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Poor Memory Performance in Aged Cynomolgus Monkeys with Hippocampal Atrophy, Depletion of Amyloid Beta 1-42 and Accumulation of Tau Proteins in Cerebrospinal Fluid

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Abstract. *Background: Due to their similarities in behavior and disease pathology to humans, non-human primate models are desirable to complement small animals as models for the study of age-related dementia. Materials and Methods: Based on their performance on delayed response task (DRT) tests of memory, aged cynomolgus monkeys were divided into two groups to compare high-performing (n=6) and low-performing (n=6) subjects. Both groups were tested for biomarkers related to Alzheimer's disease and their brains were scanned using structural magnetic resonance imaging. Results: The subjects with poor DRT performance had evidence of atrophy in the hippocampus and cortical areas, significantly lower cerebrospinal fluid levels of amyloid beta amino acid 1-42 ($p<0.001$) and higher cerebrospinal fluid total tau levels ($p<0.05$) compared to the group performing well on the DRT tests. Conclusion: Old, memory-impaired Cynomolgus monkeys may be useful as a spontaneous non-human primate model for investigations of age-related neurodegenerative diseases.*

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As one of the first neurodegenerative diseases to be characterized (1, 2), models of Alzheimer's disease (AD) has been extensively studied using many different animal models (3, 4), including animals genetically-modified to develop the pathological hallmarks of AD, such as amyloid beta (Aβ) plaques and neurofibrillary tangles (NFTs) (5, 6).

An ideal model of AD in animals must exhibit the current criteria established by the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's disease and Related Disorders Association. These characteristics include cognitive impairments and biomarker profiles similar to those seen in patients with AD (7, 8). A major advantage provided by non-human primate (NHP) models of age-related dementia is the observation that these animals exhibit signs of disease that mimic the signs seen in humans, especially cognitive decline (9, 10). Particularly if a proportion of the aged population spontaneously develops age-associated dementias, NHPs may represent extremely valuable spontaneous models.

In an animal model, progression from mild cognitive impairment (MCI) to AD-like dementia should be confirmed by the presence of biomarkers that reflect the pathological processes in the brain. For example, cerebrospinal fluid (CSF) levels of amyloid beta 1-42 ($A\beta_{42}$) should fall, and total tau proteins (t-tau) and phosphorylated tau proteins (p-tau) should be elevated (2, 7, 11). As an integral part of the clinical assessment of patients with suspected AD, a structural assessment using magnetic resonance imaging (MRI) is used as a valid diagnostic tool for MCI and progressive neurodegeneration (12). Similar morphological changes should be present in a viable animal model. Plaques of Aβ have been found to develop spontaneously in aged NHPs, including the rhesus monkey (13-15), the cynomolgus monkey (16), great apes (17) and the vervet monkey (18). Additionally, all six tau protein isoforms that are biomarkers for AD in the human brain have also been identified in brains of NHPs (19).

Studies have identified significant age-related changes in Aβ and tau protein levels in mouse lemurs (20) and chimpanzees (17). However, the majority of naturally-developing beta amyloid plaques in aged NHPs have not been linked to significant neuronal injury or to development of NFTs (21). Although studies of rhesus monkeys have provided the greatest insight into cognitive aging processes, investigations of other NHP species, with their wide variety of reproductive, morphological, and behavioral adaptations, can also shed light on factors that underlie age-related cognitive changes in our own species (9).

Several cognitive assessments in NHPs have been successfully adapted from human neuropsychology (10). The delayed response tasks (DRT) are simple, well-established tests (22-24) that are easy to administer and suitable for assessing spatial, working and episodic memory in NHPs (9, 10, 25-30).

The aims of the present study were to identify two sub-groups of aged cynomolgus monkeys: one that performed poorly on the DRT tests and one that performed well on the same tests, and to compare CSF biomarkers associated with AD and MRI scans across groups. Dependent measures similar to those used to routinely diagnose age-related brain changes in human patients suffering from dementia were employed.

We conducted three types of DRT that have previously been used with cynomolgus monkeys (25): the short-term memory test (STMT), the long-term memory test (LTMT) and the memory load test (MLT). In a previous publication, we showed that young and aged cynomolgus monkeys differed significantly in their DRT performance and levels of $A\beta_{42}$ (28).

Materials and Methods

Subjects. Twelve aged (>20 years old; 5 male and 7 female) cynomolgus monkeys participated in the study. These animals included the six lowest performing subjects and the six highest performing subjects on the STMT (see below) from our previous work (28). The subjects' ages were determined from birth certificates for the animals born in captivity (n=8), and from dental scaling (31, 32) for animals born in the wild (n=4). The subjects were clinically healthy and tested monthly to confirm that they were tuberculosis-free. Potential gender-related differences were not investigated due to the small number of animals of each sex in each group.

Housing. The subjects were housed in the association for assessment and accreditation of laboratory animal (AAALAC)-accredited Primate Research Center (PRC) at Bogor Agricultural University (IPB; Bogor, Indonesia). Study subjects lived in pairs or social groups of various sizes and had access to both indoor and outdoor areas. For the five-month testing period, subjects were pair-housed in adjacent individual cages, which permitted restricted tactile contact. The adjacent cages consisted of two joined individual cages - approximately 150×75×50 cm (W×L×H) in size. The animals' diet consisted of fruit and standard monkey chow pellets (Harlan® 2050 Teklad Global 20% Protein Primate Diet; Indianapolis, IA, USA) provided twice-a-day.

Tap water was provided *ad libitum* throughout the experiment. To prevent hunger from being an influencing factor in the cognitive tests (where the subjects had to retrieve desirable food items), subjects were not deprived of food during testing. All of the test procedures, as well as subject housing conditions (before, during, and after the experiment), were approved by the PRC Institutional Animal Care and Use Committee (IACUC) under license PRC IPB 13-11-IR.

Study design. Memory tests were used as a tool to select for study subjects with cognitive impairments similar to those displayed by human patients identified to be suffering from severe memory impairment and cognitive decline. A DRT was selected to categorize subjects for further comparisons of biomarker levels and structural parameters.

Among the three DRTs utilized in the present study, the STMT was chosen for technical reasons; it is not particularly time-consuming and it provides easily-interpretable results; and because our previous work (28) had shown that performance on this task had the strongest correlation with circulating $A\beta_{42}$ of the three DRTs that we conducted. The six animals (from the 18 aged monkeys tested in Darusman *et al*. 2013 (28)) that performed the worst on the STMT were designated as the low-performer group and were compared to the six aged animals that performed the best on the STMT; designated as the High-performer group (Table I). CSF biomarker analysis, MRI scans and further cognitive assessments were conducted for all 12 subjects in both groups. Subjects were tested on the STMT in November 2012 and were sampled for CSF in January 2013. MRI scans were conducted in February 2013 and further DRT tests were conducted in March-May 2013.

DRT assessment. A total of 24 STMT trials were used to screen the 18 aged animals. The 12 selected animals were further assessed in 21 LTMT trials and 30 MLT trials each. Prior to testing, the subjects were habituated to the procedures and the experimenter; they voluntarily sat down and faced the experimenter once the test stimuli were prepared. The results of the tests have previously been shown to have a high degree of interobserver reliability (average Cohen's kappa coefficient = 83%) (28).

The DRTs were carried out according to a previously established protocol (for a detailed description of the DRT procedures and training/habituation, see Darusman *et al*. 2013 (28)). Briefly, the subjects were presented with a tray with identical cups. Food items ('baits') were hidden within the inverted cups, which the subjects

Table I. *The short-term memory test summary.*

No.	Tattoo	Gender	Trials	Mean correct response (%)	Source
1.	T3107	Male	24	25.01	PRC Natural breeding facility (Tinjil island)
2.	I1116	Female	24	41.67	PRC Bogor breeding facility (in situ facility)
3.	I1122	Female	24	41.67	*In situ* facility
4.	T3111	Male	24	45.84	Tinjil island
5.	C5545	Female	24	50	*In situ* facility
6.	T2800	Male	24	50	Tinjil island
7.	K44	Female	24	56.67	Tinjil island
8.	C5544	Male	24	53.16	*In situ* facility
9.	C2466	Male	24	55.3	*In situ* facility
10.	8969	Female	24	52.16	Tinjil island
11.	C5353	Male	24	56.67	*In situ* facility
12.	C5306	Male	24	53.16	*In situ* facility
13.	T3232	Male	24	58.34	Tinjil island
14.	10749	Female	24	58.34	*In situ* facility
15.	C1980	Female	24	58.34	*In situ* facility
16.	C0168	Female	24	62.51	*In situ* facility
17.	9661	Male	24	66.67	*In situ* facility
18.	11087	Female	24	70.84	*In situ* facility

were then tasked to retrieve following a set time delay. For the STMT, a single bait was hidden in an array of three identical cups and time delays of 0 (no delay), 30, 60 and 120 sec were applied. The subjects were allowed a single answer. For the LTMT, a single bait was again used, but all three cups were unique in design, and longer delays (0, 2, 4, 8, 12 and 24 h) were utilized. The subjects were again only allowed a single answer per trial. For the MLT, six identical cups were used and two baits were hidden, each in a separate cup; delays of 0 and 30 sec were used. For this task, the subjects were allowed two answers.

CSF. Two sampling areas were utilized for the collection of cerebrospinal fluid: the lumbar sub-arachnoid space and the cisterna magna (suboccipital area). CSF collection from the sub-occipital area was performed only if the collection from the lumbar area did not yield a large enough sample. Animals were sedated with 1 mg/kg bodyweight ketamine (Ilium Ketamil®; Troy laboratories Ltd., Glendenning, NSW, Australia) intramuscularly. The lumbar and sub-occipital areas were shaved and prepared with povidone iodine solution.

The lumbar puncture was performed by positioning the animals in lateral recumbency and a 22-gauge spinal needle was inserted into the lumbar interspace at the level of the palpated iliac crest. To perform the suboccipital puncture, the animal's neck was fully-flexed to expose a small triangular depression directly below the occipital articulation where the spinal needle was inserted perpendicularly. A 2 ml CSF sample was obtained from each subject using these techniques. Samples were stored in propylene tubes at −70˚C until further analysis.

Biomarker analysis. Concentrations of $A\beta_{42}$ in CSF samples were measured using the Invitrogen™ human $A\beta_{42}$ ELISA kit (catalog number KHB3441; Invitrogen™, Camarillo, CA, USA). The minimum detectable level of human $A\beta_{42}$ was listed at 10 pg/ml and the assay has no known crossreactivities with other Aβ species ($A\beta_{12}$, $A\beta_{20}$, $A\beta_{28}$, $A\beta_{35}$, $A\beta_{40}$) or other neurodegenerative markers, such as α-synuclein and amyloid precursor protein (APP). The t-tau and p-tau concentrations in CSF were measured using the Invitrogen™ human t-tau (catalog number KHB0042), p-tau pS396 (catalog number KHB7031) and p-tau pT231 (catalog number KHB8051) enzyme linked immunosorbent assay (ELISA) kits. The detection limits for the assays were listed at 12 pg/ml, 2 pg/ml and 0.7 units/ml, respectively. In addition, all of the p-tau kits were validated not to crossreact with non-p-tau or protein kinase A (PKA)-p-tau. Absorbance was measured at 450 nm with correction wavelengths at 540 and 570 nm. All samples were analyzed in duplicate and the intra-assay coefficients of variation (33) were 8.6%, 3.4%, 4.1% and 5.3% for $A\beta_{42}$, t-tau, pS396 and pT231, respectively.

MRI. MRI scanning was conducted at the Ciptomangunkusumo hospital (RSCM) in Jakarta, Indonesia, using a routine method for diagnosis of AD-related dementia in elderly human patients. The MRI scans were carried out in a Siemens™ Magnetom Avanto 1.5 T scanner (Erlangen, Germany) using a human knee coil. The subjects were pre-medicated with a subcutaneous atropine sulfate injection (0.025 mg/kg body weight), followed by an intramuscular injection of ketamine (10 mg/kg) for anesthesia. The injections provided 30-45 min of anesthesia. Subjects were under constant observation by veterinary staff before, during, and after the scan. Prior to scanning, the animals were wrapped in blankets to minimize motion and to provide heat insulation. During scanning, head motion was minimized by stabilizing the subject's head with foam cushions and elastic straps.

Structural T2-weighted (t2w) and T1-weighted (t1w) images were acquired when these images were obtained quickly and the subject was still under proper anesthesia, additional t1w inversion recovery (t1w-IR) images were acquired. The t1w images were acquired from the axial plane and followed a T1 scan protocol optimized for 1.5 T, using the imaging parameters: field of view (FOV) 192×256 mm^2, slice thickness 0.5 mm, base resolution 256, repetition time/echo time (TR/TE) of 2400-2800/3.5-6 ms. The t2w images were acquired in the coronal and axial plane and the

acquisition parameters were as follow: FOV (read) 135×180 mm^2, slice thickness 1.5 mm, base resolution 256, and TE −20-80 ms. The series consisted of approximately 50-55 images (25 t1w, 25-30 t2w, +11 t1w IR) for each subject. The scanning took 35-40 min for each subject.

The structural MRI images were interpreted independently by two MRI physicians from RSCM who were blinded to the identity of the subjects. The physicians scored the subjects according to criteria that distinguish between dementia of the AD type and dementia of other types (34). Two criteria that established the diagnosis of dementia of AD type are cerebral atrophy and hippocampal atrophy (12). The cerebral atrophy criterion was defined as a widening of the cortical sulci and Sylvian fissures of both cerebral hemispheres in proportion to the cranial space, while the hippocampal atrophy criterion was defined as an amorphous shape and reduction in size.

Other criteria relate to the diagnosis of dementia of other types, such as hypo/hyperintense lesion at intracerebral regions, ventricles (third, fourth, and lateral ventricles) lesion, absent or presentation of the midlineshift, lesion at infratentorial region (pons, cerebellum, and cerebellar-pontine angle), orbital condition (bilateral orbits, ocular bulbs, and optic nerves) and signs of skull fracture and/or associated soft tissue injury. Among other differential diagnoses of dementia of AD type, vascular dementia (VaD) is regarded as mandatory for differentiating the diagnosis of other types of dementia in humans by a structural MRI (12, 35), which are indicated by hypo- or hyperintense lesioninthe intracerebral regions, specifically in the white matter. Other types of dementia, such as multiple system atrophy (MSA) can be diagnosed by atrophy at several parts of the infratentorial region, such as the pons, cerebellum and cerebellar-pontine angle (36). Physical injury related to memory problems can be diagnosed by possible lesions at bilateral orbits, optic nerve condition, and signs of skull fracture and associated soft tissue damage.

Scoring and data analysis. The primary dependent variable for performance assessment in the DRT was the percentage of correct responses, defined as the percentage of answers in the trials that resulted in the recovery of bait. In all tests, 'no response', when the subject did not choose any of the cups at all, was recorded and was distinguished from an incorrect response (the selection of a cup that did not result in the recovery of a bait). In order to obtain an overview of the combined performance on the DRTs, especially for correlations with biomarkers, an expression for the overall performance on the STMT, LTMT and MLT was created: The total DRT performance was calculated for each monkey as the unweighted combined average retrieval accuracy in all three tests. Since LTMT performance may also relate to memory consolidation (37), a separate analysis was performed where retrieval accuracy (percent correct) was plotted as a function of the length of the delay. Normal memory consolidation should be evident as a U-shaped curve, with peak performances at time points with 0 and 24 h delays.

t-Tests were applied to test for differences across the two groups (low-performers *vs*. high-performers) in biomarker concentrations and DRT performances. Pearson's product-moment correlations were used to assess for relationships between biomarker levels and DRT scores. Fischer's exact test was used for the MRI data, focusing on differences in the distribution of positive diagnoses of possible AD type dementia between the groups. *p*-Values less than 0.05 were considered significant.

Results

Selection of high and low performers. A total of 18 aged cynomolgus monkeys of both sexes were tested on the STMT (Table I). The mean percentage correct responses from low performers (subjects number 1 through 6) was 42.4% while the mean for the high performers group (numbers 13-18) was 62.5%. The performance of the selected group of low performers differed significantly from the performance of the selected group of high performers.

DRT assessment. The percentage of correct responses for low performers was significantly lower than that for the high performers in the LTMT ($p<0.001$) and MLT tests ($p<0.01$) (Table II). The highest incidence of no response in the LTMT occurred in the low-performance group after a 4-h delay (9.5%), while in the high-performance group, it occurred at the 6-h delay (3.2%). The retrieval accuracy of the low performers followed an inverse function [$y=24.75+(57.8/(t+1))$], while the retrieval accuracy of the high performers was better-fitted to a quadratic function ($y=96.99-5.35t+0.194t2$). The quadratic function was not significant ($p=0.077$, two-tailed test).

Biomarker analysis. Results of the biomarker analyses are presented in Table III. There were significantly lower CSF levels of $A\beta_{42}$ ($p<0.001$) and higher CSF levels of t-tau ($p<0.05$) in low performers than in high performers (Figure 1). However, Levene's test for the t-tau data showed that the variance across subject groups was not equal: one of the low performers had the highest t-tau value (908.1±60.11 pg/ml). No significant differences were found in the levels of p-tau (pS396, $p=0.057$ and pT231, $p=0.064$) across groups (Figure 2). Levene's test showed that the variance of pT231 levels in the low performers was significantly higher ($p<0.01$) than that in the high performers (Figure 2).

Correlations among biomarkers and between biomarkers and the DRT. $A\beta_{42}$ levels only correlated significantly (negatively) with t-tau levels ($r=-0.684$, $p<0.05$). t-Tau was significantly positively correlated with pS396 ($r=0.729$, $p<0.01$), but not with pT231, even though pS396 and pT231 were significantly positively correlated with one another ($r=0.617$, $p<0.05$).

Between biomarkers and DRT, the CSF $A\beta_{42}$ was significantly positively correlated with all DRT tests (Figure 3), while the other biomarkers were not significantly correlated with any DRT test. $A\beta_{42}$ was significantly positively correlated with STMT ($r=0.903$, $p<0.001$), LTMT ($r=0.718$, $p<0.01$), MLT ($r=0.646$, $p<0.05$) and total DRT ($r=0.877$, $p<0.001$).

MRI. The MRI scans were performed at a hospital, and, as far as we are aware of, this was the first use of this standard MRI setup for NHP examination. The equipment produced

Table II. *Delayed response task assessment summary.*

Group	Values	Mean correct response (%)	
		Long-term memory test	Memory load test
Low-performers	Mean	39.6	37.8
	Standard deviation	8.4	2.7
	Trials	126	180
	N	6	6
High-performers	Mean	76.9	44.4
	Standard deviation	14.2	4.1
	Trials	126	180
	N	6	6

Table III. *Comparison of Alzheimer's disease-associated biomarkers.*

Group	Values	$A\beta_{42}$ (pg/ml)	t-tau (pg/ml)	pS396 (pg/ml)	pT231 (units/ml)
Low performers	Mean	258.8	496.3	44.1	2.4
	Standard deviation	77.1	235	13.9	1.4
	N	6	6	6	6
High performers	Mean	454.7	227.8	29.2	1.2
	Standard deviation	45.5	79.2	9.6	0.29
	N	6	6	6	6

$A\beta_{42}$: Amyloid beta amino acid 1-42; t-tau: total tau; pS396: phosphorylated tau serine 396 (pg/ml); pT231: phosphorylated tau threonine 231 (U/ml).

adequate image quality for gross structure analysis of the NHP brains and allowed for assessment according to the criteria, which are routinely used at this hospital to diagnose the possibility of dementia of AD and non-AD types. The slice thickness of the images was not optimized in the present study and the equipment did not allow for a more thorough structural examination of the white matter, grey matter and CSF.

All low performers (n=6), but no high performers (n=0) had indications of atrophy in the hippocampus (Fischer's exact test: $p<0.01$). Five low performers also had additional indications of cerebral atrophy by cortical sulci widening, while no high performers did (Fischer exact test: $p<0.05$) (Table IV) with a summary of all section numbers listed in Table V. Figure 4 displays examples of the sections of cortical sulci widening and hippocampal atrophy from the t1 IR MRI images of low performers compared to high performers.

None of the subjects (n=12) showed any signs of dementia of the non-AD type. There were no hypo/hyperintense lesions in intra-cerebral regions and no lesions in the ventricles (third, fourth, and lateral), or in the infratentorial region (pons, cerebellum, and cerebellar-pontine angle).

Discussion

DRT assessment. The low performers performed worse than the high performers on all of DRT tasks. The DRT assesses memory functions, including spatial, working and episodic memory, the types of memory most affected in human AD type dementia (10). The findings suggest that the use of the STMT as the selection test to divide low and high performing subjects on DRT was appropriate, allowing us also to predict subjects' memory problems on the MLT and LTMT. Furthermore, it suggests that the short-term memory problems in low performing, aged cynomolgus monkeys may serve as a good predictor of memory deficits associated with DRT assessment

Cognitive tests for diagnosing AD in humans are designed to assess the function of particular cognitive domains; primarily memory (spatial, working, and episodic among others), executive decision making, attention and visuospatial ability (7, 8, 38, 39). The ability to recall the positions of the baited cups can be used to assess subjects' spatial memory. The various time delays between stimulus presentation and the subject's decision that were employed in all of the DRT memory tests in the present study, should provide insight into possible shifts in visual-passive memory to active-working memory (40). Episodic memory relates to the ability to learn and retain new information and impaired episodic memory provides clear evidence of cognitive decline, as it is most commonly seen in the progression of MCI to AD in humans (7, 8). The longer time delays that are employed in the LTMT evaluate memory processing and the incorporation of a stimulus as a signal to be stored and retrieved at a later time. Instead of having a storage problem (being forgetful), the most prominent symptom among patients in early stages of AD (predementia or prodomal AD) is impaired episodic memory (7). This means that the information or stimulus cannot be correctly interpreted. Patients with prodomal AD demonstrate lower performance on tasks that assess new learning, recall, retention and abstract reasoning (41, 42).

In addition, for assessing working memory and episodic memory, the LTMT also functions as a test of memory consolidation (37). The inverse (U-shaped) function of percentage of correct responses by time delay in the low performers suggests that memory did not recover after the time delay, while the quadratic function observed for high performers suggests that memory seemed to recover. However, the quadratic function was not statistically significant, possibly indicating weak recovery or consolidated memory in the group of high performers.

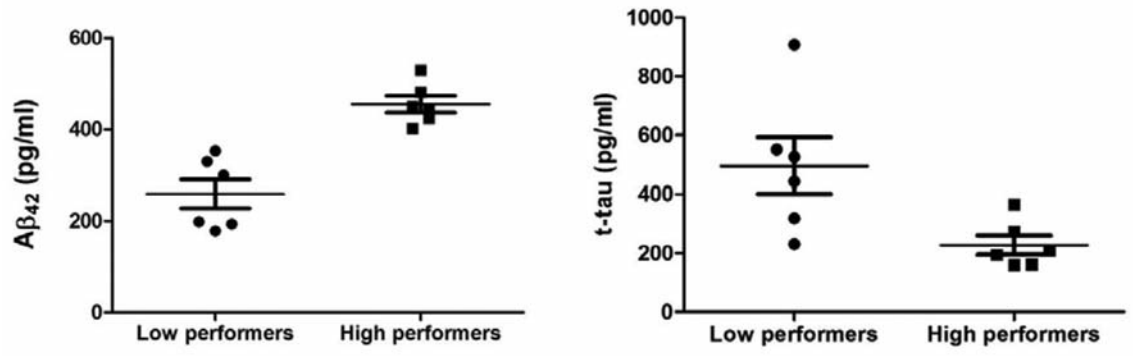

Figure 1. *Cerebrospinal concentrations of amyloid beta amino acid 1-42 ($A\beta_{42}$) (A) and total tau (t-tau) (B) in the two groups. Each point represents one subject. The solid horizontal line indicates the mean value of the group and the error bars represent the standard error of mean. The highest t-tau level was observed in one of the low performers.*

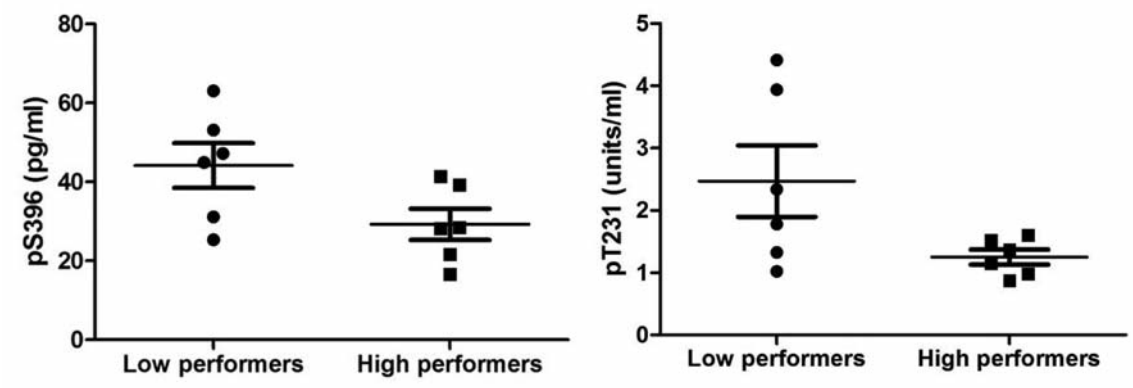

Figure 2. *The phosphorylated tau serine 396 (pS396) (A) and phosphorylated tau threonine 231 (pT231) (B) protein concentrations in the cerebrospinal fluid of the two groups. Each point represents one monkey. The solid horizontal line indicates the mean value of the group, and the error bars represent the standard error of mean.*

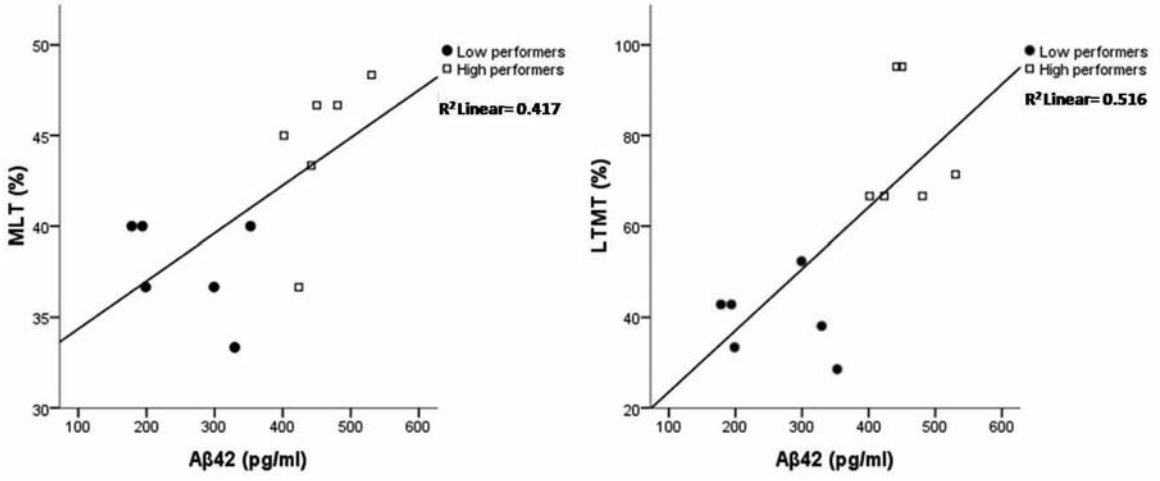

Figure 3. *Correlation of correct responses in the memory load test (MLT) and long-term memory test (LTMT) in the two groups with amyloid beta amino acid 1-42 ($A\beta_{42}$) in cerebrospinal fluid. Each point represents one subject.*

Table IV. *Magnetic resonance imaging examination.*

Group	Criteria for diagnosis of Alzheimer's disease		Criteria for differential diagnoses		
	Cerebral atrophy	Hippocampal atrophy	Vascular dementia	Multiple system atrophy	Injuries
Low performer's N	5	6	0	0	0
High performers N	0	0	0	0	0

Table V. *Slide summary from each subject's T1 and T2 magnetic resonance imaging (MRI) images.*

Subject's ID	MRI images (number of slides)						
	t1W-ax	t1W- IR	t2w-cor	t2W-ax	t1W-sag	t2-cor	t2-sag
T3311	11	11	19	11	120	64	-
I1166	17	11	17	15	9	-	-
I1112	18	11	15	15	-	-	-
9661	-	11	19	64	64	-	96
T3107	13	11	14	13	-	-	-
T2800	11	11	14	11	9	-	-
T3232	11	11	14	11	9	-	-
C1980	11	11	11	13	9	-	-
11085	14	11	14	14	9	-	-
C5545	14	11	14	14	9	-	-
C0168	13	11	14	13	9	-	-
10749	13	11	14	13	9	-	-

t1W-ax: T1 weighted axial; t1W- IR: T1 weighted inversion recovery; t2w-cor: T2 weighted coronal; t2W-ax: T2 weighted axial; t1W-sag: T1 weighted sagital; t2-cor: T2 coronal; t2-sag: T2 sagital.

The subjects in the low-performer group failed to respond (they did not choose any cup) more frequently than high performers. The onset of no response answers began at the 4-h delay for low performers, while high performers did not exhibit this behavior until the 6-h delay. These results can be interpreted to suggest that low performers had encoding problems; failing to interpret the tray with the three unique cups that comprised the LTMT set up as a test stimulus. Consequently, the monkeys did not respond to this 'un-encoded stimulus'. Attempts to choose cups following long delays, even when the wrong cups were chosen, clearly indicate that the monkeys were responding to a stimulus that they recognized (they remembered the procedure, even if they did not remember the exact location of the bait within a cup). These findings suggest that high performers have fewer problems encoding the relevant stimuli in this procedure because they know what to do in response to the presentation of the LTMT apparatus.

Biomarker analysis. Although the CSF levels of $A\beta_{42}$ and t-tau proteins were significantly different across performance groups, only the between-group differences in $A\beta_{42}$ levels were similar to those of human patients with AD when compared to normal subjects. The percentage elevation in t-tau in low-performer macaques was much smaller than that seen for human patients with AD (43). The CSF level of $A\beta_{42}$ was positively correlated with performance in all of the DRT, while the other biomarkers had no predictive value in the DRTs. Evidence suggests that the build-up of $A\beta_{42}$ (44) and tau proteins in the brain is associated with neuronal injury (45-47), and the accumulation of $A\beta_{42}$ in the brain correlates with low levels of amyloid in the circulation. Studies by Fagan *et al.* (48) and Fosberg *et al.* (49) described how circulating $A\beta_{42}$ levels reflect, in inverse proportion, fibrillar $A\beta_{42}$ levels, and consequently, the amyloid plaque load in the brain. Low CSF levels of $A\beta_{42}$ may indicate a higher occurrence of plaques that sequester the $A\beta_{42}$ peptide in the brain parenchyma, resulting in reduced availability of Aβ that can diffuse into CSF (11). $A\beta_{42}$ has been found to be the most abundant species of Aβ in amyloid plaques, which led to the development of assays for this Aβ isoform (11, 50).

However, low circulating amyloid levels are not exclusively tied to AD, but are also associated with other neurodegenerative diseases, such as amyloid angiopathy, Lewy body dementia (LBD), frontotemporal dementia (FTD), Creutzfeldt-Jakob's disease and Parkinson's disease (50). Therefore, other

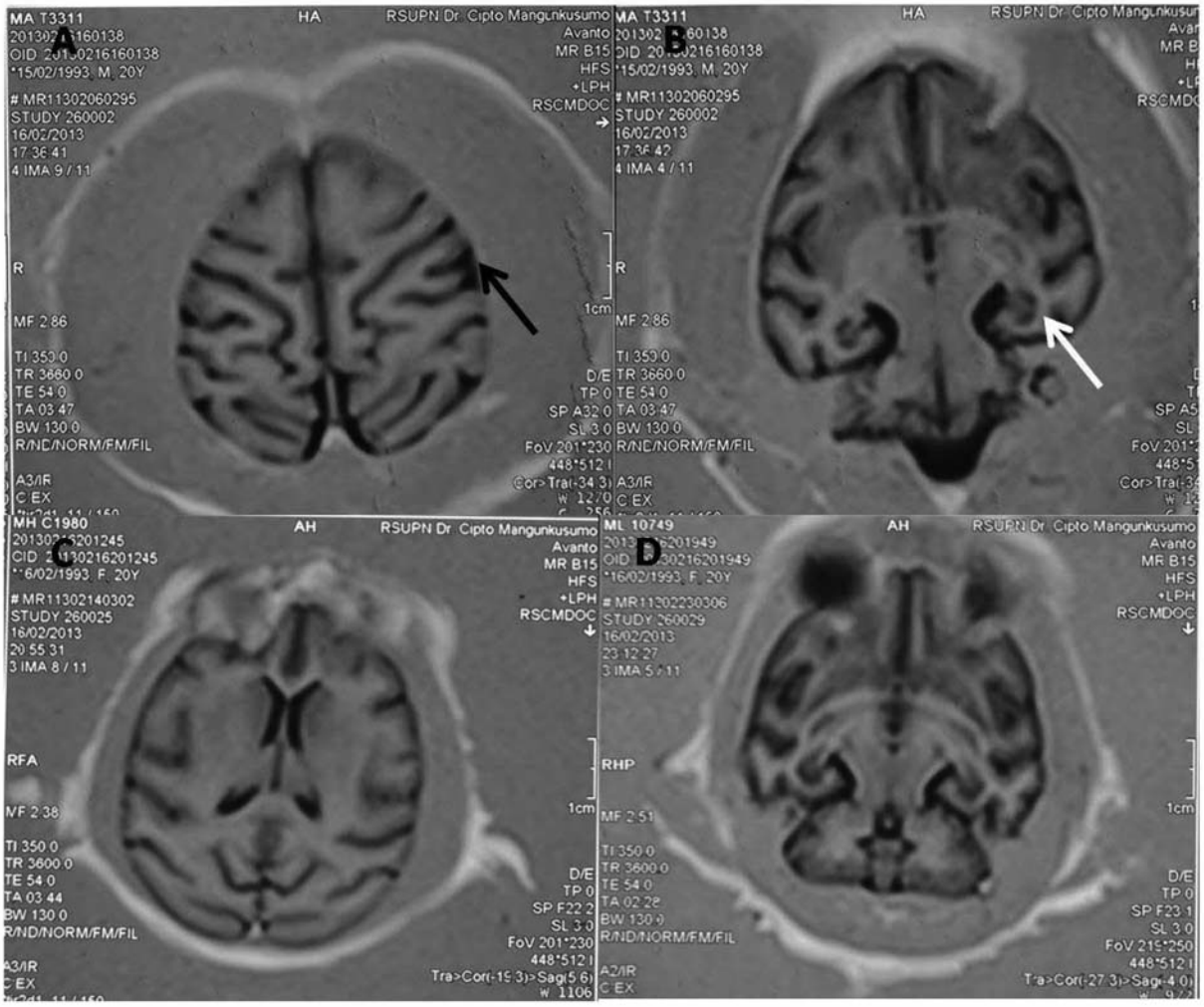

Figure 4. *T1-weighted inversion recovery images were recorded from low performers (A and B) with indication of widening of sulci (black arrow) and hippocampal atrophy (white arrow), and high performers (C and D).*

biomarkers are needed for differential diagnosis of AD, emphasizing on measurements of t-tau and p-tau.

t-Tau levels were significantly higher in low performers compared with high performers, suggesting the possibility of a neuronal injury process in low performers with high t-tau. High CSF levels of tau proteins, both t-tau and p-tau, reflect the deterioration rate of neuronal integrity and axonal degeneration (11, 46, 50). In relation to AD, hyperphosphorylated tau is the principal component of paired helical filaments, which form NFTs, neurophil threads and senile plaques neuritis (11). However, increases of CSF t-tau are also seen in patients with acute disorders, such as stroke or brain trauma; Creutzfeldt-Jakob's disease (51); FTD and LBD; and occasionally in depression, alcoholic dementia and Parkinson's disease (50).

The p-tau levels remain unchanged in several diseases where t-tau levels tend to be elevated (11), therefore p-tau is considered to be a more specific biomarker for AD, with a 92% rate in discriminating patients with AD from non-demented individuals (7). Additionally, high CSF p-tau levels are associated with a fast progression from MCI to AD, rapid cognitive decline in AD and indicative of the rate of hippocampal atrophy (2, 11).

Onset of amyloid plaque and NFT formation occurs at different time points (2). The plaques, indicated by low CSF levels of $A\beta_{42}$, develop earlier in pre-clinical AD, potentially in the absence of any indications of dementia (clinical dementia rating=0). On the other hand, NFTs, indicated by high CSF levels of t-tau and p-tau, develop later, at the very mild to mild AD stage (2). In the present study, we found that the low-performing subjects presented with lower CSF levels of $A\beta_{42}$ and higher t-tau, compared to the high-performing group. However, the p-tau levels were not

significantly different between the groups. Since p-tau is regarded as the most specific biomarker of NFTs, the findings were not able to confirm NFT formation. The absence of a significant difference between p-tau levels means that we cannot conclusively rule-out a difference in NFT formation between the high- and low-performing groups of subjects.

The pS396 levels may suggest that NFT progression had not yet reached the intracellular stage, but was still in the earlier stages, the punctate or fibrillar stages. Any early-stage NFT formation (21, 52) should have been evident in the level of pT231 in the low-performing subjects. As this was not the case, the memory decline in the low-performing group cannot be attributed to neuronal damage stemming from NFT formation, similar to that seen in human AD. These findings are in line with studies of other aged NHPs, where amyloid plaques have been found, but significant tauopathy is unusual (21).

However, there is another possible explanation that could be related to the properties of pT231, which are also observed in the last stage of NFT (53). The pT231 immunoreactive properties can be found on extracellular NFTs due to particular epitopes that may be lost or masked during the evolution of the lesion (53, 54). This finding suggests that the appearance of pT231 in early and late progression of NFT formation brought potential bias to our interpretation, regardless of detectable NFT formation or other specific p-tau indicative of early NFT estimation. Another effect that could mask a difference in NFT formation between the two subject groups is that pT231 can be seen diffusely throughout the dendritic tree and soma in normal aging animals (55). Overall then, the predictive power of this biomarker is probably low.

MRI. Although in rhesus monkeys the relationship between the amyloid burden and dementia-related cognitive impairments has been described as debatable (15, 56), we have, in a previous study, found a correlation between delayed response performance and $A\beta_{42}$ concentrations in cynomolgus monkeys (28). In the present study, these results received further support from structural MRI with indications of atrophy of the hippocampus in low-performing monkeys. Applied to the present subjects, structural MRI diagnostic criteria of dementia in human were consistent with structural abnormalities in the low performers that resembled those found in humans suffering from AD-type dementia. Signs of cortical sulci widening, indicative of atrophy of the cortex and hippocampal atrophy are both regarded as sensitive markers of the progressive form of AD (12).

In a comparison of cortical sulci in humans and cynomolgus monkeys, a developmental MRI study by Sawada *et al*. described the homologies between the primary sulci and gyri of the two species (57). Several differences were also observed, including fewer sulci and gyri located in the neocortical region in humans, and the earlier emergence of the superior temporal, cingulate and collateral sulci in cynomolgus monkeys. The sulci are known to be located partly in cortical regions specialized for cognition, recognition and language in humans, but not entirely in monkeys (57-59).

Experimental brain lesions in monkeys have demonstrated the importance of the hippocampus and hippocampal formation (medial temporal lobe) in the types of memory disorders that is also seen in AD and neurodegenerative disease, including memory consolidation, associative memory function, declarative memory and recognition memory (10, 60-62). Experimentally-induced lesions and degeneration of the medial temporal lobe and prefrontal cortex are associated with impaired delayed response performance in aged rhesus monkeys (62-64). MRI studies on human and rhesus monkey brains reveal how degenerative diseases affect the hippocampal formation, which contributes to an age-related cognitive decline and suggests that interventions can preserve cognitive health (65).

MRI can enhance the understanding of morphological changes in mild cognitive impairment and the relation of these changes to cognitive deficits. This is important for the development of diagnostic, preventive and therapeutic strategies. MCI may develop to AD and other severe forms of dementia and MRI provide evidence of the etiologies of the various dementia types (12, 35). As such atrophy of the medial temporal lobe seems to be a more important predictor in MCI than small-vessel lesions (66). In the present study, none of the low performers with indications of hippocampal atrophy had evidence of vascular problems, suggesting similar etiology of the progression of MCI-type cognitive impairment in humans and cynomolgus monkeys.

Based on the MRI criteria applied to the subjects of the present study, none of the high-performing subjects had lesions associated with dementia in other studies, as described in an MRI study of rhesus monkeys (67). As in humans, gray matter volume decreases with age in chimpanzees and rhesus monkeys, as described by Chen *et al*., who found that the volume of gray matter and white matter in the forebrain area was decreased up to 5% in aged monkeys (68). Koo *et al*. also reported that the thickness in several cortical areas changed with aging (69). The losses in the volumes of the white and grey matter forebrain did not seem to correlate with the cognitive decline in a study by Wisco and co-workers (70).

However, structural changes, which were related to aging and cognitive decline in rhesus monkeys, were described by Alexander *et al*. as a loss of gray matter in several cortical areas, such as the prefrontal cortex, portions of the temporal cortex and the visual cortex (71). The losses were found to correlate with declines in working memory, as measured by delayed response performance. Age-related reductions in the volume of the dorsal prefrontal and anterior cortices have

also been related to behavioral changes in a study by Shamy and co-workers (72). Memory decline by aging was found to be mainly caused by degenerative and reparative changes of the myelin (67). The degenerative changes related to de-myelination of axons and loss of synapses, while the reparative changes related to re-myelination leading to shorter internode formation and the accompanying increase of paranodes and oligodendrocytes.

In conclusion, aged cynomolgus monkeys with poor DRT performance were found to have low levels of $A\beta_{42}$ and high levels of t-tau in their CSF. In addition, their brains exhibited structural changes comparable to those seen in human patients suffering from age-related dementia, implying that aged cynomolgus monkeys suffer from spontaneous age-related neurodegenerative disease.

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