and our results may be due to the difference in animal model chosen and the brain region analyzed. Therefore, more research is needed to determine how PV-containing interneuron density changes in the macaque model. The density of PV GABAergic interneurons in the prefrontal cortex has been shown to relate to behaviors such as working memory, attention, and cognitive flexibility (Ferguson and Gao, 2018). It has also been shown that diminished levels of PV interneurons in the prefrontal cortex (PFC) are found in the pathology of many psychiatric disease (Ferguson and Gao, 2018). This is important to note, since anatomical brain abnormalities in some psychiatric disorders have been found to be associated with aging (Koutsouleris, et al., 2013). Furthermore, the density of these PV interneurons surrounded by perineuronal nets has been shown to play a role in the development of neurological diseases (Wen, et al., 2018). PNNs that surround these PV-containing interneurons act as an ion buffering system, playing a critical role in cell functioning and protection, specifically from oxidative stress (Wen, et al., 2018).

The density of perineuronal nets surrounding neurons is proposed to be another important neuroanatomical correlate of brain aging. Perineuronal nets surround neurons and act as ion buffers, functioning in a variety of ways including neuronal protection (Wen, et al., 2018). PNNs have also been shown to be involved in controlling plasticity and memory, with one study

showing their removal leading to restored memory and plasticity in an Alzheimer's disease model (Foscarin, et al., 2017). In our research, it was discovered that age did not have an effect on the density of PNNs in the retrosplenial cortex. Unlike Ueno and colleagues that proposed that there is a higher density of PNNs surrounding neurons in the sensory cortex as the mouse brain ages, this was not seen in our experiment (Ueno, et al., 2018). Again, this discrepancy could be due to the use of different animal models and the differences between sensory brain regions and the retrosplenial cortex, which is central in cognitive processing. In addition, due to unforeseen limitations from the coronavirus pandemic, we were not able to process all of the data that was collected and did not have as large of a sample size as originally planned. Therefore, in order to definitively conclude how perineuronal net density changes in the aging macaque brain, more research with a larger sample size is needed.

While the overall density of PNNs was not significantly changed in the aged macaque, there was a significant difference in the proportion of PV neurons expressing PNNs in the retrosplenial cortex. Adult macaques had a significantly greater proportion of PNNs surrounding PV-containing neurons compared to the aged macaques. These results might reflect a possible deterioration of PNNs surrounding PV neurons with age. In other literature, it has been shown that the density of PV-neurons associated with PNNs decreases in cortical areas, such as the retrosplenial cortex (RSC), in aged mice compared to adult mice (Ueno, et al., 2019). This result supports our finding that the proportion of PV neurons expressing PNNs decreases with age. However, contradictory evidence has also shown that adolescent and adult rats have more PV-containing interneurons surrounded by PNNs in the medial pre-frontal cortex (mPFC) compared to juveniles (Baker, et al., 2017). The information gained from our experiment can be used for

further research about the cause of this apparent decrease in the proportion of PNNs surrounding PV neurons that is accompanied with aging.

Results of Relationships to Behavior:

One effective way to better understand an animal's cognition is to compare individual differences in behavior with neuroanatomical data. The three behavioral tasks used in this experiment were the delayed non-matching to sample (DNMS), object discrimination (OD), and delayed response (DR) tasks. The DNMS task is used to evaluate an animals' recognition memory, specifically with non-spatial stimuli (Rapp and Amaral, 1991). The OD task tests reward association memory (Hara, et al., 2012). Finally, the DR task tests short-term spatial memory. By comparing the density of PV neurons surrounded by PNNs to the percent correct responses for each behavioral task, we are able to gain information about which behaviors are affected by PV-PNN surrounded neurons. This allows for possible identification of pathological mechanisms underlying neurological diseases and disorders.

The major finding from this study is that the proportion of PV-neurons surrounded by PNNs in the RSC is greater in adult monkeys compared to aged monkeys. This indicates that an unknown mechanism of aging might be decreasing this proportion in the retrosplenial cortex (RSC). Another important finding from our study is that there is a significant relationship between the density of PV neurons surrounded by PNNs and the percent correct of DNMS task responses. The proportions of PNN-surrounded PV neurons were compared to the percentage of correct responses in all three of the cognitive tasks: delayed nonmatching to sample (DNMS), delayed response (DR), and object discrimination (OD). Only the DNMS task showed a significant increase in percentage of correct responses as a function of increasing proportions of

PV neurons that were expressing PNNs. The specificity in these relationships indicate that perineuronal nets have an impact on PV neurons with regards to non-spatial recognition memory, the cognitive function evaluated in the DNMS task. However, it is important to note that only the retrosplenial cortex was examined in this experiment, and that the density of these neurons could vary in other regions of the brain and could correlate with the other behaviors used in this battery. In other research, the knockout of PNNs in the perirhinal cortex of the mouse brain leads to enhanced recognition memory (Romberg, et al., 2013). While Romberg's study did not specifically look at PNNs surrounding PV neurons in the retrosplenial cortex, it is interesting to note that the destruction of PNNs showed a causal improvement in recognition memory and synaptic plasticity in the perirhinal cortex. These two major findings may be because PV neurons expressing PNNs are found in greater abundance in the retrosplenial cortex than in other regions of the brain.

Conclusions:

Taking all of the results of this present study, we suggest that aging has an effect on the proportion of PV-containing neurons surrounded by PNNs. In the data, it was shown that aged macaques had a significantly lower proportion of PNNs surrounded-PV-containing neurons compared to adult macaques. The density, however, of perineuronal nets (PNNs) and of parvalbumin (PV)-containing interneurons did not change with age in the retrosplenial cortex (RSC) of the macaque model. These data suggest that there may be a relationship between the density of PV neurons surrounded by PNNs and the monkey's performance on certain cognitive tasks. The delayed non-matching to sample task (DNMS) showed a significant relationship between percentage of correct responses on the task and the density of PV neurons surrounded

by PNNs. Based on these findings, it could be proposed that PV neurons surrounded by PNNs are involved in recognition memory of non-spatial stimuli, what was tested in the DNMS task. Additionally, the greater proportion of PV interneurons surrounded by PNNs within the retrosplenial cortex of the adult macaque suggest that age may play a role in this proportional degradation.

The information gained from this experiment can be used for future research to determine if there is, for certain, a change in the density of perineuronal nets with regards to aging. Since there was a decreased proportion of PNN-surrounded PV neurons associated with aging, it is possible that part of the normal aging process involves this mechanism in the RSC. Since macaques do not develop neurodegenerative diseases, like humans do, it is still unknown if the densities of parvalbumin (PV) neurons, perineuronal nets (PNNs), or PV neurons surrounded by PNNs change in the retrosplenial cortex of an aging human brain (Squire and Zola-Morgan, 1988). However, at the same time, macaques do experience age-related cognitive decline, like humans (Comrie, et al., 2018). Therefore, these data could be used to guide future experiments aimed at learning more about the brain-aging process. By doing so, this will allow us to learn more about the human aging brain, and how cognition declines naturally throughout one's life.

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