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Ryan Martin April 10, 2017

Memory Assessment in a Nonhuman Primate Model of Alzheimer's Disease Using a Visual Paired-Comparison Task

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Abstract

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By Ryan Martin

Importance: There is currently no animal model for Alzheimer's disease that fully recapitulates the etiologic, cognitive and pathological characteristics of the disease.

Objective: To determine if there is a transgenic nonhuman primate model of AD (AD monkey) recapitulates memory deficits present in human AD.

Measures: Visual Paired-Comparison task and quantitative PCR.

Subjects: The experimental group consists of two female AD monkeys aged between 9 and 10 years that contain the Swedish and Indiana mutations in βAPP under the control of human polyubiquitin promoter (APPswe/ind). Six macaques (3 males and 3 females) aged between 5 and 6 years were used as control. For molecular analysis, two female macaques aged between 9 and 10 years were used.

Results: Transgenic macaques displayed a significantly lower novelty preference on the Object-in-Place VPC task. All other differences in VPC tasks were not statistically significant. Up-regulation of the APP transcript in lymphocytes was observed in both AD monkeys. Down-regulation of ApoE, CX3CR1, and CXCL8 transcripts was observed in both AD monkeys.

Conclusion: AD monkeys show altered looking behavior on spatial-relational memory tasks, preferring familiar to novel stimuli. This altered looking behavior may be due to the increased expression of APP. A VPC task utilizing more visually similar stimuli than the VPC Color-Delay is required to determine if recognition memory is impaired.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by a decline in cognitive function and the development of dementia (Reitz and Mayeux, 2015). In the United States there are approximately 5.5 million individuals diagnosed with late-onset AD alone, and the global number of affected individuals is expected to double in the next 20 years (Tosto and Reitz, 2016; Weiner et al., 2013). With no current cure and a rising population of AD patients, the $215 billion dollars spent by the U.S. per year on treatment for the disease will only increase with time (Reitz, 2015). As a result, there is a growing need for more innovative approaches to better understand such a prevalent and fatal disease.

Given the growing need for an effective treatment and the ethical concerns regarding invasive and novel approaches to the treatment of AD in humans, the importance of a better animal model is clear. Rodent models present an approximation to AD in several aspects yet fail to completely replicate the pathology. Rodent models carrying the mutant APP transgene exhibit excessive Aβ deposition, however, they fail to develop neuropathology such as neurofibrillary tangles (NFTs), and are therefore an incomplete model of AD (Kokjohn & Roher, 2009). Attempts such as the triple transgenic mouse model of AD have been successful in producing excess amounts of Aβ excess and NFTs, yet they do so by significantly deviating from the etiology of familial AD and therefore also fail to successfully model the disease (Oddo et al., 2003). Due to the limitations of the currently available animal models in recapitulating human conditions, many promising therapeutic attempts have failed once reaching human trials. Perhaps the most notable therapeutic strategy has been active immunization against Aβ 42 that removed amyloid plaques and improved cognitive functioning in transgenic mice, yet developed meningoencephalitis in some patients and showed little evidence of improved cognitive state

(Holmes et al., 2008; Orgogozo et al., 2003). More recent trials of passive immunization have shown some promise, but there are potential side effects that often are not predicted by rodent models (Jucker, 2010; Sevigny et al., 2016). Ultimately, the difference between animal models and patients may likely due to the fact that rodents are too evolutionarily distant from humans to replicate AD. In addition to differences in lifespan and neuroanatomy, rodents also differ from primates in the extent of age-regulated neuronal genes (Kokjohn & Roher, 2009; Loerch, et al, 2008). Furthermore, injection of plaque-equivalent concentrations of fibrillar Aβ into the brains of aged Rhesus monkeys produced NFTs, neuronal degeneration, and microglial proliferation, yet the same injection into rat brains produced no detectable pathology (Guela et al., 1998). Additionally, this toxicity was observed to be greater in higher order primates than lower order primates. While transgenic mouse models have served to advance the understanding AD development, a nonhuman primate model may capture human AD more closely than other animal models.

Three AD monkeys have been produced by overexpressing mutant human β-amyloid precursor protein (βAPP) with the Swedish and Indiana mutations (APPswe/ind) regulated by human polyubiquitin promoter; two are females (AD1 and AD2) and one is a male (AD3, with mosaic transgene). As previous works have illustrated, a βAPP transgenic animal model results in the accumulation of Aβ deposits with age, as seen in human AD (Calhoun et al., 1998). Transgenic rodent models carrying APPswe/ind have shown substantially elevated Aβ levels compared to the wild-type murine Aβ, as well as to other AD related mutations (Chirshti et al., 2001; Folkesson et al., 2007). In addition, previous measurements in all three AD monkeys have revealed substantially elevated total levels of βAPP in lymphocytes, as well as heightened levels of Aβ to a lesser degree. Furthermore, longitudinal measurement of Aβ42 in cerebrospinal fluid

(CSF) at the age of 24 – 48 months (juvenile to young adult) has revealed elevated levels in two of the three AD monkeys. Behavioral tasks administered during adolescence (1-18 months) revealed a slight reduction in spatial memory at 18 months of age indicating a possible initiation of AD pathology in the medial temporal lobe, specifically the hippocampus (unpublished work). This potential early presentation of disease pathology is in line with the understanding of ubiquitously promoted adolescent models of disease. Given their initial biological and cognitive test scores, these animals appear to be a promising model for AD, however further testing is required to determine if the full AD behavioral phenotype develops.

Pathologically, AD is characterized by gross cortical atrophy and ventricular dilation, as well as intracellular accumulation of neurofibrillary tangles (NFTs) and extracellular amyloid-β protein (AβPs) (Giri et al., 2016; Tosto and Reitz, 2016). AβPs may be present throughout the AD brain as either diffuse or neuritic plaques, however, the latter are more closely associated with cognitive impairment (Terry et al., 1994). AβP has been found to spread throughout the brain in a prion-like mechanism by utilizing corruptive protein templating (Jucker & Walker, 2011). According to the amyloid cascade hypothesis, AβPs are responsible for the initiation of AD pathology and the subsequent accumulation of NFTs (Haass, & Selkoe, 2007; Hardy & Higgins, 1992). Despite this key role, the progression of NFT pathology correlates more closely with decline in cognitive function than AβP pathology (Arriagada et al., 1992; Sabbagh et al., 2010). The earliest formation of AD pathology appears in the locus coeruleus/subcoeruleus complex; however, the overall progression of NFT pathology is typically classified as 6 stages beginning in the medial temporal lobe (Braak & Braak, 1995; Braak & Del Tredici, 2011). Stages I and II, the transentorhinal stages, characterize the beginning of the disease and are clinically silent. In Stage I pathology, tauopathy is confined to the transentorhinal region and

begins in the pre-α layer. Stage II extends into the entorhinal region, beginning in the pre-α layer and extending down to the pri-α layer as the disease progresses (Braak et al., 2006). Stages III and IV are known as the limbic stages that correspond to the early, prodromal state of cognitive impairment (Braak & Braak, 1995). Stage III tauopathy extends into the fusiform and lingual gyri of the neocortex (Braak et al., 2006). The entorhinal and transentorhinal cortex continues to accumulate tau and then begins to thin. The CA1 subfield of the hippocampus develops band-like changes in dendritic structure as pathology progresses; the CA2 is considerably burdened with neurons containing tau, whereas CA3 and CA4 are moderately affected (Braak et al., 2006). In stage IV, tau pathology broadens into neocortical association areas and affected areas continue to worsen. The neocortical stages, stages V and VI, describe the most advanced stages of AD, at which point NFT-induced lesions have reached the neocortex and pathology has extensively affected the medial temporal lobe structures (Braak & Braak, 1995). Stage V describes the spreading of tau pathology from the medial temporal lobe into frontal, superolateral, and occipital regions to affect higher-order association areas (Braak et al., 2006). Lastly, in stage VI the tau pathology reaches the primary and secondary neocortical areas, as well as the striate area of the occipital lobe. The orderly progression of neurodegeneration in AD patients underlies the systematic decline in mental capacity progressing through the three clinical stages of psychopathology: preclinical, mild cognitive impairment (MCI), and dementia (Alzheimer's Association, 2014).

Given that the early stages of AD tau pathology preferentially target the medial temporal lobe structures, deficits in recognition, spatial-location, and spatial-relation memory associated with damage to relevant structures may be utilized to assess progression of pathology according to its predicted trajectory. Many cognitive tests that are used clinically to assess the state of

mental deterioration – such as the Mini-Mental State Examination and the Alzheimer's Disease Assessment Scale – have been shown to be effective in differentiating between the deficits associated with AD (Swainson et al., 2001). However, the visual paired-comparison (VPC) task has recently shown its effectiveness in predicting advancement from MCI to AD as well as predicting progression from an unimpaired state to MCI (Zola et al., 2013). The VPC task assesses recognition-based memory by measuring the time spent viewing either a novel or a familiar visual stimulus. When memory is intact, the subject will preferentially examine the novel stimulus; however, as memory decreases the novel and familiar viewing times approach equal proportions (Snyder et al., 2008). The various components of the behavioral assay may be modified to allow investigators to examine the specific types of memory, such as spatial memory. Lastly, the format of this test offers an objective assessment of memory without depending on complex instructions or an understanding of language, which allows the task to be administered both human and nonhuman primate subjects without significant modification of the task or methodology (Richmond et al., 2004).

The VPC task also allows differentiation between impairments in different components of memory; the VPC Color-Delay task assesses memory capacity and retention ability as a function of time. It has been shown to identify impairments after the hippocampus, subicular complex, and parahippocampal areas TH/TF are damaged (Manns et al., 2000; Pascalis & Bachevalier, 1999). The Spatial-Location task assesses memory of object-location associations, which requires an egocentric frame of reference. This task has been shown to reveal impairments in spatial memory due to lesions in the hippocampus and TH/TF of the parahippocampal area (Bachevalier and Nemanic, 2008). The Object-in-Place task assesses memory for spatial relations between objects using an allocentric frame of reference (Blue et al., 2014). The Object-

in-Place task, along with the Object-Replace functioning as a control task, has demonstrated the ability to identify perirhinal-specific lesions (Bachevalier and Nemanic, 2008). Additionally, the VPC task is superior to similar memory tests, such as the DNMS task, in that it does not permit behavioral strategies to compensate for cognitive deficits (Pascalis & Bachevalier, 1999).

In addition to cognitive tasks, biomarkers have been utilized for early and preclinical detection of AD (Bateman et al., 2012). In addition to the more common CSF biomarkers, plasma biomarkers found in peripheral lymphocytes can also provide an accurate, though indirect, measure of AD (Kalman et al., 2005). Chemokines CX3CR1 and CXCL8 are involved in the proinflammatory signaling involved in AD pathology, demonstrating their potential as additional biomarkers (Baggiolini et al., 1994; Corrêa et al., 2011). Lastly, apolipoprotein E (ApoE) is involved in metabolism and deposition of Aβ making it a clear target as a biomarker indicating AD pathology (Liu et al., 2013).

The current study used the VPC task in combination with blood biomarkers to determine if a pathological memory deficit is present in AD monkeys with human βAPP mutations. Four variations of the task were used to assess recognition and spatial memory. Spatial memory tasks were further differentiated as either allocentric or egocentric spatial memory tasks. Four blood biomarkers were evaluated in lymphocyte samples: ApoE, CX3CR1, CXCL8, and SOD2. Expression levels were determined by using quantitative PCR (qPCR).

Methods

The methods presented in this study have been reviewed and approved by Emory University's Institutional Animal Care and Use Committee.

Subjects

Subjects in this study consisted of ten adult Rhesus macaques (*Macaca mulatta*) in total. The AD group consisted of two adult female monkeys aged between 9 and 10 years old. AD monkeys were generated with lentiviral gene delivery in mature oocytes followed by *in vitro* fertilization and embryo transfer into surrogate females. AD monkeys carry human βAPP with the Swedish K670N/M671L + Indiana V717F (APPswe/ind) mutations regulated by the human polyubiquitin C promoter. All subjects were housed individually and kept on twelve-hour light-dark cycle and given access to environmental enrichment. The behavioral task control group is comprised of six adult macaques, three males and three females, aged between 5 and 6 years at the time of their assessment (Blue, Kazama, & Bachevalier, 2013). The biomarker control group consisted of two wild-type (WT) females aged between 9 and 10 years which were housed in the same room as the AD monkeys.

Equipment and Arrangement

All testing involving the VPC task was conducted in a quiet, isolated testing room designed to minimize visible and audible distractions. A white noise generator was used to further mask any noise, and a tarp was used to isolate the monkey from the researcher present in the same room. Animals were seated in an adjustable, Plexiglass primate testing chair. The chair was positioned so that animals were seated 60 cm from a computer monitor on which the visual stimuli were presented. Eye movements were detected using an infrared camera and processed with Tobii eye-tracking software. An additional camera was mounted on a tripod behind the monitor in order to ensure the monkey's eye movements were consistent with those reported by Tobii.

Visual Paired-Comparison Tasks

All trials began with the familiarization phase, in which the animal was presented with a novel visual stimulus. The familiarization phase was completed when the subject spent either 30 cumulative seconds looking at the image or when the 5-minute cutoff time had been reached. When the cutoff time was used, the delay did not begin until after the animal had looked at the image again in order to ensure that the time separating familiarization and comparison was only due to the delay period. For the delay period, a blank screen was presented for a time specific to the particular variant of the VPC task. Following the delay, the familiar stimulus was presented alongside a novel stimulus for a comparison phase. The comparison phase consisted of two components lasting 5 seconds each, which were separated by a 5-second delay. Novel and familiar stimuli switched positions during the second component of the comparison phase to prevent any subject side-bias from influencing the data. Upon completion of each trial, the monkey was given a food reward regardless of stimulus preference. Novelty preference was determined during the comparison phase by using the percentage of time spent looking at the novel stimulus out of the total time looking at novel and familiar stimuli.

A minimum of 10 trials were completed for each variant of the VPC task. The behavioral criteria to a trial as valid required that the subject look at the stimulus for a minimum of 10 seconds during the familiarization phase and a minimum of 1 second for the comparison phase. Furthermore, trials were not counted when subjects looked at either side for more than 90% of the time during the comparison phase.

VPC Color-Delay

The Color-Delay variant of the VPC task was designed to test recognition memory of a single, color image and decay of this memory as a function of time. Preferential viewing of the novel stimulus was used to imply that recognition memory was intact. The task presented a color image during the familiarization phase (Figure 1A). Following the familiarization phase, four different time delays were pseudorandomly presented: 10, 30, 60, and 120 seconds. Each time delay was repeated a minimum of 10 times. The novel image of the comparison phase was selected for equal size and saliency as the familiar image.

VPC Spatial-Location

The Spatial-Location variant of the VPC task was designed to test memory for the location of a stimulus. The task utilized only one stimulus, a color image for both the familiarization and the comparison phase of each trial (Figure 1B). The initial location of the image presented in the familiarization phase was randomly selected. This location of the image was maintained during the comparison phase; however, an identical image was presented in a random location in addition to the familiar image.

VPC Object-in-Place

The Object-in-Place task was designed to test allocentric memory of object-place relations. During the familiarization phase, a single stimulus composed of five images was presented. The five images were equally sized and evenly distributed around the center of the screen (Figure 1C). During the comparison phase of the task, all original images were present in both the novel and familiar stimuli. The novel stimulus, however, had three out of the five

images rearranged, so that they occupied different locations but maintained the same distribution. The familiar stimulus remained unaltered.

VPC Object-Replace

The VPC Object-Replace task functioned as a control for the Object-in-Place task to ensure that impaired performance was not due to deficits in perceptual abilities, attentional processes, or novelty preference. As in the Object-in-Place task, the initial stimulus presented during the familiarization phase consisted of five equally sized and spaced images. During the comparison phase, however, the novel stimulus had three out of the five images replaced with novel images (Figure 1D). The familiar stimulus was unaltered.

RNA Isolation

Peripheral blood was collected from the two AD monkeys, as well as two control monkeys not used in behavioral testing; all animals were aged 9 to 10 years old. Lymphocytes were then isolated from blood samples and resuspended in 500 μL of Trizol (Invitrogen). A phenol-chloroform extraction was performed by adding 100 μL of chloroform to Trizol homogenates. Samples were then centrifuged at 12,000 x g for 10 min at 4°C. The aqueous layer was removed for RNA precipitation overnight with isopropanol at -20°C. Precipitated RNA was pelleted at 12,000 x g for 30 min at 4°C. Pelleted RNA was washed twice w/ 75% ethanol then dissolved in RNase/DNase water.

Quantitative PCR

Total RNA was extracted by using an RNeasy Mini Kit (Qiagen, Chatsworth, CA) and reverse-transcribed as instructed by the manufacturer (High-Capacity cDNA Reverse

Transcription Kit; Applied Biosystems, Norwalk, CT). Q-PCR was performed by using 2× Power SYBR® Green PCR Master Mix (Applied Biosystems), with specific primers and cDNA samples. The CFX96 Real-Time System (Bio-Rad) was used, and cDNA samples were first incubated at 96°C for 3 min followed by 45 cycles; at 95°C for 10 second and 62°C for 30 sec. The specific primers used for qPCR were: APP Mut, (APP Mut. F2: GCCCTGCTGCCGACCGAG and APP Mut R1: TGTTTCTTCTTCAGCATCACCAA) ApoE (ApoE-F: GGGTCGCTTTTGGGATTACC and ApoE-R: CTCATCCATCAGCGTCGTCA), CXCL8 (CXCL8-F: GGAAGGAACCATCTCGCTCT and CXCL8-R: GCAAAACTGCACCTTCACACA), CX3CR1 (CX3CR1-F1: AAAACGAATGCCTTGGTGAC and CX3CR1-R1: AGGAAAAACACGACGACCAC), SOD2 (SOD2-F1: GATCCACTGCAAGGAACAACAG and SOD2-R1: CAGGCCTGACATTTTTATACTGAAGGT), and UBC (UBC-F: CCACTCTGCACTTGGTCCTG and UBC-R: CCAGTTGGGAATGCAACAACTTTA).

Statistical Analysis

For the VPC Color-Delay task, a two-way repeated measures ANOVA was used with Delay as the repeated within-subject factor and Group as the between subjects factor to compare familiarization time, total time looking at stimuli during the comparison phase, and novelty preferences for the color stimuli. Planned independent t-tests were run to compare novelty preference between AD and control groups at each delay separately, and one-sample t-tests compared the performance at each delay to chance for each group individually.

For the VPC tasks, novelty preference on the three types of spatial VPC tasks were compared using a Group [AD, Control] X Task-Type [Spatial-Location, Object-Replace, and Object-in-Place] repeated measures ANOVA. Additional independent-samples t-tests were

conducted to compare novelty preference in control and AD groups for each spatial task type separately, and one-sample t-tests compared the scores of each group to chance levels (50%). Effect sizes are reported as Cohen's D (d_{Cohen}) for all t-tests and partial eta squared (η_p^2) for all ANOVAs.

For expression level analysis, individual ΔΔCq values were calculated by taking the difference in average WT monkey and AD monkey Cq values. Independent sample t-tests were performed to compare each AD monkey's biomarker expression to the WT control.

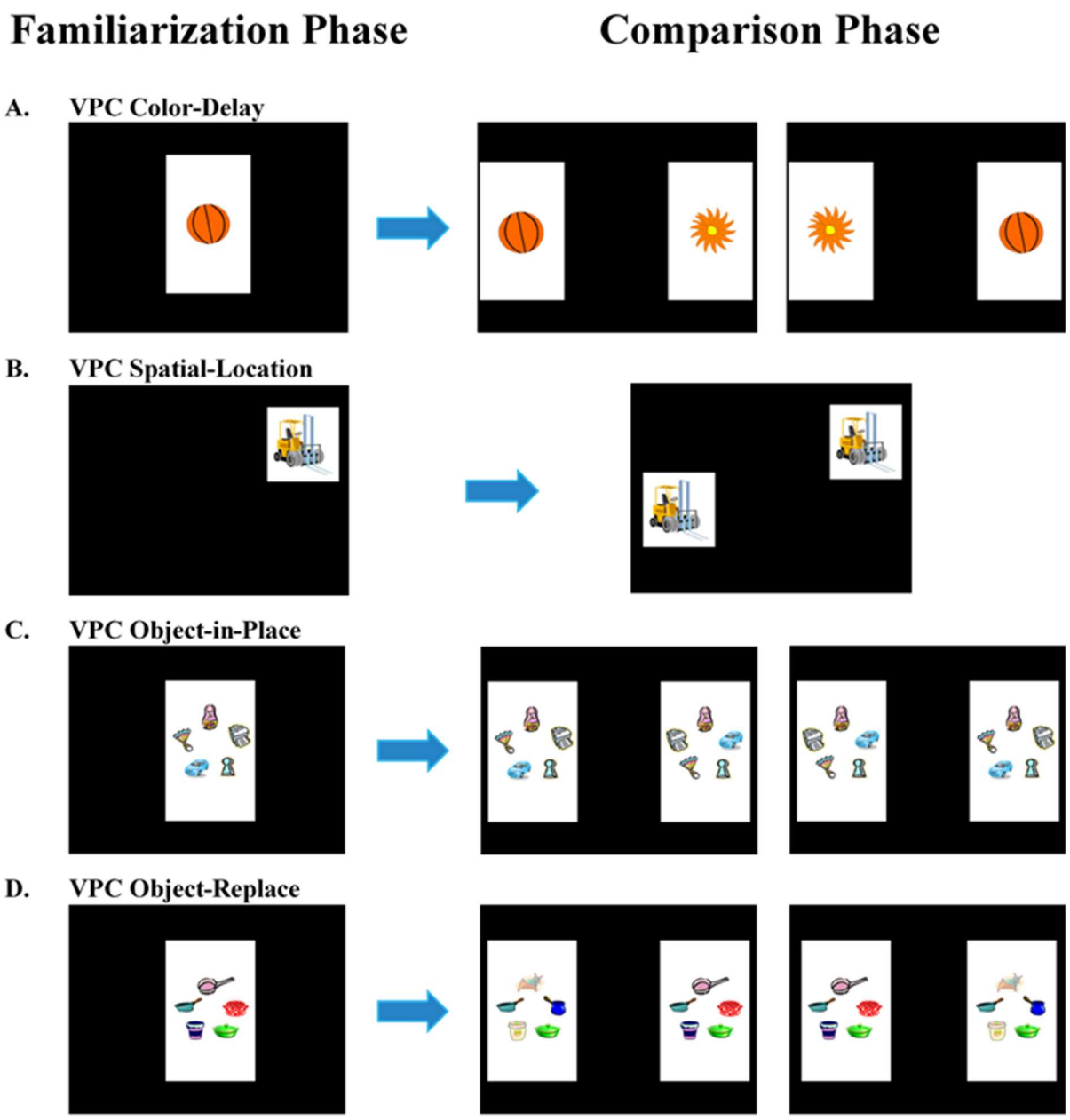

Figure 1. Examples of the different stimuli presented in each component of the VPC task. (**A**) VPC Color-delay utilizes a single image for the familiarization period and a single novel image of similar saliency for the comparison phase. (**B**) VPC Spatial-Location task only uses a single image for each trial; during the comparison phase, the image reappears in its original location as well as a random new location. (**C**) VPC Object-in-Place uses five grouped images to make up a stimulus. In the Object-in-Place task the position of 3 out of 5 images are rearranged to produce the novel stimulus. (**D**) VPC Object-Replace functions as a control task for the Object-in-Place task, and similarly presents 5 grouped images in a single visual stimulus. Unlike the Object-in-Place task, however, this task switches out 3 out of the 5 original images to create the novel stimulus. In tasks **A**, **C**, and **D** the position of the novel and familiar stimulus alternate to avoid a side-looking bias.

Results

VPC Color-Delays Task

The average novelty preferences of AD and control groups are shown for each of the 4 delays in Figure 2. A repeated measures ANOVA was conducted to compare the effect of delay length on novelty preference in control and AD groups in the Color-Delay task. There was not a significant effect on delay length, [Delay: $F_{\text{Sphericity Assumed}}(3,18) = 0.54$; $p = 0.66$; $\eta_p^2 = 0.083$; Delay X Group: $F_{\text{Sphericity Assumed}}(3,18) = 1.61$, $p = 0.22$, $\eta_p^2 = 0.212$]. This indicates that the length of the delay separating the familiarization phase from the comparison phase in the Color-Delay task did not have an effect on the novelty preference of either the AD or the control group. One sample t-tests showed no difference in novelty preference from chance in the AD group at all delays [10s: $t(1)=3.66$, $p=0.17$, $d_{\text{Cohen}}=2.58$ and 30s: $t(1)=3.86$, $p=0.16$, $d_{\text{Cohen}}=2.73$ and 60s: $t(1)=2.91$, $p=0.21$, $d_{\text{Cohen}}=2.06$ and 120s: $t(1)=6.24$, $p=0.10$, $d_{\text{Cohen}}=4.41$]. However, One sample t-tests showed a difference in novelty preference from chance in the Control group at all delays [10s: $t(5)=13.38$, $p=0.00$, $d_{\text{Cohen}}=5.46$ and 30s: $t(5)=11.42$, $p=0.00$, $d_{\text{Cohen}}=4.66$ and 60s: $t(5)=10.77$, $p=0.00$, $d_{\text{Cohen}}=4.39$ and 120s: $t(5)=6.08$, $p=0.0020$, $d_{\text{Cohen}}=2.48$]. When compared individually to the Control group for novelty preference using repeated measures ANOVAs, neither AD1[Delay: $F_{\text{Sphericity Assumed}}(3,27) = 0.21$; $p = 0.89$; $\eta_p^2 = 0.22$; Delay X Group: $F_{\text{Sphericity Assumed}}(3,17) = 0.23$, $p = 0.88$, $\eta_p^2 = 0.024$] nor AD2 [Delay: $F_{\text{Sphericity Assumed}}(3,39) = 0.55$; $p = 0.650$; $\eta_p^2 = 0.041$; Delay X Group: $F_{\text{Sphericity Assumed}}(3,39) = 0.64$, $p = 0.47$, $\eta_p^2 = 0.047$] were different from the Control group. However, when each AD monkey was individually compared to chance novelty preference using a one sample t-test, AD1 [10s: $p = 0.17$, 30s: $p = 0.36$, 60s: $p = 0.022$, 120s: $p = 0.94$] performed significantly above chance at the 60 second delay, while AD2 was significantly above chance at all delays [10s: $p = 0.00$, 30s: $p = 0.0010$, 60s: $p = 0.00$, 120s: $p = 0.00$] (Figure

2B). A repeated measures ANOVA revealed a difference between control and AD groups for Familiarization Time to reach the desired cumulative 30 seconds of looking at the stimulus in the Color-Delay task [Delay: $F_{\text{Sphericity Assumed}}(3,18)=4.80$, $p=0.013$, $\eta_p^2=0.44$; Delay X Group: $F_{\text{Sphericity Assumed}}(3,18)=1.33$, $p=1.13$, $\eta_p^2=0.36$. Planned group comparisons of Familiarization for each delay revealed a significant group difference at the 30s delay [30s: $t(6)=-5.50$, $p=0.0020$, $d_{\text{Cohen}}=-4.49$]. Repeated measures ANOVA revealed no difference between Control and AD groups for Total Looking Time during the comparison phase [Delay: $F_{\text{Sphericity Assumed}}(3,18)=1.02$, $p=0.41$, $\eta_p^2=0.146$; Delay X Group: $F_{\text{Sphericity Assumed}}(3,18)=0.29$, $p=0.83$, $\eta_p^2=0.046$.

VPC Spatial Tasks

Independent-samples t-tests were conducted to compare spatial memory in control and AD monkeys for the Spatial-Location, Object-Replace, and Object-in-Place tasks. As shown in Figure 3, there was not a significant difference between control and AD groups in novelty preference for the Spatial-Location task [Delay 5s: $t(6)=0.090$, $p=0.93$, $d_{\text{Cohen}}=0.067$]. A one sample t-test of the Control [$t(5)=5.010$, $p=0.004$, $d_{\text{Cohen}}=2.05$] and AD group [$t(1)=10.27$, $p=0.062$, $d_{\text{Cohen}}=7.26$] showed a difference from chance in novelty preference in the Control group but not the AD group (Figure 3A). When compared individually, independent samples t-tests revealed that AD1 [$t(14)= 0.13$, $p = 0.90$, $d_{\text{Cohen}}=0.30$] and AD2 [$t(14)= -0.061$, $p = 0.95$, $d_{\text{Cohen}}= -0.072$] were not different from the Control group on the Spatial-Location task (Figure 3B). Additionally, individual one sample t-tests revealed that AD1 [$p = 0.35$] was not different from chance, while AD2 [$p = 0.036$] was significantly different from chance in novelty preference (Figure 3B). An independent samples t-test revealed no difference in Familiarization [$t(6)=-0.174$, $p=0.87$, $d_{\text{Cohen}}=-0.13$], however there was a difference in Total Looking Time [$t(6)=4.48$, $p=0.004$, $d_{\text{Cohen}}=4.86$] between AD and Control groups.

For the Object-Replace task, as seen in Figure 4, there was not a significant difference in novelty preference when the groups were compared in an independent sample t-test [t(6)=-1.74, p=0.13, d_{Cohen}=-1.42]. One sample t-tests demonstrated a difference from chance for novelty preference in both Control [t(5)=9.10, p=0.00, d_{Cohen}=3.71] and AD groups [t(1)=4.28, p=0.15, d_{Cohen}=3.03]. When compared individually, independent samples t tests revealed that neither AD1 [t(13) = -0.12, p = 0.91, d_{Cohen}= -0.40] nor AD2 [t(15)= -0.13, p = 0.90, d_{Cohen}= -0.27] was significantly different from the control (Figure 4B). Additionally, a one sample t-tests revealed that AD1 [p = 0.165] did not differ from chance in novelty preference, while AD2 [p = 0.021] was different from chance in novelty preference. Independent t-tests for Familiarization [t(6)=-0643.68, p=0.53, d_{Cohen}=-0.51] and Total Looking Time [t(6)=-1.06, p=0.33, d_{Cohen}=-1.24] revealed no difference between the Control and AD groups.

As Figure 5 shows, novelty preference on the Object-in-Place task was significantly difference for the Control and AD groups in an independent sample t-test [t(6)=3.85, p=0.0080, d_{Cohen}=3.02]. One sample t-tests of Control [t(5)=3.80, p=0.013, d_{Cohen}=1.55] and AD groups [t(1)=-2.81, p=0.218, d_{Cohen}=-1.99] showed a significant difference from chance for novelty preference in the Control group but not the AD group (Figure 5B). When compared individually, independent samples t-tests revealed that AD1 [t(15)= 0.059, p= 0.059, d_{Cohen}= 3.54] was not different from the Control group, while AD2 [t(15)= 2.43, p = 0.028, d_{Cohen}= 2.50] was significantly different (Figure 5B). Additionally, a one sample t-tests revealed that AD1 [p = 0.14] and AD2 [p = 0.22] were not significantly different from chance in novelty preference. Independent t-tests for Familiarization [t(6)=-0.19, p=0.85, d_{Cohen}=-0.15] and Total Looking Time [t(6)=-1.99, p=0.094, d_{Cohen}=-1.49] revealed no significant difference for the Object-in-Place task.

qPCR

Expression data shown in Figure 6 were obtained from three monkeys: two AD and one WT control. The data were normalized to the WT monkey. The following normalized levels were observed in ΔΔCq of AD1: ApoE=1.10, APP=5.58, CX3CR1=0.42, CXCL8=0.26, and SOD2=0.732. The levels for AD2 ΔΔCq were: ApoE=0.68, APP=7.26, CX3CR1=1.10, CXCL8=0.27, and SOD2=0.74. Independent t-tests on Cq revealed that both AD monkeys compared to the WT monkey had different expression of APP [AD1: $t(2)=24.28$, $p=0.0020$, $d_{Cohen}=34.34$ and AD2: $t(2)=24.37$, $p=0.0020$, $d_{Cohen}=34.46$] and ApoE [AD1: $t(2)=-4.68$, $p=0.043$ $d_{Cohen}=-6.61$, and AD2: $t(2)=-10.62$, $p=0.0090$, $d_{Cohen}=-15.01$]. However, neither AD monkey was different from the WT monkey when examining CXCL8 [AD1: $t(2)=1.63$, $p=0.25$, $d_{Cohen}=2.30$ and AD2: $t(2)=2.64$, $p=0.119$, $d_{Cohen}=3.73$], and only AD1 differed from WT in CX3CR1 expression [AD1: $t(2)=-24.50$, $p=0.002$, $d_{Cohen}=-34.65$ and AD2: $t(3)=-0.312$, $p=0.775$, $d_{Cohen}=-0.36$]. There was no difference in SOD2 expression when AD monkeys were compared WT [AD1: $t(2)=1.14$, $p=0.37$, $d_{Cohen}=1.61$ and AD2: $t(2)=1.07$, $p=0.40$, $d_{Cohen}=1.51$].

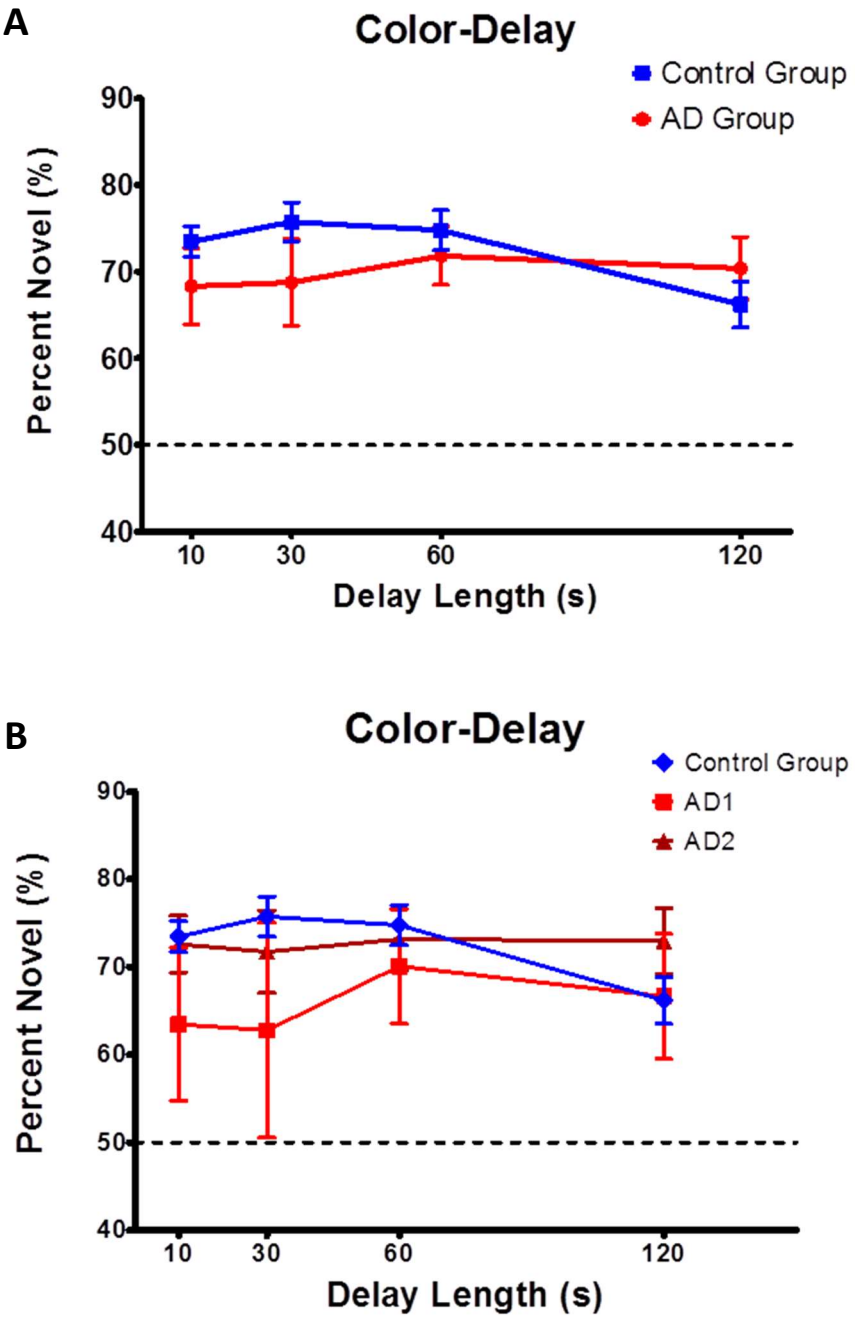

Figure 2. Mean percentage of time spent looking at the novel stimulus during novel-familiar comparison of the VPC task for color stimuli. Percent novelty for control group compared to (**A**) grouped AD animals (A) and monkeys AD1 and AD2 shown separately (B). While the AD group has a lower mean novelty preference for 10, 30, and 60s, there was no significant difference between the two groups at any time. Likewise, the individual monkeys AD1 and AD2 were not different at any delay length. All scores were significantly different from chance. Note that the dashed line represents chance and the vertical error bars represent SEM.

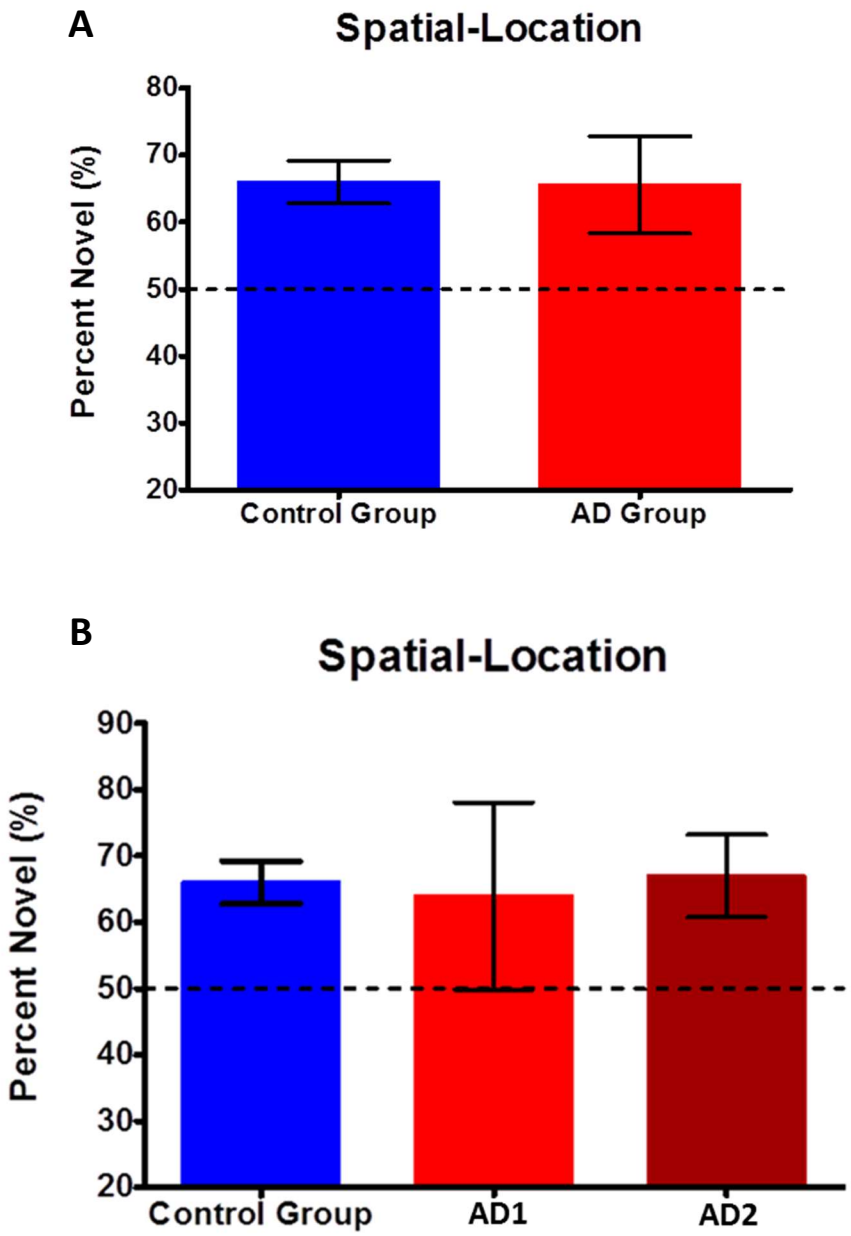

Figure 3. Mean percentage of time subjects spent looking at novel stimuli in tasks utilizing spatial memory with AD animals grouped and shown separately. (**A**) The AD group was not different from the Control group in novelty preference and was above chance novelty preference. (**B**) Neither AD1 nor AD2 differ from the Control. AD1 was not significantly different from chance, while AD2 was different. The dashed line represents chance (50%) and the vertical error bars represent SEM.

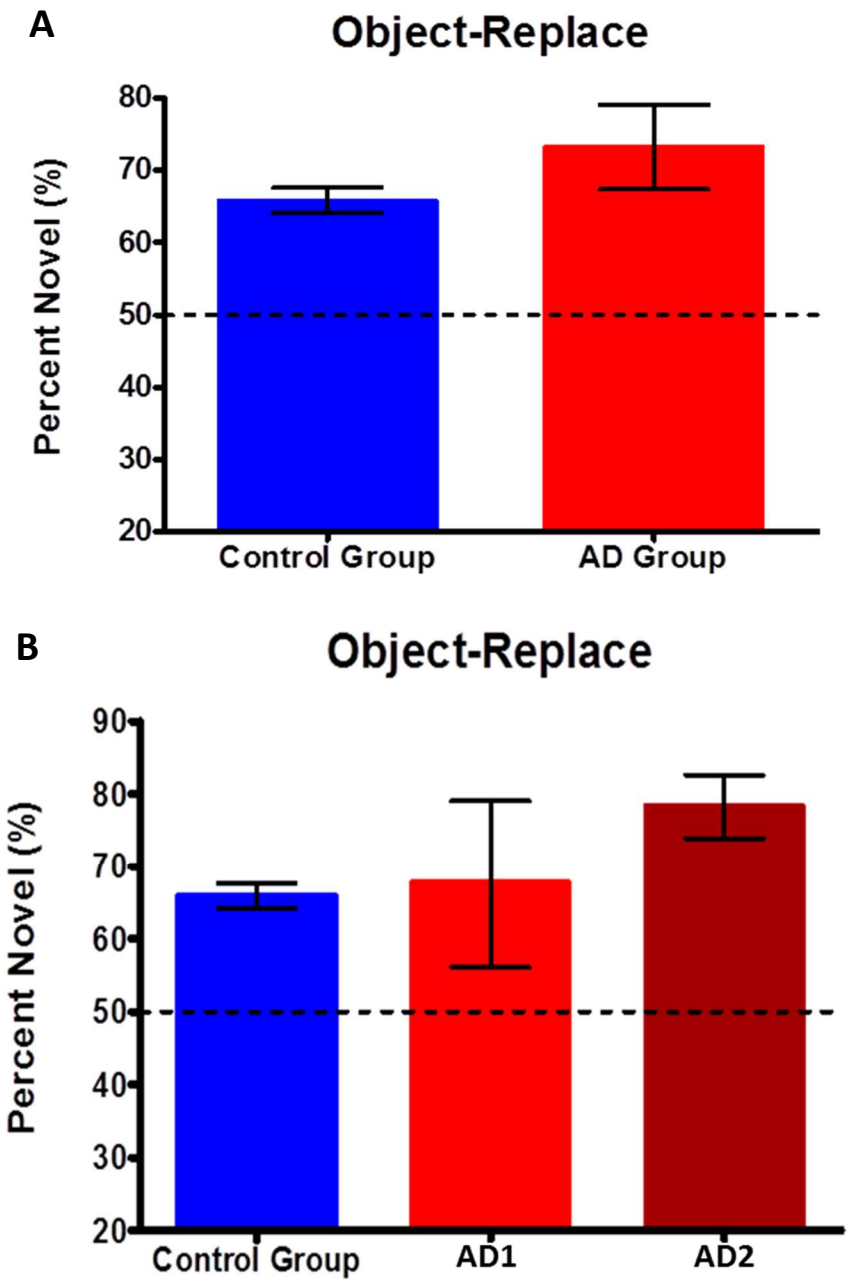

Figure 4. Mean percentage of time subjects spent looking at novel stimuli in Object-Replace task. (**A**) The AD group did not differ from the Control in novelty preference and performed above chance. (**B**) Neither AD monkey differed from the Control group, however AD1 did not differ from chance novelty preference, while AD2 did. The dashed line represents chance (50%), the vertical error bars represent SEM.

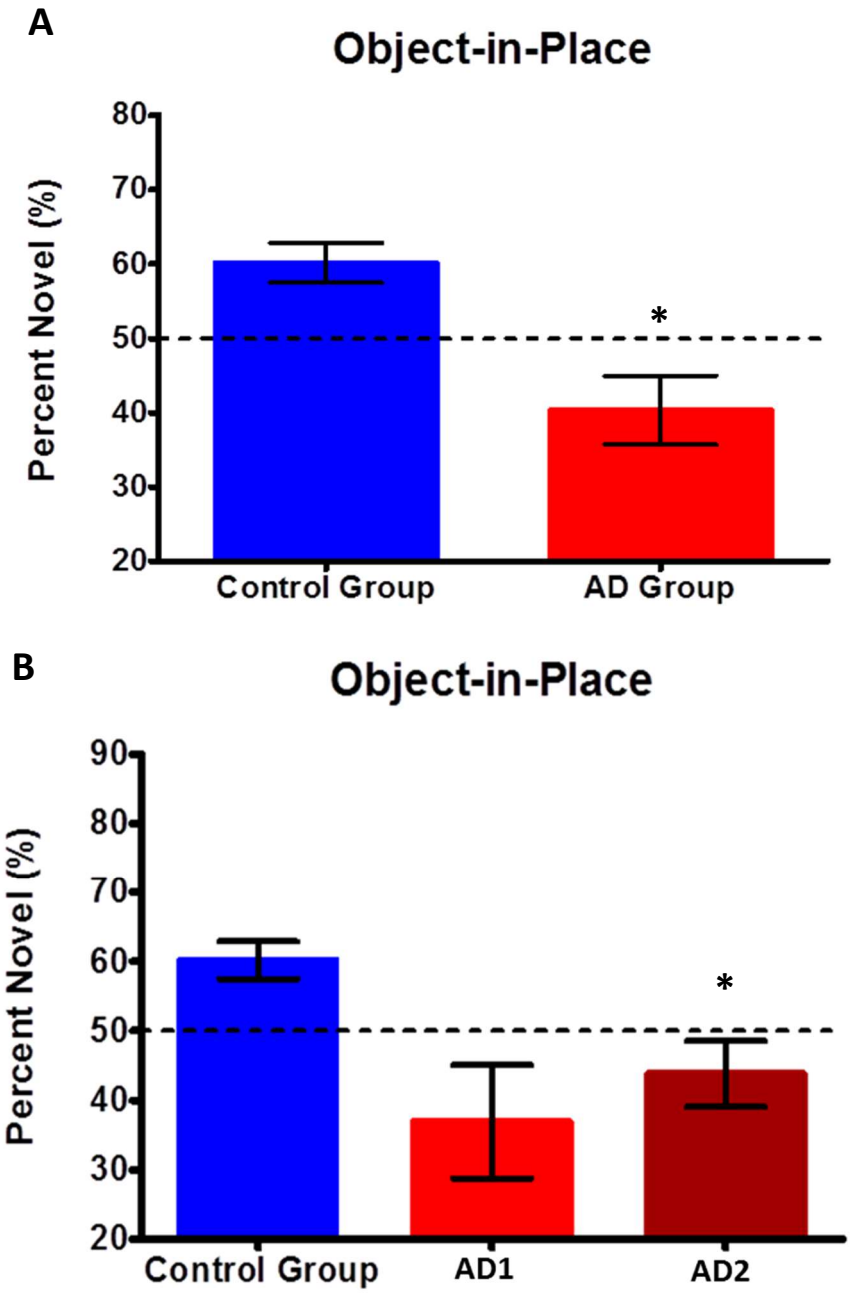

Figure 5. Mean percentage of time subjects spent looking at novel stimuli in the Object-in-Place task. (**A**) The AD group spent significantly less time looking at novel stimuli compared to the Control group. The AD group did not differ from chance novelty preference.
The task revealed a significantly higher novelty preference in the control group compared to the (**B**) AD1 and AD2 are not significantly different from chance novelty preference. The dashed line represents chance (50%), the vertical error bars signifies SEM, and the asterisk represents significant difference ($p < 0.05$) from the control group.

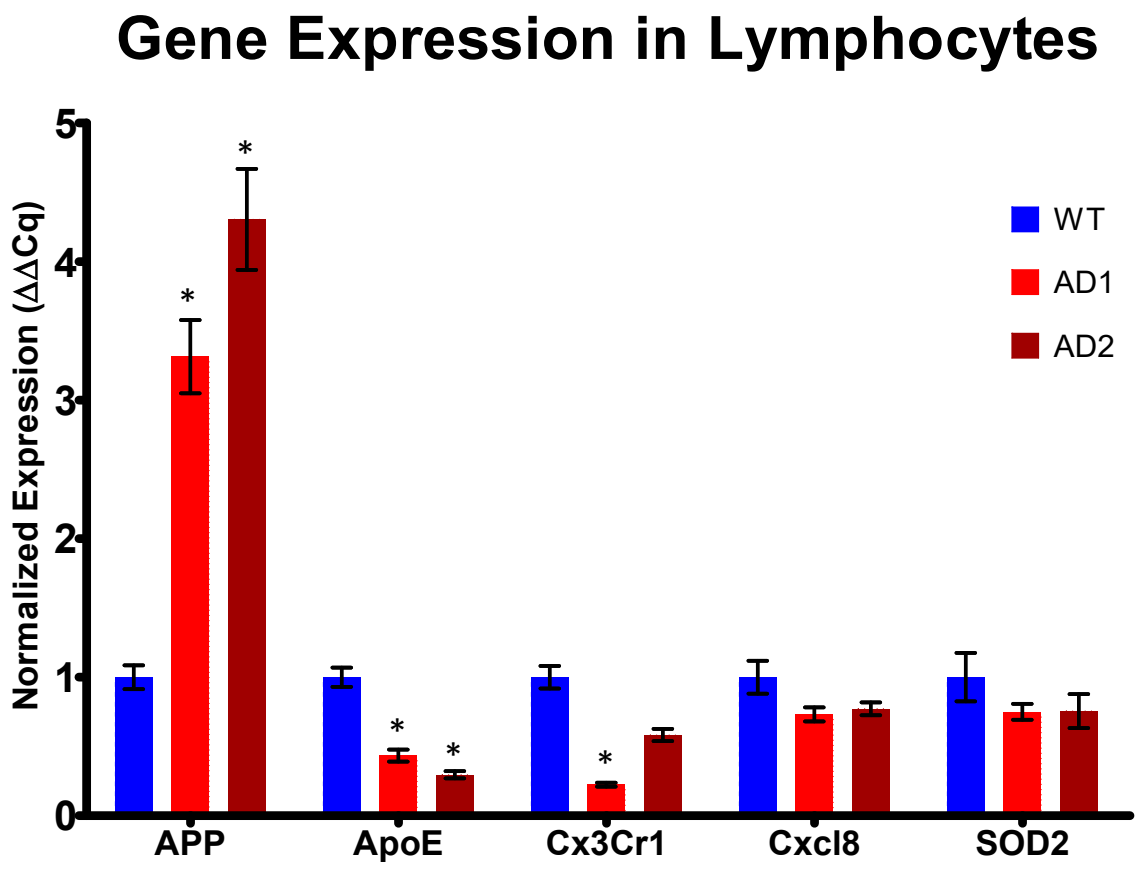

Figure 6. Normalized RNA expression of genes corresponding to biomarkers obtained from lymphocytes of AD and control monkeys. Expression levels were normalized to a WT control monkey. ApoE, APP, CX3CR1 and CXCL8 were examined due to diagnostic relevance, while SOD2 was chosen as a negative control. Overexpression of the APP transcript as well as reduced expression of ApoE in lymphocytes was observed in both AD monkeys. Reduced expression of CX3CR1 was observed only in AD1. Asterisks represent significant difference ($p < 0.05$) from WT monkey. Error bars represent SEM. Asterisks represent statistically significant difference from WT control monkey.

Discussion

The visual paired-comparison task was used to assess the memory of two transgenic Rhesus macaques that expressed the human mutant Swedish and Indiana mutations in βAPP under the control of a human polyubiquitin promoter. The percentage of total time during the

comparison phase spent looking at the novel image was used as a measure of memory. A slight impairment was detected in the VPC Spatial-Location task at 18 months of age during a previous unpublished assessment. In line with the previous findings, these monkeys presented a significant difference on one of the spatial variations of the VPC task. AD monkeys had significantly lower novelty preference on the VPC Object-in-Place task compared to the control monkeys (Figure 5). However, this performance was different from chance viewing behavior. When examined separately, AD2 had significantly different novelty preference from the control. While AD1 had an lower mean percent novelty, the monkey also had a considerably larger variation. The source of AD1's inconsistent VPC results may possibly be due to behavioral issues, including a lack of cooperativity when beginning and completing the tasks; testing sessions of this were of often shorter than AD2 due this apparent lack of cooperativity. However, both AD monkeys' performance on the Object-Replace task indicates that the Object-in-Place results are not due to visual deficits, difficulty remembering the five grouped stimuli, attentional deficits, or altered novelty preference due to the conditions of the task. It can therefore be said that overall the effect observed in the AD group is specific to allocentric memory, while egocentric memory, as seen in the unimpaired Spatial-Location task, is unaffected.

The differing performances on these tasks may be explained by their respective neural correlates. Egocentric processing only requires a subset of the brain structures necessary for allocentric processing, and does not engage the hippocampus to the same extent (Zaehle et al., 2007). As a result of this information, it is therefore reasonable to conclude that the AD monkeys may have some type of damage to the hippocampus. Hippocampal dysfunction was likely not detected in the Spatial-Location task given its utilization of the parahippocampal areas TH/TF and reduced dependence on the hippocampus relative to the Object-in-Place task (Bachevalier

and Nemanic, 2008; Zaehle et al., 2007). Similarly, the Color-Delay task incorporates a wider range of medial temporal lobe structures including the hippocampus, subicular complex and parahippocampal areas TH/TF (Manns et al., 2000; Pascalis & Bachevalier, 1999). While the Braak stages indicate that the AD monkeys should possess tau pathology in the parahippocampal gyrus prior to the hippocampus, there are four possible explanations. First, it is possible that the pathology has taken a different path, either due to the genetic manipulations used to produce the model, as a result of a species-specific variation in pathology, or due to a variation in pathology which naturally occurs in humans (Murray et al., 2011; von Gunten et al., 2006). Second, and more likely, the Color-Delay task may not have been demanding enough given the incorporation of several structures and their ability to interact and compensate. Therefore, the difference in AD and Control groups on the Color-Delay task may become significant as more visually ambiguous or complex stimuli are paired during the comparison phase, therefore increasing the demand on recognition memory. Third, the Object-in-Place task may be more sensitive to pathological changes of AD than the Color-Delay task. Fourth, it is possible that the altered performance on the VPC spatial tasks is due to the presence of Aβ rather than NFTs. Memory deficits due to Aβ have been observed in transgenic mouse models lacking tauopathy (Hsiao et al., 1996; Lesné et al., 2006). Additionally, Aβ deposition has been found to play a role in memory deficits, though to a lesser extent than tau; this impairment resulting from Aβ is most prominent in the preclinical and early stages of AD which would be consistent with the suspected state of the AD monkeys (Chételat et al., 2012). While it cannot be stated that the AD monkeys currently suffer the same behavioral impairments observed in some animal models or AD patients, the abnormal results obtained from the spatial memory tasks imply a possible emergence of cognitive impairments which will manifest at a later age.

Our findings are supported by VPC task results conducted on human AD patients (Crutcher et al., 2009; Zola et al., 2013). While these tests have been effective in identifying progression to MCI or AD, they rely exclusively on black and white stimuli and a time delay of 10 seconds or 2 minutes before the comparison phase. This version of the VPC task tests recognition memory as the Color-Delay task administered to the AD monkeys does, however, the lack of visual cues from color along with the increased time delay produce a more challenging task for subjects than the one administered in our study. Assuming the AD monkeys are at an equivalent stage of pathology, this fact is in line with the previous statement that the difficulty of the Color-Delay task administered to the monkeys may not be challenging enough to identify a deficit. Of course, the lack of variation within the VPC task administered to humans can neither confirm nor reject the possibility that the Object-in-Place task is able to provide a diagnosis at an even earlier stage than the black and white task. Furthermore, the time constraints did not permit the completion of the VPC Black-&-White Easy or Black-&-White Hard components. However, other VPC studies on macaques have shown that it is possible to reveal an impairment in recognition memory after switching from color to complex or ambiguous black and white stimuli (Zeamer & Bachevalier, 2013).

While these additional variations of the VPC task were intended to be part of this study, the time constraints did not allow for the tasks' completion. Additionally, the male AD macaque, ROn12, could not be included in the study due to timing issues. Following this study, the Black-&-White VPC task will be completed for the two AD females. Additionally, the AD male, ROn12, will be tested in an attempt to get a more complete view of the effects of the mutant APP transgene. However, given that the AD3 could be a genetic mosaic with heterogenous expression

pattern throughout the body, it is uncertain whether or not his VPC results would be consistent with the two female AD monkeys.

When analyzing expression levels in the lymphocytes of the AD and control monkeys, five biomarkers were used; SOD2 was selected as a negative control with no significant difference in expression levels between AD and WT control monkeys. APP expression was up-regulated with at least a five-fold increase in both AD1 and AD2, suggesting the overexpression of the mutant APPswe/ind transgenes (Figure 6). The increased expression of APP indicates that the transgene was successfully integrated into the genome of the AD monkeys, suggesting a possible link to the altered behavior on the Spatial-Location task; elevated expression of APP has been reported in human AD patients and animal models that develop AD pathogenesis (Babić et al., 2014; Weissmann et al., 2016; Wu et al., 2016). ApoE was also different between the WT control and AD monkeys; expression of the gene was significantly lower in AD monkeys. Decreased expression of ApoE has been shown in humans to be an effective biomarker for human AD patients (Doecke et al., 2012). This provides further evidence that the AD monkeys have begun developing AD pathology. CX3CR1 and CXCL8 are inflammatory biomarkers for AD which were found to have slightly lower mean expression levels in AD monkeys when compared to the WT control; however, only AD1's expression of CX3CR1 was significantly different, and neither were significantly different for CXCL8 expression (Figure 6). This may have been due to the small number of subjects involved, the question of whether peripheral blood cells such as lymphocytes are best for assessing neuroinflammation, or the possibility that the changes of these inflammatory response genes had yet to be fully activated at early stage AD.

Strengths of this study are the sensitivity of the VPC task in assessing memory deficits. As previously discussed, the format of this test does not depend on verbal communication. The

VPC task results can therefore be translated between human and nonhuman primates without significant confounding variables, such as a clear explanation of what is expected. Rather, human subjects were simply told to look at the screen as if they were watching television (Zola et al., 2013).

The weaknesses of this study are the low sample size. Due to the fact that only two AD animals could be tested in this study, any individual factors present in the animals could not be completely accounted for. In particular, AD1 demonstrated lack of interest in the VPC task and an overall lack of cooperativity. However, attentional deficits have also been detected in human AD patients (Oken et al., 1994; Perry & Hodges, 1999). This behavior likely contributed to the considerably larger variance across testing when compared to the other AD monkey, AD2. This concern may be minimized in the future by running additional trials to offset incorporative events that passed the testing criteria. An additional weakness is that both AD subjects are female. Evidence suggests that women have a higher prevalence of AD and progression occurs more quickly (Li & Singh, 2014). While this does not necessarily impact our findings based on AD females, being able to assess a male AD monkey, would provide a better perspective of whether AD monkeys are a relevant model for AD.

Overall, this study compiles further evidence that AD monkeys with mutant APPswe/ind transgene under the regulation of human polyubiquitin promoter produces characteristic visual behavior and novelty preference. The VPC Object-in-Place task, which selectively targets the hippocampus, revealed significantly reduced novelty preference in transgenic monkeys compared to controls. While mean scores for recognition memory on the Color-Delay task were below the control scores, the differences were not significant; this may have been due a lack of difficulty in the task as well as the small sample size. When examining gene expression pattern

in lymphocytes, both AD1 and AD2 presented overexpression of APP. The elevated expression of APP provides further evidence that the transgenic monkeys are likely to progress in AD pathology and develop significant memory deficits over time.

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