Multimodal analyses of a non-human primate model harboring mutant amyloid precursor protein transgenes driven by the human EF1α promoter.

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A B S T R A C T

Alzheimer’s disease (AD) is the leading cause of dementia which afflicts tens of millions of people worldwide. Despite many scientific progresses to dissect the AD’s molecular basis from studies on various mouse models, it has been suffered from evolutionary species differences. Here, we report generation of a non-human primate (NHP), common marmoset model ubiquitously expressing Amyloid-beta precursor protein (APP) transgenes with the Swedish (KM670/671NL) and Indiana (V717F) mutations. The transgene integration of generated two transgenic marmosets (TG1&TG2) was thoroughly investigated by genomic PCR, whole-genome sequencing, and fluorescence in situ hybridization. By reprogramming, we confirmed the validity of transgene expression in induced neurons in vitro. Moreover, we discovered structural changes in specific brain regions of transgenic marmosets (TG1, 7 years old, although β-plaque-like structures in TG1 brain at 7 years old, although β-plaque-like structures in TG1 brain at 7 years old, although β-plaque-like structures in TG1 brain at 7 years old, although β-plaque-like structures in TG1 brain at 7 years old, although β-plaque-like structures in TG1 brain at 7 years old, although β-plaque-like structures in TG1 brain at 7 years old, although β-plaque-like structures in TG1 brain at 7 years old, although β-plaque-like structures in TG1 brain at 7 years old, although β-plaque-like structures in TG1 brain at 7 years old, although β-plaque-like structures in TG1 brain at 7 years old. Thus, it may be beneficial for drug development and further disease modeling by combination with other genetically engineered models and disease-inducing approaches.

1. Introduction

Alzheimer’s disease (AD) is a progressive, intractable, and devastating neurodegenerative disorder which is the most common cause of dementia in elderly patients (Barker et al., 2002). The main pathological hallmarks of AD were first described by Alois Alzheimer in 1907, causing the formation of senile plaques and neurofibrillary tangles.
1906 (Hippius and Neundorfer, 2003): extracellular amyloid-β (Aβ) plaques plaques called senile plaques, neurofibrillary tangles mainly consisted of misfolded and hyper-phosphorylated microtubule associated protein tau (MAPT), and eventually neuronal loss. At present, there is no available treatment to prevent the neuronal loss posed by AD, in spite of the existence of Food and Drug Administration (FDA)-approved six medications (Rivastigmine, Galantamine, Donepezil, Memantine, Memantine combined with Donepezil, and Tacrine) for providing symptomatic relief (Ali et al., 2019; Barker et al., 2002). More recently, FDA has made an accelerated approval to the anti-amyloid monoclonal human IgG1 antibody aducanumab (Mullard, 2021), but the long-term efficacy of the antibody-based therapy is yet under debate.

Mutations in three genes, including Amyloid precursor protein (APP), Presenilin-1 (PSEN1) and Presenilin-2 (PSEN2) genes, are responsible for early-onset familial AD (Guerreiro et al., 2012). Although significant efforts have been made with the generation and characterization of a variety of AD mouse models expressing their mutant form, including transgenic mice with mutant APP, PSEN1, and/or MAPT genes (Drummond and Winanskiw, 2017), and mutant App knock-in ones (Saito et al., 2014), these models have been suffered from evolutionary differences between mice and humans. For example, differences in the anatomical cerebral structure may be critical for the elucidation of the AD pathological progression: tau lesion initially occurs from the hippocampus and entorhinal cortex. In particular, the endogenous murine Ap reported seem difficult to aggregate by itself (Saito et al., 2014). Moreover, a relatively short life span in mice (approximately two years) may make it difficult to detect late-onset intravital phenotypes.

The common marmoset (marmoset; Callithrix jacchus), a small non-human primate species, harbors various features advantageous for biomedical and preclinical research, owing to its physiological and anatomical similarities to those of humans (Izpisua Belmonte et al., 2012; Kishi et al., 2014; Okano et al., 2012; Okano, 2021). In addition, the comparatively long lifespan of marmosets (10–15 years) may facilitate studies on late-onset diseases using the animal model, while the values of long-lifespan animals for the investigations of late-onset diseases have not yet been proven. Recently, we and other groups developed the technology of production of gene-modified marmosets by lentiviral transduction or genome editing technology, which is advantageous for disease modeling and evolutionary studies (Heide et al., 2020; Murayama et al., 2020; Park et al., 2016; Sasaki et al., 2009; Sato et al., 2016). Developmental experiments were summarized in Table 1.

CSF was collected by suboccipital (WT marmosets) or lumbar (TG1 marmoset) puncture using a 27 G butterfly needle under anesthesia by intramuscular injection of 30–50 mg/kg ketamine and 2.4-4 mg/kg xylazine. We could not perform CSF collection by suboccipital puncture for TG1 marmoset due to the regional edema of the animal, which resulted from currently unknown reasons. The levels of neurofilament light chain (NFL) in CSF were quantified using R-PLEX Neurofilament L Antibody Set (F217X-3; Mesa Scale Discovery) as previously described (Kasuga et al., 2022).

Positive control mice of AD-related pathology used in immunohistological analyses were male and 18 months old. One APP23 mouse and one Tau P301L mouse were used.

2.2. Cell culture of ESCs/iPSCs and iN cells

A marmoset WT ESC line, No. 40 (CMES40) (Sasaki et al., 2005) was used in the present study. In addition, an iPSC line from TG2 was established as described previously (Nakajima et al., 2019; Yoshimatsu et al., 2021). These cells were cultured as described previously (Yoshimatsu et al., 2019). In brief, ESCs/iPSCs were cultured on 3Gy-irradiated mouse embryonic fibroblasts (MEFs) in ES medium (ESM) consisting of 1x KnockOut DMEM (Thermo Fisher) supplemented with 20% KnockOut Serum Replacement (Thermo Fisher), 1mL-glutamine (Nakalai Tesque), 1% non-essential amino acids (Sigma), 0.2mM 2-mercaptoethanol (Sigma) and 10ng/mL fibroblast growth factor 2 (Peprotech). For high-efficient differentiation of neuronal cells from the marmoset ESCs/iPSCs, we used the NEUROG2-overexpression system as described previously (Yoshimatsu et al., 2019). The transgenic ESC/iPSC lines generated in the current study will be distributed by the corresponding authors upon request.

2.3. Genomic analyses

Genotyping PCR and Southern blotting were performed as described previously (Yoshimatsu et al., 2019). For PCR detection of hKO, two primer sets as following: hKO-1 (gagagatccccgactacttcaagagcttcagg) and hKO-2 (gagagatccccgactacttcaagagcttcagg), 146 bp amplicon were used. For Southern blotting, we used EcoRI (Takara Bio) for genomic digestion, and the entire oocyte donors and surrogate mothers. The surrogate mothers were maintained with vasectomized adult male marmosets. WT male marmosets older than 1.5-years old were used as sperm donors. These marmosets were purchased from CLEA Japan, Inc. (Tokyo, Japan) and kept in CIEA.

Oocyte and sperm collection, and in vitro fertilization (IVF) was performed as previously described. In brief, for oocyte collection, the oocyte donors whose plasma progesterone levels were monitored were intramuscularly injected with recombinant human follicle-stimulating hormone (FSH, 25 IU; Fuji Pharma) for 9 days, followed by the intramuscular injection of human chorionic gonadotropin (hCG, 75 IU; ASKA Pharmaceutical) on day 10. On day 11 (16–20 h after the hCG injection), the hormone-treated female marmosets were pre-anesthetized with 0.04 mg/kg medetomidine (Nippon Zenyaku), 0.40 mg/kg butorphanol (Meiji Seika Pharma) and 0.40 mg/kg midazolam (Astellas Pharma). The oocytes were surgically collected from the anesthetized animals by inhalational anesthesia with isoflurane (Sumitomo Dainippon Pharma) during the operation. The collected oocytes were incubated in Porcine Oocyte Medium (Research Institute for the Functional Peptides) at 38°C. For IVF, sperms were collected from healthy male marmosets. For insemination, each oocyte was incubated with 3.6 × 10⁴ sperms in a drop of TYH medium (LSI Medience) for 16 h at 38°C. Lentiviral transduction for pronuclear stage embryos using pLent6-EF-APP (sw/ind)–2AKO (Supplementary sequence), followed by embryo transfer to surrogate mothers was performed as described previously (Sasaki et al., 2009a, 2009b; Sato et al., 2016). Developmental experiments were summarized in Table 1.

No. H27–306(4)), CIEA (approval No. 11028, 14029, 15020, 16019, 17029, 18031, 19033, 20049). Animal care was conducted in accordance with the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals (2011).
Fig. 1. Generation of APP-transgenic marmosets. (A) Graphical schematic of the lentiviral vector. Truncated RSV promoter (P_{RSV}), 5′ long terminal repeat (LTR), packaging signal (φ), RRE, cPPT and ΔU3/3′LTR were lentiviral sequences for transduction (Miyoshi et al., 1998). Woodchuck Hepatitis Virus posttranscriptional regulatory element (WPRE) was inserted into the downstream of the transgene for enhancement of lentiviral titer and transgene expression. (B) Phase contrast (Phase) and orange fluorescence images of the early-stage embryos with lentiviral transduction (gray-circled) and without. Scale bars, 100 µm. (C) Macroscopic images of neonatal APP-TG marmosets. (D) Genomic PCR analysis of APP-TG marmosets. Used primers are described in Materials and Methods. (E) Southern blotting of APP-TG marmosets. EcoRI was used for genomic digestion, and the entire hKO sequence as the DNA probe. (F-G) Transgene integration sites and quantification of transgene-mapped reads in WGS analysis. (H) RT-PCR analysis of the transgene expression in APP-TG-derived fibroblasts.
sequence of hKO (657 bp) was as the DNA probe (digoxigenin labeled). For WGS, we performed DNaseq (2x150bp configuration). NGS library preparation and sequencing were performed by GENEWIZ Japan (Saitama, Japan). As a reference, we combined the sequence of pLenti6-FF-AP(sw/ind)-2AKO (Supplementary sequence) with cj1700 marmoset genome assembly (https://www.ncbi.nlm.nih.gov/assembly/GCF_009663435.1/), and used bowtie2 to make a mapping index and mapping of respective reads on the reference sequence with the lentiviral transgene sequence. We deposited the raw and processed WGS data in NCBI GEO (accession: GSE194005).

Fluorescence in situ hybridization (FISH) analysis was performed with technical support from Chromosome Science Labo (Hokkaido, Japan). In brief, we supplemented thymidine (300 µg/ml) with technical support from Chromosome Science Labo (Hokkaido, Japan). In brief, we supplemented thymidine (300 µg/ml) with technical support from Chromosome Science Labo (Hokkaido, Japan).

Table 1

| Virus-injectedembryos | Developed to |
|------------------------|-------------|
|                        | 2-cell | 4-cell | 8-cell | Morula | Blasto-syst | ET (recipient) | Birth |
| APP(UUC)+MPT (1:10)    | 10     | 10     | 9      | 4      | 4           | 3(2)           | 0     |
| APP(UUC)+MPT (1:1)     | 6      | 4      | 4      | 2      | 0           | 0              | 0     |
| APP(UUC)               | 3      | 3      | 3      | 3      | 2           | 2(1)           | 0     |
| MAPT                   | 3      | 3      | 3      | 3      | 0           | 0              | 0     |
| APP(EF1α)              | 65     | 61     | 59     | 52(ET-4) | 28(ET-4) | 2(ET-1) | 9 (7) | 2     |

For immunohistochemistry, we used a total of 10 µm thickness and subsequent immunostaining following deparaffinization. We used following primary antibodies: anti-β-Amyloid 1–16 antibody clone 6E10 (BioLegend, #803001, 1 mg/ml) at a 1:250 dilution, and Phospho-Tau (Ser202, Thr205) Monoclonal Antibody (AT8) (Thermo Fisher Scientific, #MN1020) at a 1:1000 dilution. In addition, Biotinylated anti-Mouse IgG (Jackson ImmunoResearch, #115–065–146) at a 1:250 dilution was used as a secondary antibody. Following primary and secondary immunostaining, sections were treated with signal development by ABC Peroxidase Standard Staining Kit (Thermo Fisher Scientific, #32020) and ImmPACT® DAB Substrate, Peroxidase (HRP) (Vector Laboratories, #SK-4105) according to manufacturers’ instructions, followed by counter-staining with hematoxylin and mounting with MOUNT-QUICK (Daigo Sangyo, #DM01).

2.4. ELISAs for Aβs

Marmoset ES/iPSC-derived neurons were maintained until analysis (day 12) in 12-well plates. Medium was fully changed with 500 µl/well of fresh medium 48 h before the harvest. The collected medium was centrifuged to remove cellular debris and stored at −80°C until analysis. The remaining neuronal cells were lysed in RIPA buffer and protein concentration was measured by BCA Protein assay (Pierce).

For marmoset endogenous and human Aβ ELISA (Aβ sequence is identical between marmoset and human), adult marmoset cortices were homogenized with 5 volumes of TBS and centrifuged at 175,000 x g for 30 min. The supernatants were used as soluble fractions for Aβ measurement. The pellet was washed with TBS and resuspended in an equal volume of 5 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0 and rotated at room temperature overnight. The GuHCl suspensions were used as insoluble fraction and diluted by 1% blocking solution (MesoScale Discovery, MSD) for Aβ measurement. Aβ40 and Aβ42 levels in the cortical lysates were measured using commercial kits, Human βAmyloid (1–40) ELISA kit II (Wako, #298-64601) and Human βAmyloid (1–42) ELISA Kit High Sensitive (Wako, #296-64401), respectively, according to the manufacturer’s protocol. Each Aβ concentration was normalized by weight of the cortices.

2.5. Postmortem analyses

For immunohistochemistry, we fixed a half of the brain samples with a 4% paraformaldehyde solution in PBS followed by paraffin-embedding, coronal sectioning (10 µm thickness) and subsequent immunostaining following deparaffinization. We used following primary antibodies: anti-

2.6. Statistical analysis

Except MRI, PET and RNA-seq analyses, all data are expressed as mean ± s.e.m. Differences between means were compared using the Student’s t-test. Differences were considered significant at p < 0.05.

2.7. Longitudinal analysis of APP-transgenic marmosets

We performed longitudinal structural MRI for evaluating APP-transgenic marmosets. From the age of 3 years old, MRI was conducted 3 times per year in total. To evaluate whether any differences existed as aged, we also acquired data from 12 control age-matched marmosets from the age of 3 years old per year. Intramuscular injection of 0.1 mg/kg of atropine sulfate (Mitsubishi Tanabe Pharma Corporation, Osaka, Japan), and 12 mg/kg of alfaxalone (Jurox Pty. Ltd., Rutherford, NSW, Australia) were administered before the MRI experiment. After performing intratracheal intubation, the anesthesia state was maintained using administration of mixture of oxygen, nitrogen, and 1.0–2.0% isoflurane (Pfizer, NY, USA) via an artificial respirator, SN-580–7 (Shinano, Tokyo Japan).

All data were obtained on 7.0 T Biospec 70/16 scanner system (Bruker BioSpin GmbH; Ettingen, Germany) equipped with actively shielded gradients at a maximum strength of 700 mT/m, using a bespoke 4-channel phased array surface coil developed for acquisition of a marmoset head data (Takashima Seisakujo Ltd., Tokyo, Japan) as the receiver coil, and a conventional linear polarized birdcage resonator with inner diameter 72 mm (Bruker BioSpin) as the transmit coil.

MT images were acquired with following parameters: Fast Low Angle Shot with/without an off-resonance frequency set bandwidth (pulse strength 7.0 µm, number of pulse = 16, bandwidth: 300 Hz, off-set frequency: 1500 Hz) repetition time/echo time: 2600 ms/ 2.86 ms, flip angle 70°, image matrix 128 x 128 x 54, in plane resolution 0.39 mm x 0.39 mm and slice thickness 0.6 mm).
2.8. Statistical analysis for structural MRI data

MTR maps were calculated using the formula: MTR = (Mss-M0)/M0, where Mss and M0 are the signal intensities obtained with and without the signal MT saturation. MTR maps were classified into gray matter, white matter, and cerebrospinal fluid. The spatially normalized segmented data based on the stereotaxic coordinate (Hikishima et al., 2011) were registered and normalized across the individual differences (Ashburner and Friston, 2000). These data were smoothed by convolving with an isotropic Gaussian kernel of full width at half maximum eight times the voxel size.

To account for the longitudinal nature of this study, we used a two-way repeated-measures analysis of variance (ANOVA) statistical model (i.e., flexible factorial design). To show the differences between TGs and controls x 3 Times (3 years old, 4 years old, and 5 years old) with Group and Time specified fixed factors. Family Wise Error correction for multiple comparisons at cluster level was applied and regarded as significant a p value of less than 0.05. All procedures described to conduct voxel-based analysis was performed using the SPM12, by using MATLAB 2016a (MathWorks, Natick, MA, USA). Detected significant different regions were identified by use of the marmoset brain atlas for MRI analysis was performed using the SPM12, by using MATLAB 2016a (MathWorks, Natick, MA, USA). Extracted significant clusters detected by VBA and measured mean MTR values were compared with the growth trajectories delineated with the mean value of control data.

3. Resting state fMRI in awake states

Awake fMRI was performed for two marmosets (TG1, 7.74 years old, and the control male; 7.12 years old) in this study. We performed surgery on each subject to attach the underlying components for a headpost to the center of their skull. Training was conducted to acclimate the subjects to the experimental environment, and finally the subjects scored 1 or 2 on the behavior rating scale (Silva et al., 2015) in the experimental environment.

3.1. Data acquisition of fMRI

All scans were performed using a 9.4 T MRI Scanner (Bruker, BioSpec94/30) and ParaVision 6.0.1 (Bruker, Billerica, USA) with a gradient set capable of 660 mT/m. For transmission, the quadrature detection coil (inner diameter = 154 mm) was used as the volume coil. We used an 8-channel phase-array receiver coil for the common marmoset (Takashima seisakusho Co., Ltd, Japan). TG1 was scanned 4 times in an experiment, 170 min in total. The control subject was scanned 3 times in an experiment, in total 180 min. The resting-state fMRI (rsfMRI) were acquired using gradient-echo EPI sequence (TR = 2000 ms, TE = 16 ms, flip angle = 65°, FOV = 42.0 × 28.0 mm, matrix size = 60 × 40, 52 axial slices, resolution = 0.7 mm isotropic, number of averages = 1, 155 time points, duration = 5 min 10 s). T2 weighted Images were acquired with following parameters (TR = 4331 ms, TE = 15 ms, flip angle = 90°, FOV = 42.0 × 28.0 mm, matrix size = 120 × 80, 52 coronal slices, resolution = 0.35 × 0.35 × 0.7 mm, number of averages = 1). To perform TOPUP, two sets of spin-echo EPI sequence with an opposite phase-encoding direction (Head-Foot and Foot-Head) were scanned (TR = 5000 ms, TE = 22 ms, flip angle = 90°, FOV = 42.0 × 28.0 mm, matrix size = 60 × 40, 52 coronal slices, resolution = 0.7 mm isotropic, number of averages = 1, 6 time points, opposite phase-encoding direction).

During the MRI scan, a measuring device was attached to the subject’s calf, and SpO2 and heart rate were constantly monitored. These values were recorded every 10 min. To prevent a drop in body temperature, a tube with warm water running through it was wrapped in a cloth and placed on the subject’s abdomen during the MRI scan. In addition, an infrared camera was placed in front of the subject’s face to observe the subject constantly during the MRI scan.

3.2. MR image preprocssing

TOPUP tool in the FMRIB Software Library (FSL) was used to reduce the distortions in rsfMRI data (Andersson et al., 2003; Jenkinson et al., 2012; Smith et al., 2004). To make this correction more accurate, the voxel size was changed to 3.5 mm isotropic, which is equivalent to human using the Statistical Parametric Mapping 12 tool (SPM12) (Friston et al., 2007). Second, realignment, slice timing correction, normalization, and smoothing were performed using SPM12. The smoothing process was performed with a setting of FWHM = 2 voxels. Third, denoising and frequency filtering were performed using CONN (Whitfield-Gabrieli and Nieto-Castanon, 2012). In this case, denoising refers to the removal of covariates such as white matter (WM) and cerebrospinal fluid (CSF) signals. For frequency filtering, we used a frequency band of 0.01–0.10 Hz. After those image preprocessing, for TG1 and control subject, the FC between brain regions (Hashikawa et al., 2015; Woodward et al., 2018) were calculated from the data of each day, and then the average FC matrix was calculated. In addition, the FC matrix between the regions comprising the default mode network (DMN) (Liu et al., 2019) was extracted and compared between TG1 and control subject.

3.3. PET measurements

TG1 and TG2 were used at the age of 3.5 years 4.5 years twice. Two healthy female marmosets were also used as control at the age of 6 years. PET imaging was performed with a microPET FOCUS 220 system (Siemens Medical Solutions USA, Knoxville, TN, USA). The field of view (FOV) is a 190 mm diameter × 76 mm height, and the spatial resolution is 1.3 mm full width at half maximum at the center of FOV.

All marmosets were anesthetized with isoflurane (1–3%, to effect) throughout the PET experiment. Before radioligand injection, a transmission scan was conducted for about 20 min with a spiraling 68Ge point source to correct attenuation. List mode data were obtained for 90 min immediately after a single bolus administration of [11 C]PiB (97.1–132.3 MBq) via a vein in the hind leg. [11 C]PiB was produced by the previously described method (Maeda et al., 2007, https://doi.org/10.1523/JNEUROSCI.0673–07.2007). All list-mode data were sorted into three-dimensional sinograms, which were then Fourier-rebinned two-dimensional sinograms (frames × minutes; 5 × 1, 5 × 2, 5 × 3, 12 × 5). PET images were reconstructed with filtered back-projection using a Hanning filter cut-off at the Nyquist frequency (0.5 mm-1).

3.4. PET data analysis

All PET data analysis was performed with PMOD image analysis software (PMOD Technologies Ltd, Zurich, Switzerland). PET images were co-registered and superimposed to the individual MR image obtained with a 7 T-MRI scanner (BioSpec AVANCE-III, 200 mm-bore, Bruker, BioSpin, Rheinetten, Germany). T2-weighted MR images were obtained using a 3D rapid acquisition enhancement sequence according to the following parameters: repetition time = 2500 ms; echo time = 8.93 ms; field of view (FOV) = 26 × 35 × 25.6 mm3, and matrix size = 128 × 128 × 64. The regions of interest (ROIs) delineated on the brainstem, cerebellum, thalamus, frontal cortex, occipital cortex, and hippocampus based on the individual MR images. Radioactivity in each ROI was calculated for each frame and plotted against time. Non-invasive Logan graphical analysis (Logan et al., 1996) using the brain stem as a reference was applied to estimate DVRs and create the DVR images.

4. Results

4.1. Generation of APP-transgenic marmosets

To establish a transgenic non-human primate model of AD, we...
exploited the lentiviral transduction technology for marmoset early-stage embryos (Sasaki et al., 2009a and 2009b). As the lentivirus vector, we placed coding sequences of human APP (the predominant neuronal isoform APP695) with the Swedish (KM670/671NL in APP770, K595N/M596I in APP695) and Indiana (V717F in APP770, V642F in APP695) mutations (Fig. 1A; named APPsw/ind) which change β- and γ-cleavage susceptibilities of the APP protein, respectively (Haas et al., 1995; Murrell et al., 2000). Moreover, to visualize transgene-integrated embryos following lentiviral transduction, we decided to use the ubiquitous promoter of the human polypeptide chain elongation factor 1a gene (EF1a) (Mizushima and Nagata, 1990) and the orange fluorescent protein gene hKO whose coding sequence was linked with APPsw/ind via a self-cleaving 2A peptide sequence (2A) (Fig. 1A).

As expected, the lentivirus-transduced embryos at 8–16 cell stage showed orange fluorescence compared to other control (non-injected) ones (Fig. 1B). Following the lentiviral vector injection into 65 marmoset embryos, 9 embryos were transferred to 7 surrogate mothers’ uterus (Table 1). As the result, we obtained two live marmosets as littersmates (Fig. 1C; named APP-TG1 and TG2). There were no evident malformations and abnormal behaviors in these monkeys.

4.2. Genomic analyses of APP-transgenic marmosets

To investigate the transgene integration of TG1 and TG2, we performed genomic PCR (gPCR) analysis using specific primer pairs for hKO (hKO-1 and hKO-2). Using genomic DNA extracted from the placenta and respective hair roots of TG1 and TG2, we detected PCR bands showing transgene integration in the genome (Fig. 1D), while no bands were detected in the negative control (water) and genomic DNA from wild-type (WT) marmoset sample. Moreover, by Southern blotting using a DNA probe specific for the hKO sequence and genomic DNA extracted from ear-biopsy-derived fibroblasts of TG1 and TG2, we detected six bands in TG1, and four bands in TG2 (Fig. 1E). It is noteworthy that the four bands in TG2 showed an almost similar pattern to those in TG1. These results were further reproduced by FISH analysis (Fig. S1A-B; five fibroblast cells from each marmoset were used), the four transgene integration sites (two on Chromosome 3, one on Chromosome 4, 7 and 10) in TG2 were matched to those in TG1.

To further scrutinize the transgene integration sites in the genome of the transgenic marmosets in base-pair resolution, we performed whole-genome sequencing (WGS) analysis using the fibroblast-derived genomic DNA used in the Southern blotting analysis (Fig. 1E). As a result, we detected the very same transgene integration sites in both the transgenic marmosets, such as two sites on Chromosome 3 and one site each on Chromosome 4, 7, 10 and 17 (Fig. 1F). Considering the random nature of lentiviral transgenesis in general (Miyoshi et al., 1998a and 1998b), we inferred that this phenomenon was caused by the littermate-chimerism by placenta sharing (Benirschke and Brownhill, 1962; Gengozian et al., 1969) or these transgenic marmosets were identical twins. To investigate the two possibilities, we performed microsatellite analysis for ten markers (Takahashi et al., 2014). We found all the markers completely matched (Fig. S2), which strongly suggests that the two transgenic marmosets were identical twins. Moreover, in the WGS analysis, we quantified the percentage of reads mapped to the transgene sequence (0.000478% in TG1 and 0.000154% in TG2; Fig. 1G). We note that the discrepancy of the results between FISH and WGS analyses (one of the transgene sites was on Chromosome 16 (in FISH) or 17 (in WGS)) may be caused by the incomplete assembly of the currently-available genomic reference (cJ700) of the marmoset. These data suggested that the TG1 and TG2 marmosets were identical twins – but the mosaicism of transgenic cells differed between the two animals, TG1 harbored more (approximately 3 folds) transgenic cells than that of TG2.

4.3. Validation of the APP transgenes

To assess the transgene expression, we performed reverse-transcription PCR (RT-PCR) analysis using the hKO-1 primer set and total RNA extracted from ear-biopsy-derived fibroblasts of TG1 and TG2. As a result, the fibroblasts showed the transgene expression (Fig. 1H).

To further investigate the transgene expression in neuronal cells in vitro, we exploited the reprogramming technology of induced pluripotent stem cells (iPSCs) (Nakajima et al., 2019; Yoshimatsu et al., 2021). We obtained one iPSC clone from the TG2 marmoset, and the iPSCs showed the transgene expression similar to that of TG2 fibroblasts (Fig. S3A). Moreover, using the highly efficient (~ 99%) neuronal induction method with WT ESCs and TG2 iPSCs by NEUROG2 overexpression (Yoshimatsu et al., 2019), we found ~10% TG2 iPSC-derived neuronal cells showed orange fluorescence (Fig. S3B), while we could not detect any orange fluorescence in original TG1 and TG2 fibroblasts and iPSCs (data not shown). Furthermore, the increase of the mutant APP expression was further confirmed by enzyme-linked immunosorbent assay (ELISA) of Aβ40 (Fig. S3C), one of the major processed forms of the APP protein by β- and γ-secretases.

4.4. Longitudinal analysis of APP-transgenic marmosets

To explore whether there were any longitudinal structural differences between APP-transgenic marmosets and controls, we conducted voxel-based analysis (VBA) with Magnetic Transfer Ratio (MTR) images (Tambasco et al., 2011). There were no significant either gray matter or white matter change due to normal aging (data not shown). On the other hand, MTR value was significantly higher at the right side of the hippocampus in APP-transgenic marmosets (N = 2) compared with age-matched control marmosets (N = 12) (p < 0.05, family wise error corrected), whereas MTR values were significantly lower at the left side of the caudate nucleus in APP-transgenic marmosets (N = 2) compared with age-matched control marmosets (N = 12) (p < 0.05, family wise error corrected). Also, MTR values were significantly lower at the splenium of corpus callosum in the two APP-transgenic marmosets in comparison with age-matched control marmosets (N = 12) (p < 0.05, family wise error corrected) (Fig. 2A). We extracted significant clusters detected by VBA and measured mean MTR values, which were shown in Fig. 2B. Rather than MTR values being gradually changed as APP-transgenic marmosets got aged, significant differences have been already observed at the age of 36 months, and slight changes were only observed thereafter.

4.5. PET analysis of APP-transgenic marmosets

In addition to the MTR analysis described above, we performed PET analysis to investigate the evident region-specific changes of the TG brains by another methodological approach. Distribution volume ratios (DVRs) were compared between TGs and controls by PET analysis (Fig. 3). Among 5 regions of interest (ROIs) such as the cerebellum, thalamus, frontal cortex, occipital cortex, and hippocampus, the largest difference was observed in the cerebellum between controls and TG1 (Fig. 3A-B). There was an approximately 12% decrease in the DVRs compared with the average DVRs in controls (Fig. 3B).

4.6. Awake functional MRI analysis of the TG1 and WT marmosets

Functional connectivity (FC) was compared between TG1 and the control (Fig. 4A). FC between left and right dorsolateral prefrontal cortex within default mode network in TG1 was up to approximately 0.37 lower than that in control (Fig. 4B-C). In TG1, FC between dorsolateral prefrontal cortex (dPFC) regions in the right hemisphere was approximately 0.46 lower than that in control. In addition, FC in the intraparietal cortex (lateral intraparietal area of cortex; medial intraparietal area of cortex, and ventral intraparietal area of cortex), which
was major part of the posterior parietal cortex, was up to approximately 0.36 higher in control than in TG1 (Fig. 4 B-C).

4.7. Neurofilament light (NfL) concentration in cerebrospinal fluid (CSF) of TG1 and WT marmosets

It is noteworthy that CSF NfL levels were elevated in AD patients compared to those of healthy controls (Dhiman et al., 2020). From a translational perspective, we assessed whether such phenotype can also be observed in our TG and WT marmosets. We collected CSF and measured the NfL concentration from ten WT marmoset at 3–11 years old, then compared it with that of TG1 marmoset at 7 years old (before sacrifice). As summarized in Fig. S4A, we revealed that the CSF NfL level was elevated in TG1, which may result from the increased Aβ plaque-like structures (Fig. 5, described in the following section). We note that the collection methods vary between WT and TG1 marmosets, which may have affected the result. In addition, we calculated the Pearson correlation coefficient between CSF NfL and age, and the value was 0.255 in the WT marmosets (Fig. S4B).

4.8. Postmortem analysis of APP-transgenic marmosets

Because of veterinarian humane endpoint of the TG1 marmoset, euthanasia was performed at the age of 7 years. Thereafter, we investigated the AD pathology of the TG1. The TG2 marmoset was found suddenly dead after ~6 h of death which might be resulted from diffuse alveolar disorder and bacterial peritonitis (Supplementary discussion) at the same age to the endpoint of TG1. Two age-matched WT marmosets (WT1–2) were used as the controls. First, to quantify Aβ plaque-like structures in the TG1–2 and WT1–2 brains, we calculated the area positive for Aβ staining in the brain section (µm²) used for immunohistochemistry (Fig. 5A). As a result, we found increased Aβ plaque-like structures (approximately two folds) in the TG1 brain (Fig. 5B-C), although the plaque size in the marmoset brains was smaller than those in APP23 transgenic mice (~1/3; Fig. 5C), which were used as a model exhibiting obvious Aβ plaques. However, Aβ plaque-like structures were not increased in the TG2 brain. As described in the genomic analyses (Fig. 1), although TG1 and TG2 were identical twins, TG2 harbored less transgenic cells than those of TG1. Therefore, the overexpression of APP transgene in TG2 was not sufficient to induce increased Aβ plaque-like structures in the brain. Using TauP301L transgenic mice as a positive control, we next assessed tau pathology using a phosphorylated tau-specific antibody, astrogliosis, and microglia, but could not find either evident tau pathology (Fig. S5A) and glial inflammation and microglial pathology (data not shown).

To assess the transgene expression in the TG brains, we performed RNA-seq using bulk RNA derived from the prefrontal cortex of TG1–2 and two age-matched WT marmosets (WT1–2). We quantified reads mapped on the transgene sequence, and Transcripts per kilobase million (TPM) of the APP transgene was 1.01055 in the TG1 and 0.743107 in TG2, while TPM was 0 in WTs. We also explored differential expressed genes (DEGs) between TG and WT. Among top 20 DEGs (named genes), the expression of PDYN, C2CD4C and FYB2 was significantly decreased in WT, while that of TVP23B, GPA33, SCN4B and SCN1B was significantly decreased in TG (Supplementary Data 1), which may show the change in neuronal/synaptic functions by the transgene.

Moreover, using BaseSpace Correlation Engine (Illumina) (Supplementary Data 1–7), we found “inflammatory response” is the leading
change of gene ontology (GO) by using the DEGs (Supplementary Data 2). Also, by referring to a published method for interspecies transcriptomic analysis (Nakajima et al., 2021) and comparing published human AD patients’ data vs healthy control with our DEGs, we found the most overlap with a study on transcriptome of choroid plexuses of AD patients and controls (Stopa et al., 2018). Despite the difference in brain regions for obtaining RNA-seq data, 194 DEGs (significantly up/up, \( p \) value = 2.2E-36) in our data were matched with the published data (GSE110226) (Supplementary Data 5).

We also performed ELISA using fresh-frozen brain hemisphere
Fig. 4. Awake fMRI analysis. (A) The panels showed the FC matrix of all brain regions of Control and TG1 and their differences (Control – TG1). (B) The panels showed the FC matrix of Control and TG1 and their differences (Control – TG1) in the component regions of the DMN. (C) The panels showed the 3D view of the FCs of Control, TG1, and their differences (Control – TG1) in the component regions of the DMN. For the 3D view panel of difference (Control – TG1), only the parts where the difference was 0.2 or higher were displayed. The components regions of the DMN were following: A8aD, Area 8a of cortex (dorsal part); A6DR, Area 6 of cortex (dorsorostral part); PGM, Parietal area PG (medial part); A23a, Area 23a of cortex; A23b, Area 23b of cortex; A23c, Area 23c of cortex; A23V, Area 23 of cortex (ventral part); A29a-c, Area 29a-c of cortex; A29d, Area 29d of cortex; A30, Area 30 of cortex; AIP, Anterior intraparietal area of cortex; LIP, Lateral intraparietal area of cortex; MIP, Medial intraparietal area of cortex; VIP, Ventral intraparietal area of cortex.
samples of the TG1–2 and WT1–2 marmosets. Still, we could not detect an evident increase in Aβ40 and Aβ42 secretion, and Aβ42/40 ratios (Fig. S5B), which may be resulted from weak transgene expression by the EF1α promoter that may be highly silenced in the marmoset brains at the age of 7 years. In line with this, APP was not increased in TG-derived fibroblasts by western blotting (Fig. S5C).

5. Discussion

In the present study, we performed multimodal analyses of two APP-transgenic marmosets. Although we utilized the human EF1α promoter which reportedly enables ubiquitous expression in mammalian cells (Mizushima and Nagata, 1990), especially in a pluripotent (undifferentiated) state (Chen et al., 2011; Seita et al., 2019), we found the
transgene expression was very marginal in the brain of adult (assessed at 7 years old) transgenic marmosets. In previous studies using macaque monkeys, weak transgene fluorescence driven by the EF1α promoter was observed in the fetal and adult tissues of EF1α transgenic monkeys (Chan et al., 2001; Seita et al., 2019; Wolfgang et al., 2001), compared to CAG promoter (Seita et al., 2019). While comparative analysis of EF1α and CAG promoters in marmosets has not been performed so far (only between CMV and CAG promoters (Sasaki et al., 2009a and 2009b)), we infer that the EF1α is not the “best” choice for transgene expression in the species. Moreover, as described in Luo et al. that the stable EF1α promoter-driven transgene expression was achieved by knock-in of the transgene into the safe harbor locus (AAVS1) in human iPSCs (Luo et al., 2014), the positional effect of transgene integration may be also important for stable transgene expression. Because AAVS1 is well-conserved in primates, therefore approaches for knock-in in the gene locus would be advantageous for ubiquitous and strong transgene expression in marmosets.

Seita et al. recently reported the generation of transgenic macaque monkeys harboring CAG promoter-driven APP transgenes with Swedish, Artic (E618G in APP695; E693G in APP770) and Iberian mutation (I641F) (Seita et al., 2020), whose high aggregation activity was validated. The CAG promoter in the transgene showed a drastic elevation of the APP expression, Aβ secretion and Aβ plaque formation even in the brains of the transgenic fetuses (Seita et al., 2020), further longitudinal analysis using live monkeys until adult stage is necessary for the evaluation of the animal model for drug development. In the study, although the APP transgene expression driven by the EF1α promoter might be marginal and further characterization is required, the 7-year-old transgenic marmoset TG1 showed increased Aβ plaque-like structures in the brain (Fig. 5). This may be consistent with the increased AD risk in Down’s syndrome patients – the long-term marginal increase in APP expression per se causes the progress of AD pathology (Head et al., 2012). For further gene engineering or crossing using this model, we have cryopreserved hundreds of oocytes from the transgenic marmosets. These oocytes showed transgene(+) genotypes (data not shown), therefore we confirmed the germline transmission of the transgene. Next approaches, including mutating other AD responsible genes, are feasible based on the transgenic model characterized in the present study.

In PIB-PET, either TG1 or TG2 did not show the significant increase in PIB-PET values in the whole brain. One of the reasons would be the resolution of PIB-PET was not small enough to detect Aβ plaque accumulation. The size of Aβ plaque-like structures in the marmoset brains was less than 100 μm whereas the spatial resolution of PIB-PET was 1.3 mm full width at half maximum at the center of FOV. Although it was difficult to visualize Aβ accumulation in PIB-PET at certain regions, in structural MRI analysis, MTR decrease was observed in the corpus callosum and caudate, whereas MTR increase was observed in the unilateral hippocampus. A previous study in mice reported a reduction of MTR in the splenium of corpus callosum (Pract et al., 2016), which was consistent with our result. Given that the authors indicated MTR reduction in APP/PS1 mouse has been already observed at 2 months of age, which was correlated with the degree of Aβ plaque deposition (4G8) as well as astro-microgliosis (Glfap and Iba-1) (Pract et al., 2016). Meanwhile, previous studies indicated high MTR in hippocampus was possibly associated with early tau hyperphosphorylation (Bigot et al., 2014; Pérez-Torres et al., 2014). It should be also noted that significant differences were also observed in hippocampus and caudate with MTR analysis was unilateral. The asymmetry in Alzheimer’s disease has been confirmed in PET studies (Frings et al., 2015) as well as histopathological data of hippocampus (Stefanits et al., 2012). The shape asymmetry was observed in hippocampus, amygdala and caudate, and the degree of their morphometric changes was associated with disease progression (Wachinger et al., 2016). Neuroanatomical asymmetries have potential to classify symptomatic classification. The MTR asymmetry in subcortical regions needs to be studied further. There are studies showing distinct laterality alterations in Alzheimer’s disease, such as hypometabolism in PET imaging (Frings et al., 2015) and asymmetric histology in the hippocampus (Stefanits et al., 2012), which would underly the existence of laterality in the MTR values in a diseased state. Although most studies were cross-sectional studies, thus less sensitive to detect asymmetry, longitudinal studies using MTR have an advantage for presymptomatic classification, given that MTR enables the detection of extensive and possibly even early amyloid pathology (Bigot et al., 2013).

We also examined functional connectivity in awake state. FC between left and right dorsolateral prefrontal cortex was low in TG 1 compared with control. The dorsolateral cortex is one of the regions composing default mode network in marmosets (Liu et al., 2019), which is associated with executive functions including working memory (Curtis, D’Esposito, 2003). Since deficits of working memory is one of the prominent symptoms of dementia, it would be reasonable to consider that function of this area is declined. The fMRI analysis is especially important to comprehend the behavioral phenotype by higher brain functions that is an special feature of primates, which may originate from the primate-specific cellular characteristics in neural cells including neurons, astrocytes, oligodendrocytes and microglia (Geirsdottir et al., 2019). Future studies will need to conduct assessment using the working memory test battery optimized for the common marmosets (Yamazaki et al., 2016; Nakamura et al., 2018).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.neures.2022.08.008.

References

Ali, M.M., Ghouri, R.G., Ann, A.H., Akbar, A., Toheed, A., 2019. Recommendations for anti-inflammatory treatments in Alzheimer’s disease: a comprehensive review of the literature. Cereus 11, e6620.

Andersson, J.L.R., Skare, S., Ashburner, J., 2003. How to correct susceptibility distortions in spin-echo planar images: application to diffusion tensor imaging. NeuroImage 20, 870-888. https://doi.org/10.1016/S1053-8119(03)00336-7.

Ashburner, J., Friston, K.J., 2000. Voxel-based morphometry - The methods. NeuroImage 11, 805-821. https://doi.org/10.1006/nimg.2000.0582.

Barker, W.W., Luiz, C.A., Kashuba, A., Luis, M., Harwood, D.G., Loewenstein, D., Waters, C., Jimison, P., Shepherd, E., Sevush, S., Graff-Radford, N., Newland, D., Todd, M., Miller, B., Gold, M., Heilman, K., Doty, L., Goodman, I., Robinson, B., Pearl, G., Dickson, D., Duyan, R., 2002. Relative frequencies of Alzheimer disease, Lewy body, vascular and frontotemporal dementia, and hippocampal sclerosis in the State of Florida Brain Bank. Alzheimer Dis. Assoc. Disord. 16, 203–212.

Benirschke, K., Brownell, L.E., 1962. Further observations on narrow chimerism in marmosets. Cyto genetics 1, 245–257.
2014. Birth of healthy offspring following ICSI in in vitro-matured common marmoset (Callithrix jacchus) oocytes. PLoS One 9, e95560.

Tomiioka, I., Nogami, N., Nakatani, T., Owari, K., Fujita, N., Motobashi, H., Takayama, O., Takae, K., Nagai, Y., Seki, K., 2017b. Generation of transgenic marmosets using a tetracyclin-inducible transgene expression system as a neurodegenerative disease model. Biol. Reprod. 97, 772–780.

Tomiioka, I., Ishibashi, H., Minakawa, E.N., Motobashi, H.H., Takayama, O., Saito, Y., Popiel, H.A., Puentes, S., Owari, K., Nakatani, T., Nogami, N., Yamamoto, K., Noguchi, S., Yonekawa, T., Tanaka, Y., Fujita, N., Suzuki, H., Kikuchi, H., Aizawa, S., Nagano, S., Yamada, D., Nishino, I., Ichinose, N., Wada, K., Kohnaka, S., Nagai, Y., Seki, K., 2017a. Transgenic monkey model of the polyglutamine diseases recapitulating progressive neurological symptoms. eNeuro 4.

Whitfield-Gabrieli, S., Nieto-Castanon, A., 2012. Conn: a functional connectivity toolbox for correlated and anticorrelated brain networks. Brain Connect 2, 125–141. https://doi.org/10.1089/brain.2012.0073.

Wolfgang, M.J., Eiselle, S.G., Browne, M.A., Schotzko, M.L., Garthwaite, M.A., Durning, M., Ramezani, A., Hawley, R.G., Thomson, J.A., Golos, T.G., 2001. Rhesus monkey placental transgene expression after lentiviral gene transfer into preimplantation embryos. Proc. Natl. Acad. Sci. USA 98, 10728–10732.

Woodward, A., Hashikawa, T., Maeda, M., Kameko, T., Hikishima, K., Iriki, A., Okano, H., Yamaguchi, Y., 2018. Data descriptor: The Brain/MINDS 3D digital marmoset brain atlas. Sci. Data 5, 1–12. https://doi.org/10.1038/sdata.2018.9.

Yamazaki, Y., Saiki, M., Inada, M., Watanabe, S., Iriki, A., 2016. Sustained performance by common marmosets in a delayed matching to position task with variable stimulus presentations. Behav. Brain Res. 297, 277–284. https://doi.org/10.1016/J.BBR.2015.10.025.

Yoshimatsu, S., Nakamura, M., Nakajima, M., Nemoto, A., Sato, T., Sasaki, E., Shiozawa, S., Okano, H., 2019. Evaluating the efficacy of small molecules for neural differentiation of common marmoset ESCs and iPSCs. Neurosci Res.

Yoshimatsu, S., Nakajima, M., Iguchi, A., Sanozuka, T., Sato, T., Nakamura, M., Nakajima, R., Arai, E., Ishikawa, M., Imairumi, K., Watanabe, H., Okahara, J., Noce, T., Takeda, Y., Sasaki, E., Behr, R., Edamura, K., Shiozawa, S., Okano, H., 2021. Non-viral Induction of Transgene-free iPSCs from Somatic Fibroblasts of Multiple Mammalian Species. Stem Cell Rep. 16, 754–770.