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알츠하이머병 동물 모델에서 만성스트레스에 의한 우울증으로 유발된 조기 알츠하이머병 발병과 장내미생물 및 장내 타우병증에 대한 연구

A Study of Gut Microbiota and Gut Tauopathy in Depression-Induced Early Onset in Alzheimer's Disease mice

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A thesis of the degree of Doctor of Philosophy

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A Study of Gut Microbiota and Gut Tauopathy in Depression-Induced Early Onset in Alzheimer's Disease mice

by

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Abstract

Alzheimer's disease (AD) is the most prevalent form of dementia. The neuropathological hallmarks of AD are defined as the amyloid plaques whose main component is aggregated amyloid beta peptide (Aβ) and intracellular neurofibrillary tangles whose main component is hyperphosphorylated tau. Although various causative factors of AD have been reported so far, the current pharmacologic treatments cannot slow or stop the pathology of AD. Interestingly, studies have shown that major depressive disorder (depression) is associated with a greatly increased risk of developing dementia. Moreover, recent studies revealed that a bidirectional communication between the gut microbiota and the brain could play an important role in various mental illnesses. In this study, I focused on the effect of young adulthood depression on the onset of AD, AD pathology alteration in the gut, and gut microbiota profile in adult mice. 4-months-old (mo) APP/PS1 mice and their wild type (WT) littermates were exposed to social defeat-based unpredictable chronic mild stress (SUCMS) to induce depression-like behavior. Interestingly, "depressed" APP/PS1 mice exhibited earlier onset of cognitive symptoms at 5-6 mo, but the age-matched WT mouse model of depression did not develop cognitive impairment until 11 mo.

Surprisingly, although there was no increase in the number of Aβ plaques in the depressed APP/PS1 mice brain compared to APP/PS1 control mice, tau phosphorylation was significantly increased in the hippocampus 5–6 mo depressed APP/PS1 mice. Furthermore, tau phosphorylation was also increased in the ileum and colon of depressed WT mice compared to WT control mice at 11–12 mo. Interestingly, shotgun metagenomic sequencing revealed various taxonomic alterations in the gut microbiota of the depressed APP/PS1 mice. Collectively, this study implies that depression during young adulthood is an important risk factor of AD, and suggests that the alteration of gut microbiota composition and gut tauopathy could be novel targets for the treatment of AD.

Keyword : Alzheimer’ s disease, depression, gut microbiota, social defeat–based chronic mild stress (SUCMS), tauopathy

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List of Abbreviations

Aβ: amyloid beta peptide

ACD: citrate-dextrose solution

AD: Alzheimer's disease

APP: amyloid precursor protein

APP/PS1: APPSwe/PSEN1dE9 transgenic

B6: C57BL/6

BBB: Blood-brain barrier

CNS: central nervous system

CSF: cerebrospinal fluid

DAPI: 4′, 6-diamidino-2-phenylindole

Depression: major depressive disorder

ENS: enteric nervous system

FMT: fecal transplantation

FST: forced swimming test

HPA: hypothalamic-pituitary-adrenal axis

IHC: immunohistochemistry

LDB: light-dark box

mo: months-old

NOR: novel object recognition test

OFT: open field test

PBS: phosphate buffered saline

PCA: principal component analysis

PSEN: presenilin

rRNA: ribosomal RNA

SCFAs: short-chain fatty acids

SUCMS: social defeat based Unpredictable Chronic Mild Stress

WT: wild type

YMT: Y-maze test

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease which is the most common cause of dementia [1]. The number of people with dementia was estimated to be 57.4 million globally in 2019 and is expected to increase to 152.8 million in 2050 due to the constant increase in the aging population [2]. Although the U.S. Food and Drug Administration (FDA) has approved six drugs for the treatment of AD, none of them can slow or stop AD symptoms [1]. The extracellular accumulation of amyloid beta peptide (Aβ) and intracellular tau neurofibrillary tangles in the brain are the hallmark pathology of AD, which leads to neuronal cell death and severe cognitive impairment [3]. As the neurotoxic accumulation of Aβ and the tau phosphorylation begin in the brain at least 20–30 years before symptom onset [4], exposure to risk factors of AD in this Aβ/phosphorylated tau–positive asymptomatic phase may affect the progression of AD.

The risk factors of AD include non–modifiable factors such as age, sex, and genetic background, and modifiable factors such as type 2 diabetes, unhealthy lifestyle, cognitive inactivity, depression, and obesity [5, 6]. Among these risk factors, depression is known to be a physiological condition that significantly increases the risk of AD

[7]. A 25% reduction in depression prevalence could potentially reduce 827,000 AD cases worldwide [8]. Notably, patients who suffered from depression early in life are associated with a 1.7 to 3.76-fold increased risk for development of AD later in life [9-13]. Furthermore, the presence of depressive symptoms in mild cognitive impairment patients increases the risk of conversion into AD [14]. An animal study utilizing Tg2576 mice found that exposure to chronic mild stress at 4-months of age accelerated the onset of cognitive impairment and increased the Aβ and phospho-tau levels in the hippocampus [15]. Despite the existence of evidence that support a strong relationship between depression and AD, the mechanism underlying the relationship is still elusive. Several studies hypothesized that the depression-induced hypothalamic-pituitary-adrenal (HPA) axis dysregulation and activation of microglia in the brain may result in hippocampal atrophy and neurodegenerative processes [16-19]. However, there are some controversies with these hypotheses as some studies failed to find the association between depression and hippocampal volume [10, 20], or the severity of the depression symptoms and the inflammatory status [21]. Therefore, a novel approach is needed to discover the effect of depression on AD progression.

It is estimated that 4.4% of the world's population is living with

depression, and the prevalence of depression is increasing [22]. Especially, 21% of adults aged 18–29 in the US have experienced some symptom of depression during their lifetime, which is the highest percentage among adults of any other age group [23]. In addition, the COVID-19 pandemic increased the prevalence of depression symptoms in young adults [24, 25], and it is reported that those in their young adulthood are more vulnerable to depression throughout the COVID-19 pandemic [26]. Thus, I focus on depression during the young adulthood as a risk factor of AD.

Gut microbiota refers to the microorganisms that colonize the gastrointestinal tract [27]. Gut microbiota is widely dispersed throughout the esophagus to the colon, while most of the gut microbiota reside in the distal small intestine and colon [28]. A human gut microbiota metagenomic study demonstrated that there are 100–150 times more genes in the human gut than our own genome [29, 30], and the gut microbial metabolome affects the host metabolism and genetics [31–34].

Few studies have discovered that the gut microbiota composition of AD and depressive patients is altered compared to that of normal individuals. A taxonomic level analysis study showed several bacteria taxa difference between AD patients and healthy controls, such as *Bacteroides*, *Actinobacteria*, *Ruminococcus*, *Lachnospiraceae*, and

Selenomonadales [35]. In depressive patients, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Enterobacteriaceae*, *Alistipes*, *Faecalibacterium*, *Bacteroidales*, and *Lachnospiraceae* were significantly altered compared to the healthy controls [36, 37]. Furthermore, animal model studies also have shown the alteration of gut microbiota composition in AD and depression animal models compared to the control [38–40]. However, these studies adopted 16S ribosomal RNA (rRNA) gene sequencing, which only targets taxonomically informative genomic loci 16S rRNA. Thus, the taxonomic profiling of AD, depressive patients, and animal models is limited to the genus level in these studies [41]. Therefore, taxonomic profiling on the species level is needed for the precise identification of differentiated microbiota affected by AD and depression.

Shotgun metagenomic sequencing is a powerful sequencing approach that provides insight into community biodiversity and function [42, 43]. For shotgun metagenomic sequencing, all DNA extracted from the sample are fragmented and each fragment is sequenced. As it targets the entire genomic content of a sample, shotgun metagenomic sequencing can provide the species/strain level taxonomic profiling [42–44].

Recent studies have revealed that the alteration of microbiota composition can affect the brain because there is communication

between the gut microbiota and the CNS through gut–brain axis [45–47]. Gut–brain axis is a bidirectional homeostatic communication through two distinct mechanisms including the circulatory system and neural pathway. Hormone, cytokines, and neurotransmitters travel through the portal vein, while the vagus and enteric nervous systems are involved as neural pathway for the crosstalk between the gut and brain [46, 48].

The interplay between gut microbial dysbiosis and AD pathology is well defined in many studies [49–51]. Gut microbial dysbiosis increases the release of inflammatory signals in the gastrointestinal tract, which results in systemic inflammation. These inflammatory signals can cross the blood brain barrier (BBB) and increase microglial activation and neuroinflammation in the brain. These cascades trigger the increase in Aβ plaques and phosphorylated tau that lead to neurodegeneration. Furthermore, fecal transplantation (FMT) of WT mice feces to AD model mice decreased Aβ plaques and phosphorylated tau in the brain [52, 53].

Although there are many studies focusing on the changes of AD pathological hallmarks in the brain by the alteration of microbiota composition, very few studies have focused on the changes of AD pathological hallmarks in the gut. Only one study identified the existence of Aβ plaques in the intestines of APP/PS1 mice [54],

while there are no studies about the altered phosphorylated tau levels in the gut of AD model mice or patients. The gastrointestinal tract contains intrinsic neuroglial circuits which is termed the enteric nervous system (ENS) [55, 56], and previous reports have shown that tau is expressed in the gut [57]. As there is bidirectional communication between the gut and brain, confirmation of the alteration in AD pathological hallmarks in the gut is needed.

In this study, I sought to discover the effect depression in Aβ-positive young adults has on the progression of AD. I placed emphasis on the alteration of AD pathological hallmarks in the gut and gut microbiota species to reveal the connection between depression in Aβ-positive young adults and AD. Especially, the shotgun metagenomic sequencing was adopted for the in-depth analysis of the taxonomic profiling of gut microbiota on the species level.

Materials and Methods

Mice

Male amyloid precursor protein Swedish mutation (APPswe)/presenilin (PSEN1) dE9 transgenic mice (APP/PS1) with C57BL/6;C3H background and the littermate wild type (WT) mice from The Jackson Laboratory (034829, Bar Harbor, ME, USA) were used. C57BL/6 (B6) male mice used for SUCMS confirmation were from The Jackson Laboratory (000664, Bar Harbor, ME, USA). For the aggressors of social defeat, male ICR mice at 4−6 months of age from Taconic Biosciences, Inc. (Rensselaer, NY, USA) were used. The mice were maintained in a temperature and humidity−controlled facility (22±2℃, 50±5%) with a 12−hour light/dark cycle. Food and water were provided ad libitum. All procedures regarding the use and the handling of the animals were conducted as approved by the Institutional Animal Care and Use Committee (IACUC) of Korea Institute of Science and Technology (#KIST−2019−057).

Social defeat based Unpredictable Chronic Mild Stress (SUCMS)

The designed SUCMS schedule refers to a previously described and modified protocol that details social defeat and an unpredictable chronic mild stress [58, 59]. The depression group mice were

introduced to SUCMS at unexpected times for 4 weeks at 4 to 5 months of age (Fig. 1A). The stressors were applied as following: day 1−28 – exposure to an aggressive ICR mice for 5 min and reversed light/dark cycle; day 8−10, 23−28 – exposure to restraint stress for 2 hr in 50 ml conical tube; day 8−10, 18−22 – tail suspension stress for 40 min; day 23−28 – tail suspension stress twice a day for 40 min each. The selection of aggressive ICR mice was performed as described in the previous report [58].

Serum corticosterone concentration measurement

On the last day of the SUCMS schedule, mice were anesthetized with 120 mg/kg ketamine and 8 mg/kg xylazine 1 hr after the last stressor exposure. 500 μl of blood was collected from the inferior vena cava and was mixed with a 100 μl citrate−dextrose solution (ACD) to prevent coagulation. After 10 min centrifugation at 3,000 rpm, the supernatant was collected. The collected serum was 1/20 diluted in PBS, and the corticosterone concentration was measured with Corticosterone ELISA kit (ADI−900−097, Enzo Life Sciences Inc., Farmingdale, NY, USA) following the manufacturer's instructions.

Forced Swimming Test (FST)

FST was employed for the evaluation of immobility, a depressive−

like behavior in mice. In a transparent cylindrical tank (15 cm diameter × 25 cm height), 25–26℃ tap water was filled up to 18.5 cm. The mice were gently released in the water and the test lasted for 6 min. The last 4 min was manually analyzed for the duration the mouse was immobile, and immobility was calculated (immobile duration / 4 min × 100). The illuminance was maintained at 50–60 lux.

Open Field Test (OFT)

OFT was employed for the evaluation of anxiety-like behavior and locomotor. Mice were allowed to freely explore in an open-field box (Width 40 cm × Length 40 cm × Height 40 cm) for 30 min. The illuminance was maintained at 15 lux. The time spent in the center zone (20 cm × 20 cm area in the middle) and the distance moved in the box were measured with EthoVision (Noldus, Wageningen, the Netherlands).

Light-Dark Box (LDB)

LDB was employed for the measurement of anxiety-like behavior. The LDB apparatus (Width 44 cm × Length 16 cm × Height 27 cm) is divided into a lit area and a dark area (width of the lit area: dark area = 26 cm : 18 cm), with a gate between the two. The dark area

had a lid on the top to block out light. On the test day, mice were placed in the dark area of the box, and the time mice spent in the lit area was measured for 10 min. The illuminance of the lit area was maintained at 10 lux.

Novel Object Recognition test (NOR)

NOR was employed for the evaluation of recognition memory. The test was done for three consecutive days. On the first day of the test, mice were habituated in an open-field box (Width 40 cm × Length 40 cm X Height 40 cm) for 30 min. 24hr later, mice were introduced to two identical object A (familiar object) for 10 min in the open-field box. 24 hr later, one of the object A was exchanged with an object B (novel object) and mice were introduced to the two different objects for 10 min in the open-field box. Time exploring the familiar object and novel object was measured on the last day of the test, and the relative exploration time was calculated (exploration time of familiar object or novel object divided by total exploration time). Objects with different color, texture, and shape were used as object A and B. The illuminance was maintained at 15 lux.

Y-Maze Test (YMT)

YMT was employed for evaluating short-term memory and working

memory. An apparatus with three arms (35 cm length × 10 cm width × 10 cm height) positioned at a 120° angle from each other was used. The mice were placed in the end of an arm of the apparatus, and were allowed to freely explore the apparatus for 10 min. The last 8 min was analyzed to calculate the alteration percentage (number of alterations/total number of entries × 100). Every entry was counted as valid if all the four limbs were placed in the arm. The illuminance was maintained at 7 lux.

Preparation of mice brain slices

Mice were anesthetized with 120 mg/kg ketamine and 8 mg/kg xylazine and were perfused and fixed with a 4% paraformaldehyde (16005, Sigma-Aldrich, Inc., St. Louis, MO, USA) solution. The isolated brain was post-fixed in the 4% paraformaldehyde solution in 4℃ overnight and was dehydrated in 30% sucrose (84097, Sigma-Aldrich, Inc., St. Louis, MO, USA) solution in 4℃ for 4-5 days. The brain samples were then sectioned with a cryostat microtome in 40 μm thickness.

Preparation of mice gut slices

After perfusion, ileum and colon were isolated and post-fixed with 4% paraformaldehyde solution for two days. The tissues were

paraffin embedded and sectioned in 6 μm thickness. Paraffin was removed with xylene and ethanol before immunohistochemistry.

Thioflavin S staining

1% thioflavin S solution was prepared with thioflavin S powder (T1892, Sigma-Aldrich, Inc., St. Louis, MO, USA) in 80% ethanol. Fixed mice brain slices were washed with PBS and were incubated for 1 min in 70% ethanol. The brain slices were then incubated in 80% ethanol for 1 min and were transferred to 1% thioflavin S solution. After incubation in 1% thioflavin S solution for 15 min, the brain slices were incubated in 80% ethanol for 1 min, and subsequently transferred to 70% ethanol and were incubated for 1 min. After final washing with PBS, the brain slices were mounted on the silane coated slide glasses.

Immunohistochemistry (IHC)

Antigen retrieval was performed in 10mM sodium citrate solution (pH 8.5) with 0.05% tween-20 at 80℃ for 30 min. After 1hr of blocking in TBS with 0.5% triton X-100 and 2% donkey serum, the samples were incubated in primary antibodies at 4℃ overnight. The following primary antibodies were used: tau (ab254256, Abcam, Cambridge, UK) 1:200; phosphorylated tau at Ser396 (35-5300, Invitrogen,

Waltham, MA, USA) 1:200; Tuj1 (ab78078, Abcam, Cambridge, UK) 1:500. The samples were then incubated in secondary antibodies and DAPI (D9542, Sigma-Aldrich, Inc., St. Louis, MO, USA) for 2hr at room temperature. The following secondary antibodies were used: donkey anti-mouse 488 (A20210, Invitrogen, Waltham, MA, USA) 1:400, donkey anti-rabbit 594 (A21207, Invitrogen, Waltham, MA, USA) 1:400.

Western Blot

Protein was extracted from the colon and ileum in RIPA buffer (89900, Thermo Fisher Scientific, Inc. Waltham, MA, USA) with protease and phosphatase inhibitor cocktail (78440, Thermo Fisher Scientific Inc., Waltham, MA, USA). 40 μg of protein was separated by 10% SDS-PAGE gel and transferred to PVDF membrane (IPVH00010, Merck Milllipore, Darmstadt, Germany). After blocking in 5% skim milk or BSA for 1 hr, the membrane was incubated with primary antibody overnight at 4℃. primary antibodies were used: tau (sc-390476, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) 1:2000; phospho-tau Ser396 (44-752G, Invitrogen, Waltham, MA, USA) 1:1000; 6E10 (803014, BioLegend, Inc, San Diego, CA, USA) 1:2000; GAPDH (sc-365062, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) 1:1000. The membrane was then incubated with secondary

antibody in 5% skim milk or BSA for 1 hr. The following secondary antibodies were used: donkey anti-rabbit IgG secondary antibody, HRP (31458, Invitrogen, Waltham, MA, USA) 1:1000; goat anti-mouse IgG secondary antibody, HRP (31430, Invitrogen, Waltham, MA, USA) 1:1000.

Statistical analysis

The statistical analysis of behavior tests and molecular work was done with a two-way ANOVA with Sidak's multiple comparisons test, unpaired T-test, one-way ANOVA with Tukey's multiple comparisons test (Prism, GraphPad Software, San Diego, CA, USA).

Shotgun metagenomic sequencing

Fresh mice fecal sample was stored in −80℃ immediately after collection until use. A DNA kit for feces (6531050, MP biomedicals, Irvine, CA, USA) was used for DNA extraction from the fecal sample following the manufacturer’ s instructions. The quality and quantity of the extracted DNA were assessed by fluorometry (Qubit fluorometer, Invitrogen, Waltham, MA, USA) and gel electrophoresis. Briefly, 100 ng of genomic DNA from each sample was fragmented by acoustic shearing. Fragments of 350 bp were ligated to Illumina's adapters and were PCR-amplified to get the final libraries of 500-

600 bp. The libraries were quantified with 4200 TapeStation system (Agilent Technologies, Inc., Santa Clara, CA, USA) and KAPA Library Quantification Kit (KK4824, Roche, Pleasanton, CA, USA). The resulting purified libraries were applied to an Illumina flow cell for cluster generation and sequenced using 150 bp paired-end reads on an Illumina NovaSeq 6000 sequencing system (Illumina, Inc., San Diego, CA, USA) following the manufacturer's protocols.

Gut microbiota taxonomic classification and diversity analysis

Taxonomic classification was performed using mOTUs2 (v. 3.0.1) with default parameters. Proportional abundances of the taxonomic table were centered log-ratio-transformed using a zCompositions R package (v. 1.4.0-1), and zero counts were imputed by Bayesian-multiplicative replacement method. Prior to the transformation, taxa with all zeros or only one positive value were removed. Community composition was analyzed by principal component analysis (PCA) on the transformed table. Community richness was measured on rarefied taxonomic matrix to 11,000 reads. To identify bacteria specifically associated with each host and environment factors, screening at the species level was carried out using the Kruskal-Wallis test comparing the groups (WT-CTL, WT-SUCMS, APP/PS1-CTL, APP/PS1-SUCMS). Subsequently, significant taxa were analyzed

using a multivariate model with mouse age, genotype, and depression as independent variables. Statistical analyses were performed in R (v.4.1.0). All statistical tests used were two-sided and corrected for multiple testing using the Benjamini-Hochberg procedure or Dunn's test.

Results

It is widely known that stress can cause depression [60, 61]. Based on this idea, stress protocols have been broadly used to induce depression in animal models. Although unpredictable chronic mild stress has been widely used to study depression [62-64], it often fails to cause depression or is not reproducible to induce depressive symptoms [65]. Furthermore, unpredictable chronic mild stress lacks social stress factors even though stress from relationships or abuse is one of the main causes of depression [66, 67]. For the construction of an animal model of depression that reflects both social and physical stress factors, I designed 'Social defeat based Unpredictable Chronic Mild Stress (SUCMS)' which refers to a social defeat and an unpredictable chronic mild stress protocol previously described with some modifications (Fig. 1A).

To verify that the SUCMS protocol can induce depressive symptoms, I applied the SUCMS in B6 mice. As I was targeting depression during 'young adulthood', 4 mo B6 mice that are compatible to human adults of 20-30 years old were used. The SUCMS B6 mice showed decreased relative body weight (Fig. 1B; two-way ANOVA with Sidak's multiple comparisons test, $p<0.0001$, CTL N=12, SUCMS N=12) which is one of the symptoms that depressive patients exhibit

[68, 69]. Furthermore, SUCMS B6 mice exhibit increased immobility in the FST, which is the main depressive-like behavior in mice (Fig. 1C; unpaired T-test, p=0.0002, CTL N=12, SUCMS N=12). The OFT showed that the SUCMS B6 mice have a tendency to exhibit anxiety-like behavior (Fig. 1D; unpaired T-test, p=0.0672, CTL N=12, SUCMS N=12), although the locomotion was normal (Fig. 1E; unpaired T-test, not significant (n.s.), CTL N=12, SUCMS N=12). The LDB test for further confirmation of anxiety-like behavior, showed no significant difference between the groups (Fig.1 F; unpaired T-test, n.s., CTL N=12, SUCMS N=12). Also, SUCMS did not induce any cognitive and memory deficit in B6 mice (Fig. 1G, H; unpaired T-test, n.s., CTL N=12, SUCMS N=12).

Next, I applied the SUCMS in 4 mo APP/PS1 mice to verify the effect depression had on the progression of AD (Fig. 2A). The WT and APP/PS1 mice with SUCMS-induced depression both exhibited significantly decreased relative body weight (Fig. 2B; two-way ANOVA with Sidak's multiple comparisons test, WT-CTL vs. WT-SUCMS p<0.0001, APP/PS1-CTL vs. APP/PS1-SUCMS p<0.0001, WT-CTL N=15, WT-SUCMS N=17, APP/PS1-CTL N=15, APP/PS1-SUCMS N=21). Also, WT-SUCMS and APP/PS1-SUCMS mice showed increased corticosterone concentration in the serum (Fig. 2C; unpaired T-test, WT-CTL vs. WT-SUCMS

p=0.0320, APP/PS1-CTL vs. APP/PS1-SUCMS p=0.0096, WT-CTL N=6, WT-SUCMS, N=10 APP/PS1-CTL N=8, APP/PS1-SUCMS N=10). Furthermore, WT-SUCMS and APP/PS1-SUCMS mice exhibited increased immobility in the FST (Fig. 2D; unpaired T-test, WT-CTL vs. WT-SUCMS p=0.0066, APP/PS1-CTL vs. APP/PS1-SUCMS p=0.0206, WT-CTL N=21, WT-SUCMS N=19, APP/PS1-CTL N=17, APP/PS1-SUCMS N=25). These results show that the SUCMS successfully work on APP/PS1 mice and induce depressive symptoms. Interestingly, although APP/PS1 mice do not have cognitive deficit until 8-12 months [70, 71], 5-6 mo APP/PS1-SUCMS mice showed significant decrease in the novel object stiffing time percentage in the NOR test (Fig. 2E; one-way ANOVA with Tukey's multiple comparisons test, WT-CTL vs. APP/PS1-SUCMS p=0.0030, WT-SUCMS vs. APP/PS1-SUCMS p=0.0011, APP/PS1-CTL vs. APP/PS1-SUCMS p=0.0045, WT-CTL N=14, WT-SUCMS N=15, APP/PS1-CTL N=10, APP/PS1-SUCMS N=11). The YMT results also showed significantly decreased alteration percentage in APP/PS1-SUCMS mice (Fig. 2F; one-way ANOVA with Tukey's multiple comparisons test, WT-CTL vs. APP/PS1-SUCMS p=0.0071, WT-SUCMS vs. APP/PS1-SUCMS p=0.0013, APP/PS1-CTL vs. APP/PS1-SUCMS p=0.0294, WT-CTL N=12, WT-SUCMS N=14, APP/PS1-CTL N=13,

APP/PS1−SUCMS N=18). These results imply that depression during young adulthood can advance the onset of AD symptoms.

To see whether depression affects the formation of Aβ plaques in the brain, thioflavin S staining was performed. In WT−CTL and WT−SUCMS, Aβ plaques were not observed. Although APP/PS1−CTL and APP/PS1−SUCMS both showed Aβ plaques in the brain, there were no difference in the Aβ plaque number between the two groups (Fig. 3A−C; unpaired T−test, n.s., APP/PS1−CTL N=5, APP/PS1−SUCMS N=5). Some studies have revealed that tau phosphorylation is not only an important neuropathological characteristic of AD brains but is also associated with depression. Depressive patients and animal models exhibit increased phosphorylated tau in the cerebrospinal fluid (CSF) or brain [15, 72−74]. Especially, phosphorylated tau at serine 396 (S396) is increased in the hippocampus of chronic unpredictable stress depression mice model [67, 68]. Therefore, the level of phosphorylated tau at S396 was investigated in the hippocampus. Interestingly, the APP/PS1−SUCMS group showed an significantly increased number of phosphorylated tau positive cells in the hippocampus compared to WT−CTL and APP/PS1−CTL (Fig. 4A, B; one−way ANOVA with Tukey's multiple comparisons test, WT−CTL vs. APP/PS1−SUCMS p=0.0261, APP/PS1−CTL vs. APP/PS1−SUCMS p=0.0294, WT−

CTL N=4, WT-SUCMS N=4, APP/PS1-CTL N=4, APP/PS1-SUCMS N=4).

To confirm whether the pathological phenotype of AD is also observed and altered in the gut, Aβ plaques level was evaluated in the ileum and colon of the experimental animals by IHC. However, there was no significant difference in the level of Aβ plaques between APP/PS1-CTL and APP/PS1-SUCMS mice (Fig. 5A-D; unpaired T-test, n.s., ileum: APP/PS1-CTL N=4, APP/PS1-SUCMS N=6; colon: APP/PS1-CTL N=5, APP/PS1-SUCMS N=5). Next, the level of phosphorylated tau at S396 was also investigated in the ileum and colon. There was no significant difference in the level of phosphorylated tau at S396 between groups (Fig. 6A-F; one-way ANOVA with Tukey's multiple comparisons test, n.s., ileum: WT-CTL N=4, WT-SUCMS N=2, APP/PS1-CTL N=4, APP/PS1-SUCMS N=6; colon: WT-CTL N=4, WT-SUCMS N=2, APP/PS1-CTL N=5, APP/PS1-SUCMS N=5). Phosphorylated tau at S396 and Tuj1, a maker protein for neurons, was investigated by IHC. Phosphorylated tau at S396 and enteric neuron as assessed with Tuj 1 staining was co-localized in both the ileum and colon (Fig. 6G, H). I next tried to verify the effect of depression induced by SUCMS during AD progression after the onset of AD symptoms. Behavior tests were performed at 11-12 mo after mice were subject to

SUCMS at 4−5 mo (Fig. 7A). Although APP/PS1−CTL mice showed cognitive impairment as confirmed in the previous studies, I could not find any cognitive impairment aggravation in APP/PS1−SUCMS mice compared to APP/PS1−CTL mice in NOR and YMT. However, WT−SUCMS mice showed significantly decreased novel object sniffing time percentage in NOR (Fig. 7B; one−way ANOVA with Tukey's multiple comparisons test, WT−CTL vs. WT−SUCMS p=0.0332, WT−CTL vs. APP/PS1−CTL p=0.0059, WT−CTL vs. APP/PS1−SUCMS p=0.0028, WT−CTL N=12, WT−SUCMS N=11, APP/PS1−CTL N=9, APP/PS1−SUCMS N=9) and alteration percentage in YMT (Fig. 7C; one−way ANOVA with Tukey's multiple comparisons test, WT−CTL vs. WT−SUCMS p=0.0006, WT−CTL vs. APP/PS1−CTL p=0.0086, WT−CTL vs. APP/PS1−SUCMS p=0.0302, WT−CTL N=11, WT−SUCMS N=8, APP/PS1−CTL N=8, APP/PS1−SUCMS N=7). These results imply that depressive experience during young adulthood can be a critical risk factor of dementia in later life. Furthermore, FST results showed that the effect of SUCMS does not last until 11 mo of age (Fig. 7D; unpaired T−test, n.s., WT−CTL N=5, WT−SUCMS N=8, APP/PS1−CTL N=5, APP/PS1−SUCMS N=8), which shows that a single depressive episode during young adulthood can affect outbreak of dementia in later life.

Thioflavin S staining in the 11−12 mo mice brain after the SUCMS at

4-5 mo also showed no difference in Aβ plaque formation between APP/PS1-CTL and APP/PS1-SUCMS (Fig. 8A-C; unpaired T-test, n.s., APP/PS1-CTL N=3, APP/PS1-SUCMS N=3). Tauopathy in the hippocampus also showed no alteration in the 11-12 mo WT-SUCMS mice compared to WT-CTL (Fig. 9A, B; one-way ANOVA with Tukey's multiple comparisons test, n.s., WT-CTL N=4, WT-SUCMS N=4, APP/PS1-CTL N=4, APP/PS1-SUCMS N=4).

Subsequent verification of Aβ plaques levels in the gut showed no significant alteration between APP/PS1-CTL and APP/PS1-SUCMS mice (Fig. 10A-D; unpaired T-test, n.s., ileum: APP/PS1-CTL N=4, APP/PS1-SUCMS N=6; colon: APP/PS1-CTL N=5, APP/PS1-SUCMS N=5). Surprisingly, phosphorylated tau was significantly increased in the ileum of WT-SUCMS, APP/PS1-CTL, and APP/PS1-SUCMS mice compared to WT-CTL (Fig. 11A, C; unpaired T-test, WT-CTL vs. WT-SUCMS p=0.0135, WT-CTL vs. APP/PS1-CTL p=0.0052, WT-CTL vs. APP/PS1-SUCMS p=0.0036, WT-SUCMS vs. APP/PS1-SUCMS p=0.0470, WT-CTL N=5, WT-SUCMS N=7, APP/PS1-CTL N=6, APP/PS1-SUCMS N=5). Furthermore, the colon of WT-SUCMS and APP/PS1-CTL mice showed increased tau phosphorylation at S396 compared to WT-CTL (Fig. 11B, E; unpaired T-test, WT-CTL vs. WT-SUCMS p=0.0509, WT-CTL vs. APP/PS1-CTL p=0.0479, WT-CTL N=7,

WT-SUCMS N=7, APP/PS1-CTL N=6, APP/PS1-SUCMS N=5), reflecting the behavior test results. However, there was no significant difference of tau expression in the ileum (Fig. 11A, D; unpaired T-test, n.s, WT-CTL N=5, WT-SUCMS N=7, APP/PS1-CTL N=6, APP/PS1-SUCMS N=5) and colon (Fig. 11 B, F; unpaired T-test, n.s, WT-CTL N=7, WT-SUCMS N=7, APP/PS1-CTL N=6, APP/PS1-SUCMS N=5) between groups. Phosphorylated tau at S396 and Tuj1 labeled by IHC showed co-localization of phosphorylated tau at S396 and enteric neuron in both ileum and colon (Fig. 11G and H).

To confirm the effect of depression-induced early onset AD on the gut microbiota composition, shotgun metagenomic sequencing was conducted. Fecal sample collection was done at three timepoints: 4 mo (before SUCMS), 5 mo (1 week after SUCMS), and 11-20 mo (Fig. 12A). The PCA for microbial composition in all groups of 4 mo showed there is no significant difference between WT and APP/PS1 mice before SUCMS (Fig. 12B; Kruskal-Wallis test with Benjamini-Hochberg procedure, R^2=0.04124, p=0.599, WT N=11, APP/PS1 N=11). Surprisingly, WT and APP/PS1 were both significantly separated by SUCMS treatment (Fig. 12C; Kruskal-Wallis test with Benjamini-Hochberg procedure, R^2=0.016103, WT-CTL vs. WT-SUCMS p<0.01, APP/PS1-CTL vs. APP/PS1-SUCMS p<0.01, WT-

CTL N=10, WT-SUCMS N=10, APP/PS1-CTL N=10, APP/PS1-SUCMS N=8), which indicated that the community structure of microbiota differed by depression. Furthermore, WT and APP/PS1 in 11-20 mo also exhibited significantly altered microbial community with SUCMS treatment (Fig. 12D; Kruskal-Wallis test with Benjamini-Hochberg procedure, R^2=0.012813, WT-CTL vs. WT-SUCMS p<0.01, APP/PS1-CTL vs. APP/PS1-SUCMS p<0.01, WT-CTL N=10, WT-SUCMS N=10, APP/PS1-CTL N=10, APP/PS1-SUCMS N=10). Interestingly, distinct microbiota composition between WT-CTL and APP/PS1-CTL was observed in 11-20 mo while there was no difference in 4 mo and 5 mo, which implies that the AD symptom onset affected the change in microbial community structure.

For the calculation of alpha diversity measurement, observed richness and Shannon's diversity index were adopted. I found significant increase of richness in WT-SUCMS and APP/PS1-SUCMS group compared to WT-CTL in both 5 mo and 11-20 mo (Fig. 12E; Kruskal-Wallis test with Benjamini-Hochberg procedure, 5 mo: WT-CTL vs. WT-SUCMS FDR<0.1, WT-SUCMS vs. APP/PS1-CTL FDR<0.1, WT-CTL vs. APP/PS1-SUCMS FDR<0.1, WT-CTL N=10, WT-SUCMS N=10, APP/PS1-CTL N=10, APP/PS1-SUCMS N=8; 11-20 mo: WT-CTL vs. WT-SUCMS

FDR<0.1, WT-CTL vs. APP/PS1-SUCMS FDR<0.1, WT-CTL N=10, WT-SUCMS N=10, APP/PS1-CTL N=10, APP/PS1-SUCMS N=10). Although this result did not show a statistical significance, there was also a tendency of increased richness between APP/PS1-CTL and APP/PS1-SUCMS (Fig. 12E). These results indicate microbiota composition alteration in the SUCMS groups. However, there was no significant difference in Shannon's diversity between the groups at both 5 mo and 11-20 mo (Fig. 12F; Kruskal-Wallis test with Benjamini-Hochberg procedure, n.s., 5 mo: WT-CTL N=10, WT-SUCMS N=10, APP/PS1-CTL N=10, APP/PS1-SUCMS N=8; 11-20 mo: WT-CTL N=10, WT-SUCMS N=10, APP/PS1-CTL N=10, APP/PS1-SUCMS N=10).

On the species level, the abundance of the top 84 taxa were shown in the hierarchy cluster heat-map (Fig. 13). The alteration of microbiota species was analyzed based on the three different factors: age, AD phenotype, and depressive experience (Fig. 14; Kruskal-Wallis test with Benjamini-Hochberg procedure, *FDR< 0.1). *Bacteroidales bacterium M6, Bifidobacterium pseudolonum, Clostridiaceae species incertae sedis, Lachnospiraceae bacterium 3.2, Lactobacillus animalis murinus*, and *Romboutsia timonensis* were increased by aging. *Alistipes finegoldii, Bacteroidaceae species incertae sedis, Bacteroides caecimuris, Bacteroides uniformis,*

Candidatuss Arthromitus sp., *Eubacterium species incertae sedis, Lactobacillus species incertae sedis*, and *Muribaculaceae species incertae sedis* were decreased with aging. Also, *Bacteroidales bacterium M9, Eubacterium species incertae sedis, Lactobacillus animalis murinus* were increased by AD, while Muribaculum intestinale was decreased by AD. *Alistipes finegoldii, Bacteroides bouchesdurhonensis faecichinchillae, Bacteroides dorei vulgatus, Bacteroides* sp., *Bacteroides species incertae sedis, Bacteroides uniformis, Candidatus Arthromitus* sp., *Clostridiaceae species incertae sedis*, and *Ruminococcus species incertae sedis* were increased by depression. *Bacteroidales bacterium M1, Bacteroidales bacterium M12, Bacteroidales bacterium M6, Escherichia coli, Lachnoclostridium species incertae sedis, Lachnospiraceae bacterium 3.2, Lactobacillus animalis murinus, Lactobacillus species incertae sedis, Muribaculaceae species incertae sedis, Muribaculum intestinale*, and *Prevotella species incertae sedis* were decreased by depression. *Clostridiaceae species incertae sedis* was significantly increased and *Lactobacillus species incertae sedis* was significantly decreased by both aging and depression. Also, *Muribaculum intestinale* was significantly decreased by both AD and depression. Interestingly, depression highly affected the composition of microbiota and was the most important factor among the three factors.

Altered microbiota species by depression in 5 mo and 11−20 mo are listed in Table 1. In 5 mo WT mice, the amount of 7 microbiota species was increased and the amount of 8 microbiota species was decreased by depression induced by SUCMS. In 5 mo APP/PS1 mice, the amount of 6 microbiota species was increased and the amount of 11 microbiota species was decreased by depression induced by SUCMS. In 11−20 mo WT mice, the amount of 5 microbiota species was increased and the amount of 6 microbiota species was decreased by depression induced by SUCMS. In 11−20 mo APP/PS1 mice, the amount of 6 microbiota species was increased and the amount of 3 microbiota species was decreased by depression induced by SUCMS (Kruskal−Wallis test with Dunn's test). *Bacteroides dorei vulgatus, Bacteroides uniformis, Bacteroides* sp., and *Alistipes finegoldii* were commonly found and increased by SUCMS induced depression in all comparison groups, indicating that these four microbiota species are important depression−associated microbiota species.

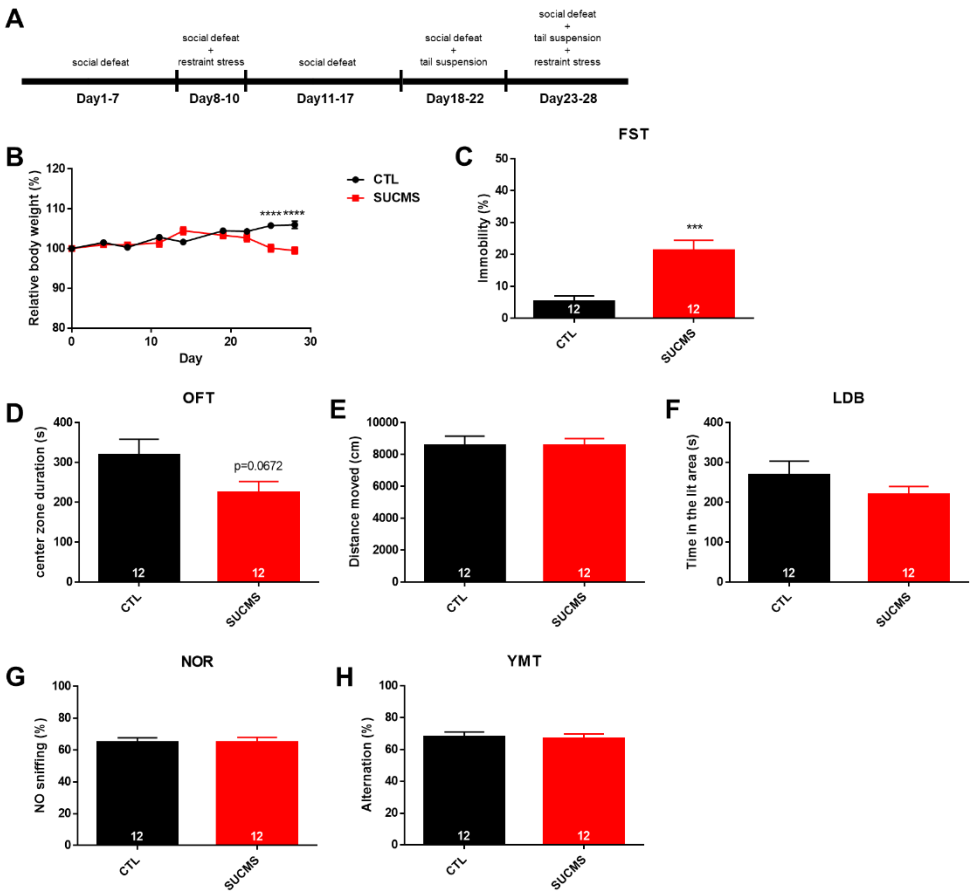

Figure 1. SUCMS-induced depressive symptoms in 5-6 mo B6 mice.

(A) Timeline of SUCMS schedule. (B) Body weight was significantly decreased by SUCMS on day 25 and 28 (two-way ANOVA with Sidak's multiple comparisons test, p<0.0001, CTL N=12, SUCMS N=12). (C) Immobility was significantly increased by SUCMS (unpaired T-test, p=0.0002, CTL N=12, SUCMS N=12). (D) Center zone duration was decreased by SUCMS, although not significant (unpaired T-test, p=0.0672, CTL N=12, SUCMS N=12). (E) There was no difference in distance moved between the control and SUCMS

mice in OFT (unpaired T-test, n.s., CTL N=12, SUCMS N=12). (F) No significant difference was found for time in the lit area between the control and SUCMS mice in LDB (unpaired T-test, n.s., CTL N=12, SUCMS N=12). (G) There was no difference in novel object (NO) sniffing time percentage between the control and SUCMS mice in NOR (unpaired T-test, n.s., CTL N=12, SUCMS N=12). (H) No significant difference was found in the alteration percentage between the control and SUCMS mice in YMT (unpaired T-test, n.s., CTL N=12, SUCMS N=12). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

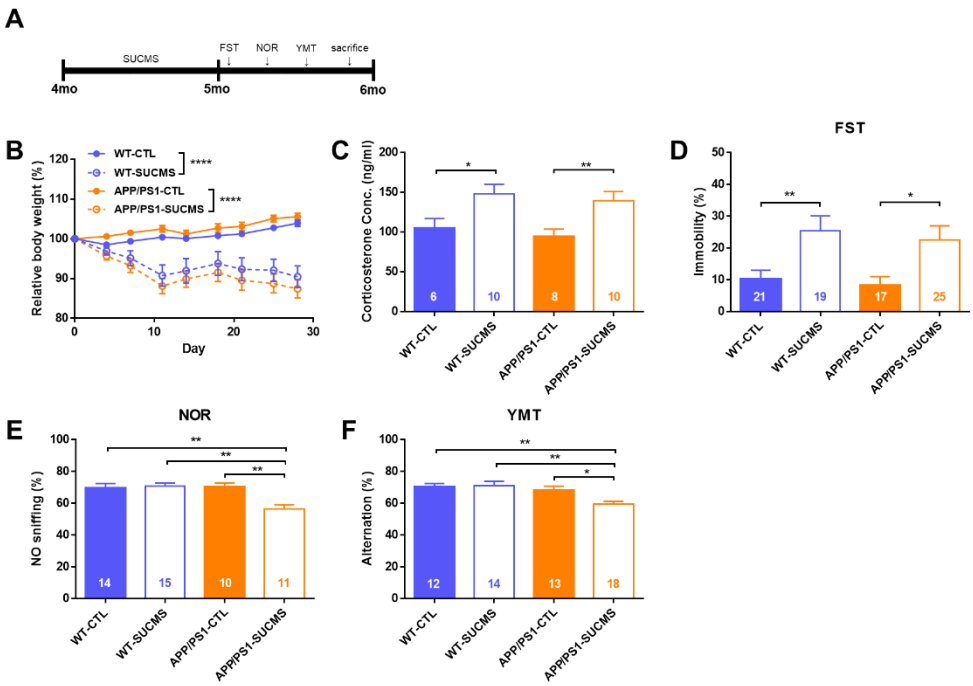

Figure 2. SUCMS-induced depression in Aβ-positive young adult caused early cognition deficit in 5-6 mo APP/PS1 mice.

(A) Timeline of SUCMS and behavior tests (B) Body weight was significantly decreased by SUCMS in both WT and APP/PS1 (two-way ANOVA with Sidak's multiple comparisons test, WT-CTL vs. WT-SUCMS p<0.0001, APP/PS1-CTL vs. APP/PS1-SUCMS p<0.0001, WT-CTL N=15, WT-SUCMS N=17, APP/PS1-CTL N=15, APP/PS1-SUCMS N=21). (C) Corticosterone concentration in the serum was significantly increased in WT-SUCMS and APP/PS1-SUCMS compared to their control groups (unpaired T-test, WT-CTL vs. WT-SUCMS p=0.0320, APP/PS1-CTL vs. APP/PS1-SUCMS p=0.0096, WT-CTL N=6, WT-SUCMS, N=10

APP/PS1−CTL N=8, APP/PS1−SUCMS N=10). (D) FST immobility was significantly increased by SUCMS in both WT and APP/PS1 (unpaired T−test, WT−CTL vs. WT−SUCMS p=0.0066, APP/PS1−CTL vs. APP/PS1−SUCMS p=0.0206, WT−CTL N=21, WT−SUCMS N=19, APP/PS1−CTL N=17, APP/PS1−SUCMS N=25). (E) APP/PS1−SUCMS mice showed significantly decreased NO sniffing time in NOR compared to other groups in (one−way ANOVA with Tukey's multiple comparisons test, WT−CTL vs. APP/PS1−SUCMS p=0.0030, WT−SUCMS vs. APP/PS1−SUCMS p=0.0011, APP/PS1−CTL vs. APP/PS1−SUCMS p=0.0045, WT−CTL N=14, WT−SUCMS N=15, APP/PS1−CTL N=10, APP/PS1−SUCMS N=11). (F) APP/PS1−SUCMS mice showed significantly decreased alteration percentage in YMT compared to other groups in (one−way ANOVA with Tukey's multiple comparisons test, WT−CTL vs. APP/PS1−SUCMS p=0.0071, WT−SUCMS vs. APP/PS1−SUCMS p=0.0013, APP/PS1−CTL vs. APP/PS1−SUCMS p=0.0294, WT−CTL N=12, WT−SUCMS N=14, APP/PS1−CTL N=13, APP/PS1−SUCMS N=18). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

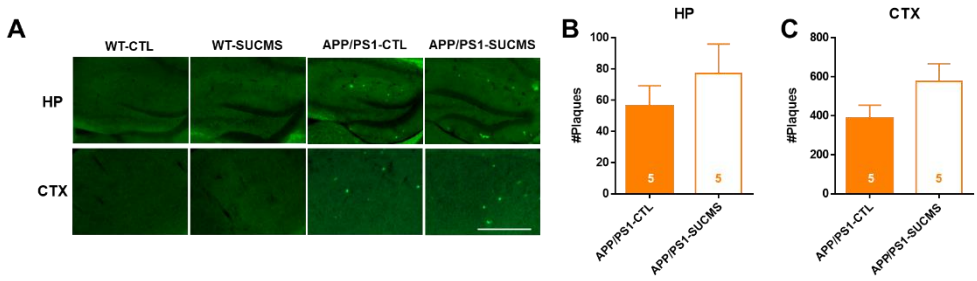

Figure 3. SUCMS-induced depression during young adulthood did not affect Aβ plaque formation in the brain of 5-6 mo APP/PS1 mice. (A) Images of thioflavin S staining in the cortex and hippocampus of 5-6 mo mice. Scale bar=1000 μm. (B, C) There was no significant plaque number difference between APP/PS1-CTL and APP/PS1-SUCMS mice in the cortex and hippocampus (unpaired T-test, n.s., APP/PS1-CTL N=5, APP/PS1-SUCMS N=5).

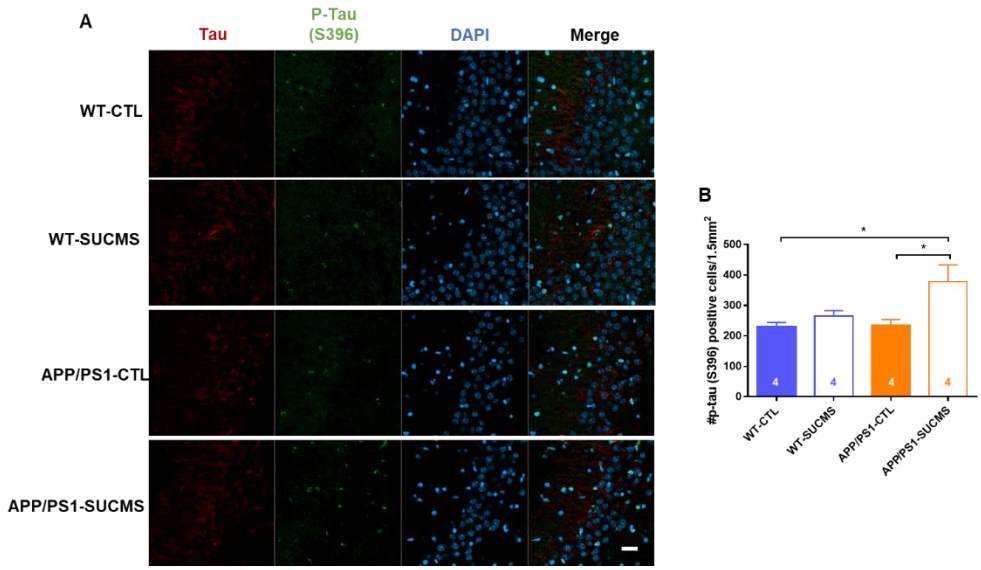

Figure 4. SUCMS-induced depression during young adulthood increased tau phosphorylation in the hippocampus of 5-6 mo APP/PS1 mice.

(A) Images of tau and p-tau S396 co-staining in the hippocampus of 5-6 mo mice. Scale bar=20 μm. (B) APP/PS1-SUCMS show increased number of p-tau positive cells in the hippocampus compared to WT-CTL and APP/PS1-CTL (one-way ANOVA with Tukey's multiple comparisons test, WT-CTL vs. APP/PS1-SUCMS p=0.0261, APP/PS1-CTL vs. APP/PS1-SUCMS p=0.0294, WT-CTL N=4, WT-SUCMS N=4, APP/PS1-CTL N=4, APP/PS1-SUCMS N=4). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

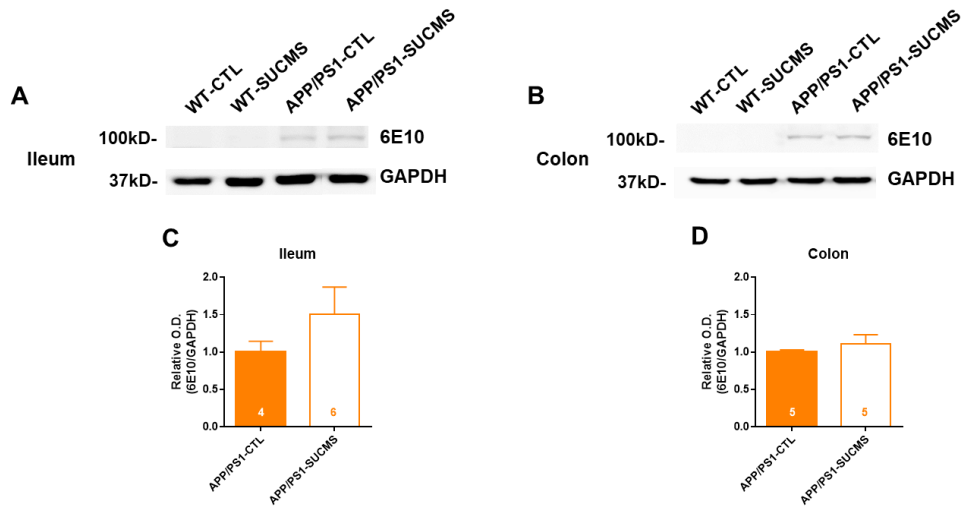

Figure 5. SUCMS-induced depression during young adulthood did not change Aβ plaque formation in the ileum and colon of 5-6 mo mice.

(A, B) Western blot image of 6E10 and GAPDH in the ileum and colon of 5-6 mo mice. (C, D) Relative O.D. of 6E10 in the ileum and colon. No significant change was found between APP/PS1-CTL and APP/PS1-SUCMS (unpaired T-test, n.s., ileum APP/PS1-CTL N=4; APP/PS1-SUCMS N=6, colon APP/PS1-CTL N=5; APP/PS1-SUCMS N=5).

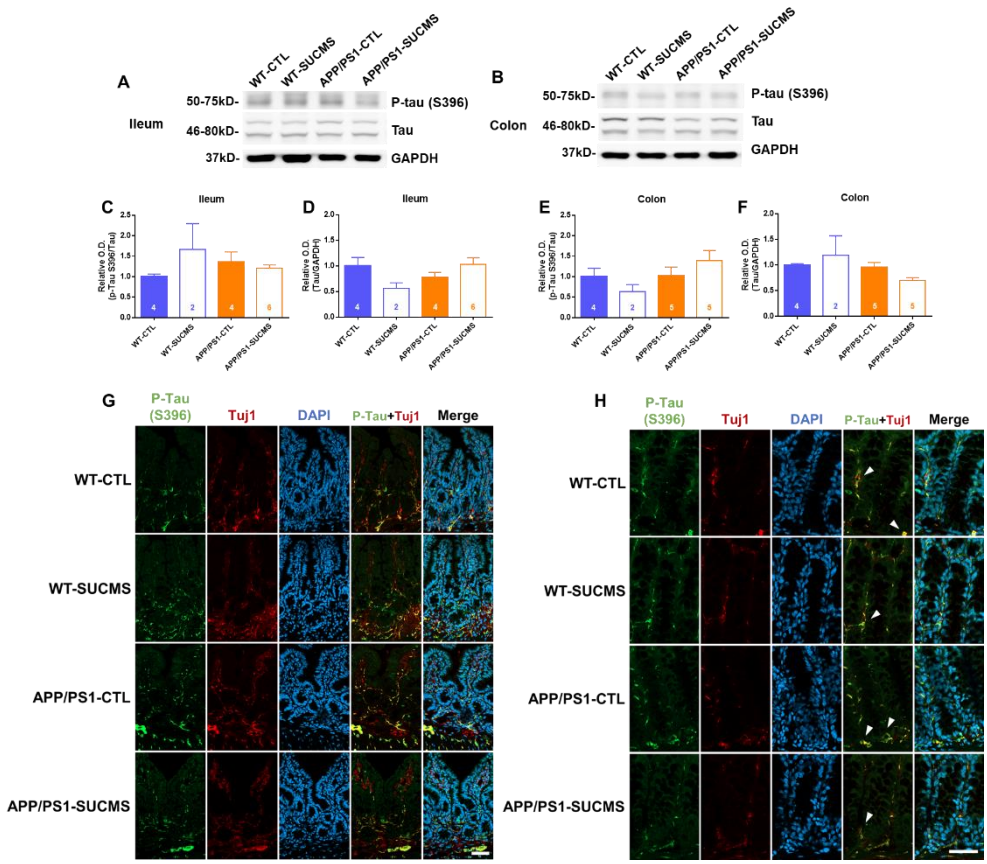

Figure 6. SUCMS-induced depression during young adulthood did not change tau phosphorylation in the ileum and colon of 5-6 mo mice. (A, B) Western blot image of p-tau (396), tau and GAPDH in the ileum and colon of 5-6 mo mice. (C-F) Relative O.D. of p-tau (396) and tau in the ileum and colon. No significant change was found between groups (one-way ANOVA with Tukey's multiple comparisons test, n.s., ileum WT-CTL N=4; WT-SUCMS N=2; APP/PS1-CTL N=4; APP/PS1-SUCMS N=6, colon WT-CTL N=4; WT-SUCMS N=2; APP/PS1-CTL N=5; APP/PS1-SUCMS N=5). (G) p-tau (396) and Tuj1 are co-localized in the ileum of 5-6 mo

mice. Scale bar=50 μm. (H) p-tau (396) and Tuj1 are co-localized in the colon of 5-6 mo mice. Scale bar=50 μm.

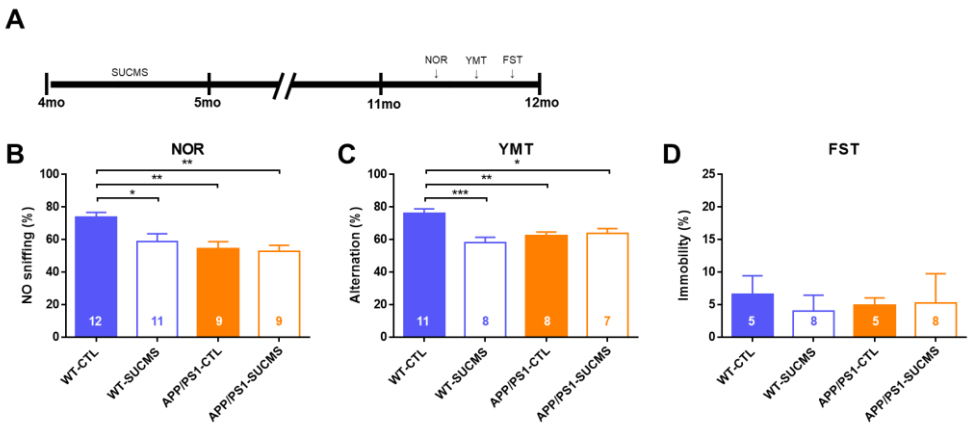

Figure 7. SUCMS-induced depression during young adulthood caused memory deficit in 11-12 mo mice.

(A) Timeline of SUCMS and behavior tests. (B) WT-SUCMS, APP/CTL and APP/PS1-SUCMS mice showed significantly decreased NO sniffing time in NOR compared to WT-CTL mice (one-way ANOVA with Tukey's multiple comparisons test, WT-CTL vs. WT-SUCMS p=0.0332, WT-CTL vs. APP/PS1-CTL p=0.0059, WT-CTL vs. APP/PS1-SUCMS p=0.0028, WT-CTL N=12, WT-SUCMS N=11, APP/PS1-CTL N=9, APP/PS1-SUCMS N=9). (C) WT-SUCMS, APP/CTL and APP/PS1-SUCMS mice showed significantly decreased alteration percentage in YMT compared to WT-CTL mice (one-way ANOVA with Tukey's multiple comparisons test, WT-CTL vs. WT-SUCMS p=0.0006, WT-CTL vs. APP/PS1-CTL p=0.0086, WT-CTL vs. APP/PS1-SUCMS p=0.0302, WT-CTL N=11, WT-SUCMS N=8, APP/PS1-CTL N=8, APP/PS1-SUCMS N=7). (D) There was no significant

immobility change in FST between groups. (unpaired T-test, n.s., WT-CTL N=5, WT-SUCMS N=8, APP/PS1-CTL N=5, APP/PS1-SUCMS N=8) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

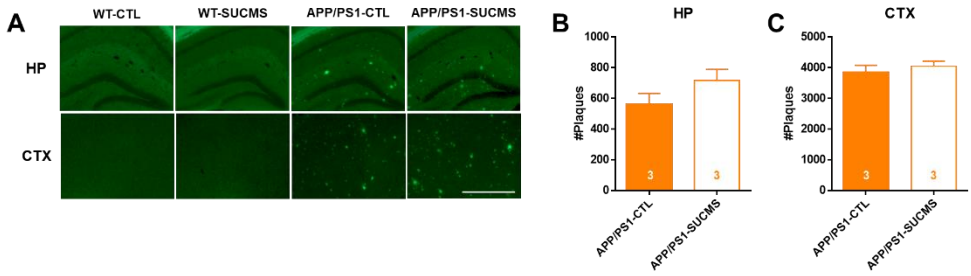

Figure 8. SUCMS-induced depression during young adulthood did not affect Aβ plaque formation in the brain of 11-12 mo APP/PS1 mice.

(A) Images of thioflavin S staining in the cortex and hippocampus of 11-12 mo mice. Scale bar=1000 μm. (B, C) There was no significant plaque number difference between APP/PS1-CTL and APP/PS1-SUCMS mice in the cortex and hippocampus (unpaired T-test, n.s., APP/PS1-CTL N=3, APP/PS1-SUCMS N=3).

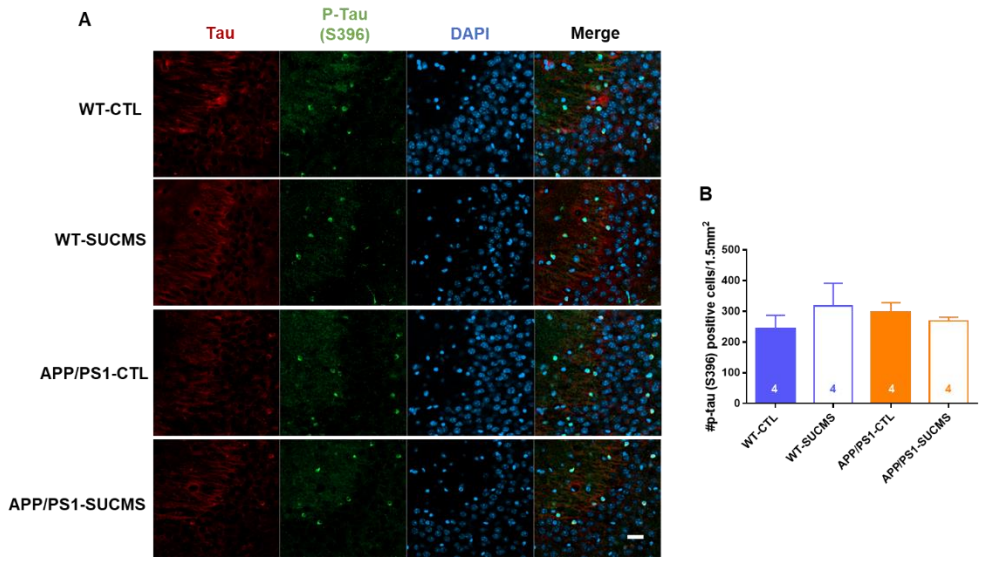

Figure 9. SUCMS-induced depression during young adulthood did not affect tau phosphorylation in the hippocampus of 11-12 mo mice.

(A) Images of tau and p-tau S396 co-staining in the hippocampus of 11-12 mo mice. Scale bar=20 μm. (B) There was no significant change in the number of p-tau positive cells between groups in the hippocampus (one-way ANOVA with Tukey's multiple comparisons test, n.s., WT-CTL N=4, WT-SUCMS N=4, APP/PS1-CTL N=4, APP/PS1-SUCMS N=4).

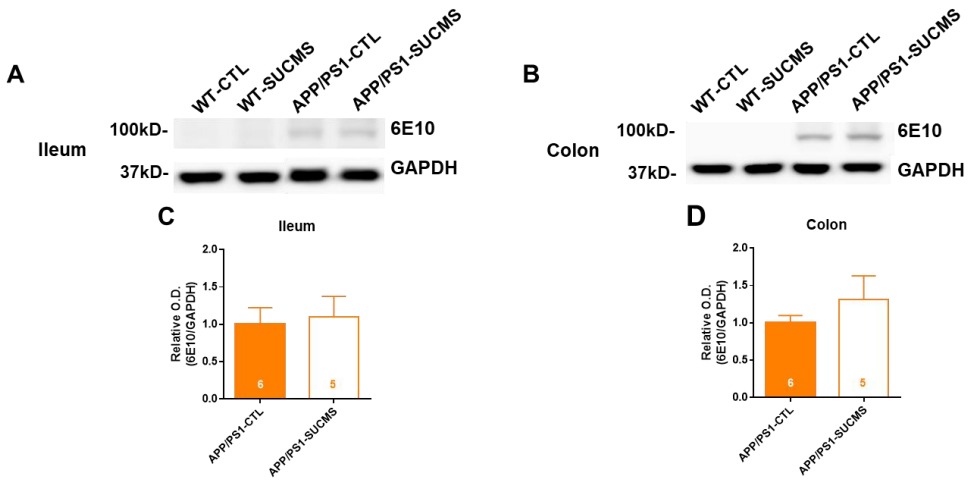

Figure 10. SUCMS-induced depression during young adulthood did not change Aβ plaque formation in the ileum and colon of 11-12 mo APP/PS1 mice.

(A, B) Western blot image of 6E10 and GAPDH in the ileum and colon of 11-12 mo mice. (C, D) Relative O.D. of 6E10 in the ileum and colon. No significant change was found between APP/PS1-CTL and APP/PS1-SUCMS (unpaired T-test, n.s., ileum APP/PS1-CTL N=4; APP/PS1-SUCMS N=6, colon APP/PS1-CTL N=5; APP/PS1-SUCMS N=5).

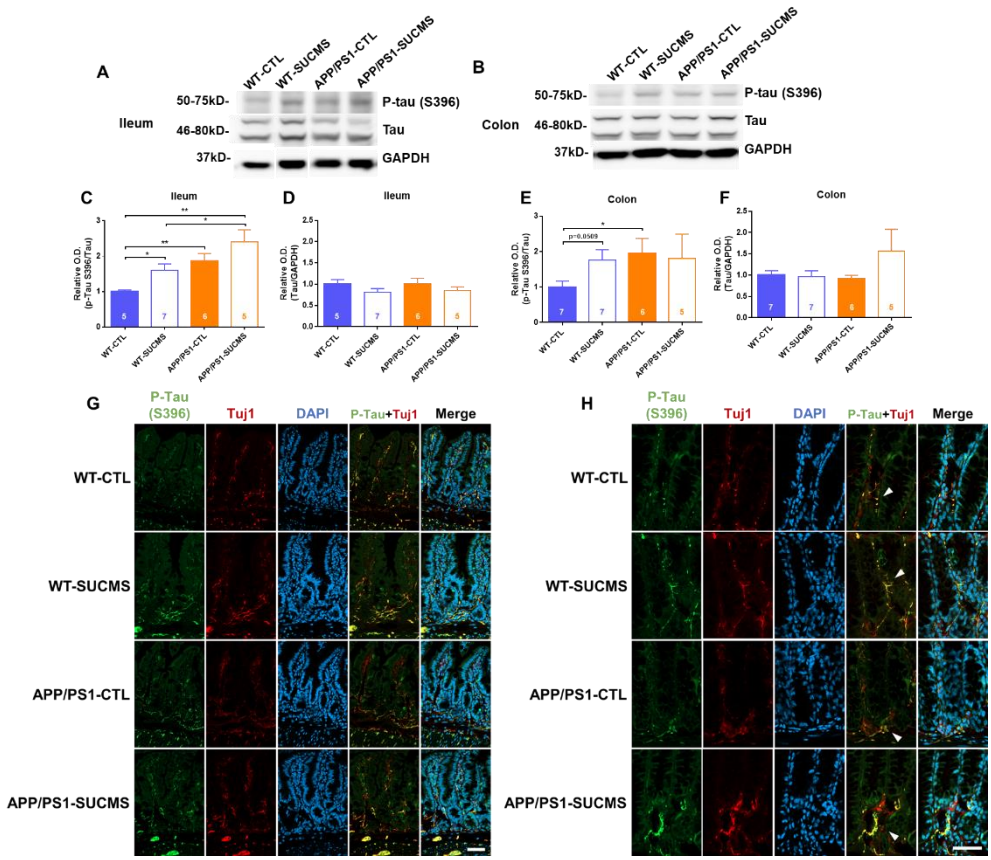

Figure 11. SUCMS-induced depression during young adulthood increased tau phosphorylation in the ileum and colon of 11-12 mo WT mice.

(A, B) Western blot image of p-tau (396), tau and GAPDH in the ileum and colon of 11-12 mo mice. (C) Relative O.D. of p-tau (396) in the ileum was significantly increased in WT-SUCMS, APP/PS1-CTL, and APP/PS1-SUCMS compared to WT-CTL. APP/PS1-SUCMS also showed increased relative O.D. of p-tau (396) in the ileum compared to WT-SUCMS (unpaired T-test, WT-CTL vs. WT-SUCMS p=0.0135, WT-CTL vs. APP/PS1-CTL p=0.0052,

WT-CTL vs. APP/PS1-SUCMS p=0.0036, WT-SUCMS vs. APP/PS1-SUCMS p=0.0470, WT-CTL N=5, WT-SUCMS N=7, APP/PS1-CTL N=6, APP/PS1-SUCMS N=5). (D) Relative O.D. of tau in the ileum. No significant change was found between groups (unpaired T-test, n.s, WT-CTL N=5, WT-SUCMS N=7, APP/PS1-CTL N=6, APP/PS1-SUCMS N=5). (E) Relative O.D. of p-tau (396) in the colon was increased in WT-SUCMS, APP/PS1-CTL compared to WT-CTL (unpaired T-test, WT-CTL vs. WT-SUCMS p=0.0509, WT-CTL vs. APP/PS1-CTL p=0.0479, WT-CTL N=7, WT-SUCMS N=7, APP/PS1-CTL N=6, APP/PS1-SUCMS N=5). (F) Relative O.D. of tau in the colon. No significant change was found between groups (unpaired T-test, n.s, WT-CTL N=7, WT-SUCMS N=7, APP/PS1-CTL N=6, APP/PS1-SUCMS N=5). (G) p-tau (396) and Tuj1 are co-localized in the ileum of 11-12 mo mice. Scale bar=50 μm. (H) p-tau (396) and Tuj1 are co-localized in the colon of 11-12 mo mice. Scale bar=50 μm. *p-value<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

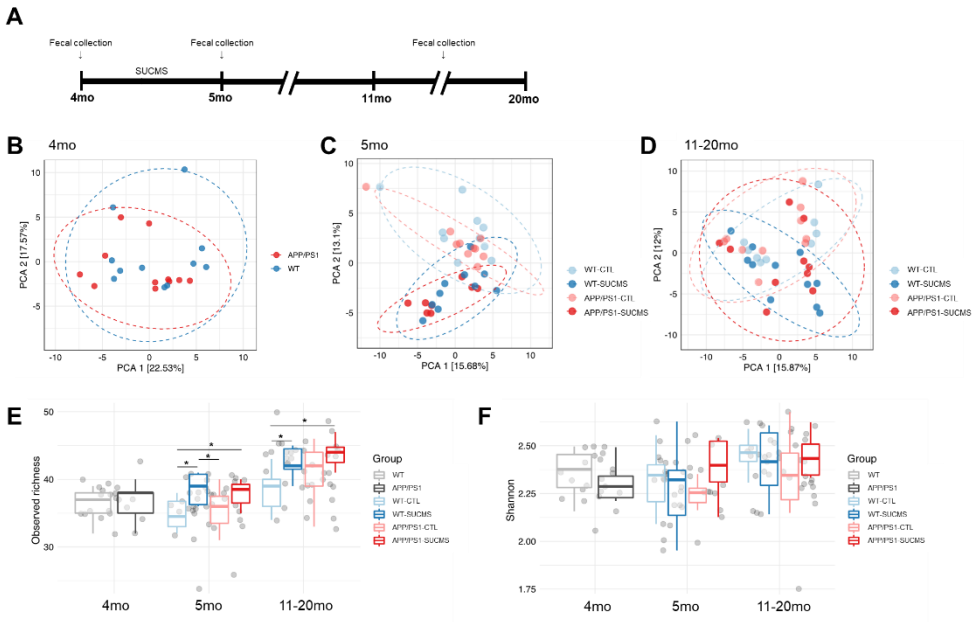

Figure 12. PCA and Alpha diversity of gut microbiota were affected by AD and depression in 5 mo and 11−20 mo mice.

(A) Timeline of fecal sample collection. (B−D) PCA plot of 4 mo, 5 mo and 11−20 mo. There is no significant difference between WT and APP/PS1 in 4 mo (Kruskal−Wallis test with Benjamini−Hochberg procedure, R^2=0.04124, p=0.599, WT N=11, APP/PS1 N=11). The control groups and SUCMS groups are significantly separated in 5 mo (Kruskal−Wallis test with Benjamini−Hochberg procedure, R^2=0.016103, WT−CTL vs. WT−SUCMS p<0.01, APP/PS1−CTL vs. APP/PS1−SUCMS p<0.01, WT−CTL N=10, WT−SUCMS N=10, APP/PS1−CTL N=10, APP/PS1−SUCMS N=8) and 11−20 mo. (Kruskal−Wallis test with Benjamini−Hochberg procedure, R^2=0.012813, WT−CTL vs. WT−SUCMS p<0.01, APP/PS1−CTL vs.

APP/PS1−SUCMS $p<0.01$, WT−CTL N=10, WT−SUCMS N=10, APP/PS1−CTL N=10, APP/PS1−SUCMS N=10). (E) Observed richness of gut microbiota species. There is a significant increase of observed richness between WT−CTL and WT−SUCMS, WT−CTL and APP/PS1−SUCMS in 5 mo (Kruskal−Wallis test with Benjamini−Hochberg procedure, WT−CTL vs. WT−SUCMS FDR<0.1, WT−SUCMS vs. APP/PS1−CTL FDR<0.1, WT−CTL vs. APP/PS1−SUCMS FDR<0.1, WT−CTL N=10, WT−SUCMS N=10, APP/PS1−CTL N=10, APP/PS1−SUCMS N=8) and 11−20 mo (Kruskal−Wallis test with Benjamini−Hochberg procedure, WT−CTL vs. WT−SUCMS FDR<0.1, WT−CTL vs. APP/PS1−SUCMS FDR<0.1, WT−CTL N=10, WT−SUCMS N=10, APP/PS1−CTL N=10, APP/PS1−SUCMS N=10). (F) Shannon' s index of gut microbiota species. There is no significant difference between groups in both 5 mo (Kruskal−Wallis test with Benjamini−Hochberg procedure, n.s., WT−CTL N=10, WT−SUCMS N=10, APP/PS1−CTL N=10, APP/PS1−SUCMS N=8) and 11−20 mo (Kruskal−Wallis test with Benjamini−Hochberg procedure, n.s., WT−CTL N=10, WT−SUCMS N=10, APP/PS1−CTL N=10, APP/PS1−SUCMS N=10).

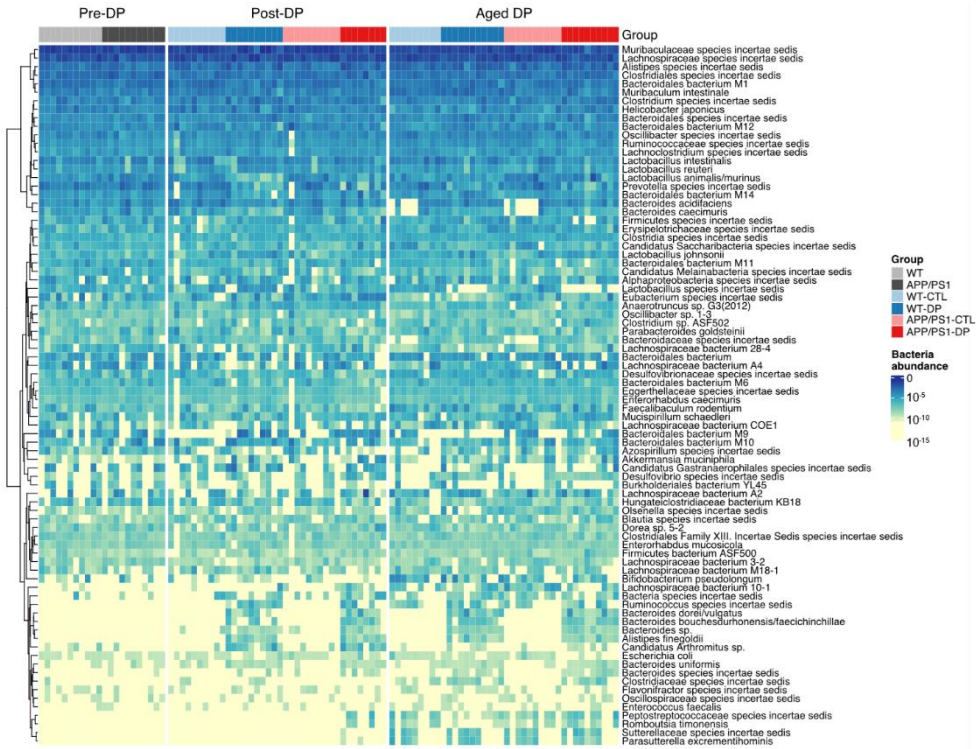

Figure 13. The hierarchy cluster heat-map showed the abundance of the top 84 gut microbiota species in 5 mo and 11-20 mo mice.

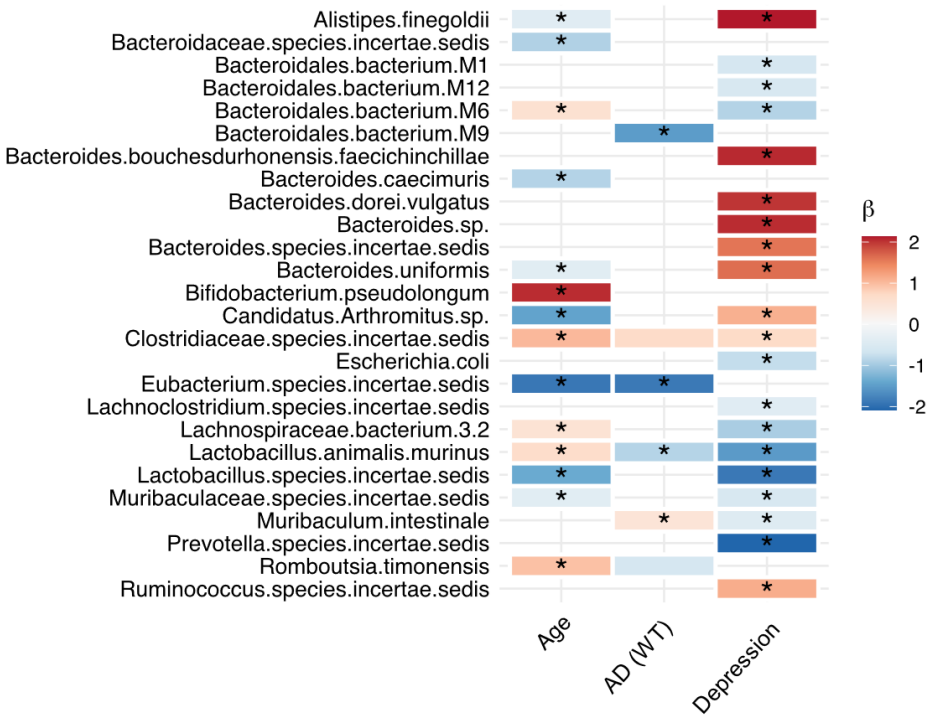

Figure 14. Gut microbiota composition was altered by age, AD, and depression.

Table of gut microbiota that are significantly increased or decreased by age, AD and depression (Kruskal–Wallis test with Benjamini–Hochberg procedure). *FDR< 0.1.

Age	Comparison	Z-score	P-value	taxa
5mo	WT-CTL vs. WT-SUCMS	-3.421	0.004	*Alistipes finegoldii*
		-2.877	0.012	*Ruminococcus species incertae sedis*
		-2.696	0.014	*Candidatus Arthromitus* sp.
		-2.555	0.032	*Bacteroidales bacterium M9*
		-2.495	0.025	*Bacteroides* sp.
		-2.334	0.039	*Bacteroides uniformis*
		-2.032	0.084	*Bacteroides dorei vulgatus*
		1.912	0.084	*Lachnospiraceae bacterium 3.2*
		1.992	0.093	*Bacteroidales bacterium M6*
		2.032	0.063	*Azospirillum species incertae sedis*
		2.294	0.065	*Bacteroidales bacterium M12*
		2.938	0.007	*Bacteroidales bacterium M1*
		2.978	0.009	*Bacteroides caecimuris*
		3.079	0.012	*Bacteroidaceae species incertae sedis*
		3.763	0.001	*Lactobacillus animalis murinus*
	APP/PS1-CTL vs. APP/PS1-SUCMS	-3.362	0.001	*Alistipes finegoldii*
		-3.002	0.008	*Bacteroides uniformis*
		-2.755	0.018	*Bacteroides bouchesdurhonensis faecichinchillae*
		-2.755	0.018	*Candidatus Arthromitus* sp.
		-2.589	0.029	*Bacteroides dorei vulgatus*
		-2.509	0.036	*Bacteroides* sp.
		2.001	0.091	*Bacteroidales bacterium M12*
		2.371	0.053	*Desulfovibrionaceae species incertae sedis*
		2.485	0.026	*Lactobacillus animalis murinus*
		2.547	0.033	*Clostridiaceae species incertae sedis*
		2.637	0.025	*Bifidobacterium pseudolongum*
		2.888	0.012	*Bacteroidales bacterium M6*
		2.888	0.023	*Muribaculaceae species incertae sedis*
		2.921	0.007	*Bacteroides caecimuris*
		2.940	0.010	*Bacteroidales bacterium M1*
		3.021	0.008	*Lachnospiraceae bacterium 3.2*
		3.215	0.008	*Azospirillum species incertae sedis*
11-20mo	WT-CTL vs. WT-SUCMS	-3.333	0.002	*Bacteroides dorei vulgatus*
		-3.324	0.001	*Bacteroides* sp.
		-2.649	0.012	*Alistipes finegoldii*
		-2.395	0.033	*Bacteroides uniformis*
		-2.390	0.025	*Bacteroides bouchesdurhonensis faecichinchillae*
		2.145	0.064	*Escherichia coli*
		2.386	0.026	*Prevotella species incertae sedis*
		2.739	0.018	*Bacteroidales bacterium M9*
		2.932	0.020	*Romboutsia timonensis*
		2.960	0.018	*Lachnoclostridium species incertae sedis*
		3.301	0.003	*Lactobacillus species incertae sedis*
	APP/PS1-CTL vs. APP/PS1-SUCMS	-3.749	0.001	*Alistipes finegoldii*
		-3.692	0.001	*Bacteroides* sp.
		-3.672	0.001	*Bacteroides dorei vulgatus*
		-3.634	0.002	*Bacteroides species incertae sedis*
		-3.271	0.006	*Bacteroides bouchesdurhonensis faecichinchillae*
		-2.984	0.009	*Bacteroides uniformis*
		3.118	0.004	*Lactobacillus species incertae sedis*
		3.194	0.008	*Prevotella species incertae sedis*
		3.443	0.002	*Escherichia coli*

Table 1. List of microbiota species altered by depression induced by SUCMS in 5 mo and 11−20 mo mice.

In 5 mo WT mice, the abundance of 7 microbiota species was

increased and the abundance of 8 microbiota species was decreased by depression induced by SUCMS. In 5 mo APP/PS1 mice, the abundance of 6 microbiota species was increased and the abundance of 11 microbiota species was decreased by depression- induced by SUCMS. In 11-20 mo WT mice, the abundance of 5 microbiota species was increased and the abundance of 6 microbiota species was decreased by depression induced by SUCMS. In 11-20 mo APP/PS1 mice, the abundance of 6 microbiota species was increased and the abundance of 3 microbiota species was decreased by depression induced by SUCMS (Kruskal-Wallis test with Dunn's test).

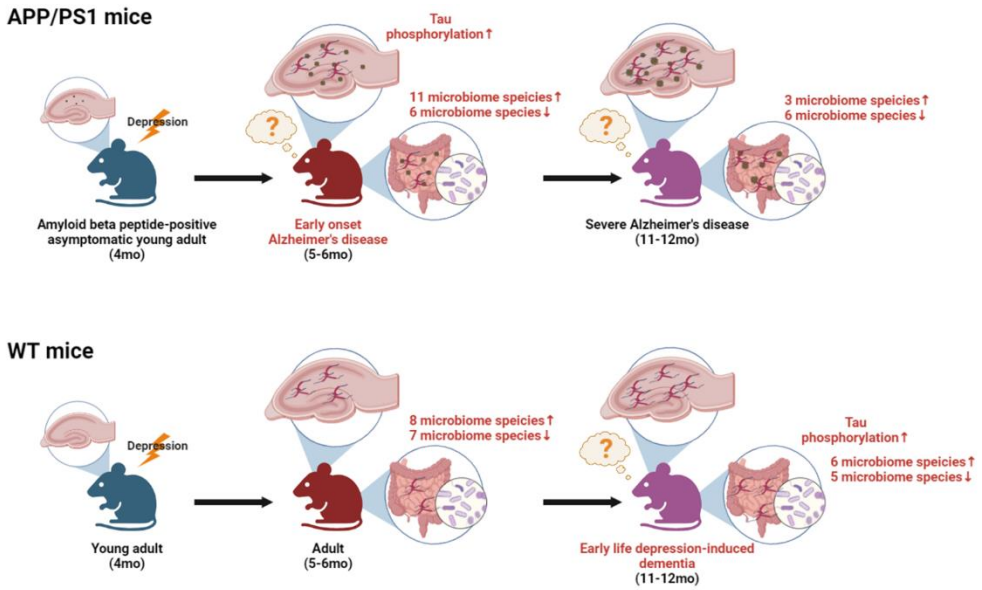

Figure 15. Graphical summary of depression-induced early onset AD in mice.

SUCMS-induced depression in Aβ-positive asymptomatic young adult caused early onset AD in 5-6 mo, accompanied by increased phosphorylated tau positive cells in the hippocampus and altered gut microbiota. In WT mice, depression induced in young adult result in dementia in later life (11-12 mo), accompanied by increased phosphorylated tau in the gut and altered gut microbiota.

Discussion

In this study, I sought to investigate whether the occurrence of depression at the Aβ-positive young adult stage affects the progression of AD. SUCMS-induced depressive APP/PS1 mice at 4 months of age showed significant cognitive deficits between 5 to 6 months of age. Considering that APP/PS1 mice normally exhibit AD symptom starting from 8 months of age, the result indicates that depression in young adult mice advanced the symptom onset of AD. Additionally, increased levels of phosphorylated tau were found in the hippocampus of 5-6 mo APP/PS1-SUCMS mice. To further identify the effect of depression on the progression of AD, depression-induced APP/PS1 and their littermates at 4 months of age were tested between 11 to 12 months of age. Although depression in Aβ-positive young adults did not aggravate the cognitive deficits in the later stages of AD, depression in normal young adult mice caused cognitive deficits later in their lives. Although the 11-12 mo mice did not show altered tau phosphorylation in the hippocampus between groups, 11-12 mo WT-SUCMS and APP/PS1-CTL mice exhibited increased tau phosphorylation in the ileum and colon. Interestingly, shotgun metagenomic sequencing revealed that the gut microbiota

composition was altered by aging, AD, and depression.

In the hypothesis of this study, it was expected that APP/PS1−SUCMS mice may exhibit severe cognitive deficits compared to APP/PS1−CTL mice at 11−12 mo. However, there was no significant difference observed between these two groups in both the NOR test and YMT. It is possible that the APP/PS1−CTL mice at this age may already have considerable memory loss as they were found to spend the same amount of time sniffing the familiar and novel objects in NOR and showed only approximately 60% of alternation in the YMT. It may have been due to a floor effect and made it hard to see the behavioral difference between the two groups.

In this study, the presence of the pathological hallmarks of AD, Aβ plaque and phosphorylated tau were investigated in the gut through western blot and IHC. The presence of Aβ plaque in the APP/PS1−CTL and APP/PS1−SUCMS mice was detected in the ileum and colon. As 6E10 antibody specifically target human Aβ sequence, the Aβ plaque observed in the ileum and colon are derived from the transgene of APP/PS1 mice. Both APP and PSEN1 transgenes in APP/PS1 mice are under control of prion protein promoter [75]. Prion protein is a cell surface protein mainly expressed in the central and peripheral nervous system [76], which includes enteric nervous system. However, the possibility that soluble from of Aβ in the brain

have transferred to the gut cannot be ignored. Further study to reveal the origin of the Aβ in the ileum and colon is needed.

In the present study, although the Aβ plaques were found in the brain and gut of APP/PS1 AD model mice, depression induced by SUCMS failed to increase the number of Aβ plaques in the brain and gut. A previous study reported a higher plasma Aβ_{40}:Aβ_{42} ratio in subjects with depression [77]. Also, AD patients with depression history showed increased hippocampal Aβ plaques [78]. On the contrary, Mackin et al. revealed that late life depression is associated with reduced cortical Aβ deposits [79]. As controversy exists in the previous studies, it is presumed that Aβ plaque is not a critical factor for depression. Further study is required to identify the relationship between Aβ and depression in the future.

On the other hand, has been reported that the presence of phosphorylated tau is essential for stress-induced depressive behaviors in mice [73], and a clinical study with depressive patients showed that depression is associated with elevated phosphorylated tau but not with Aβ plaques [80]. Furthermore, phosphorylated tau is increased in stress-induced depression animal models [72, 73]. The present study also showed increased tau phosphorylation at S396 in the hippocampus of young adult depression-induced early onset AD mice, indicating that depression is associated with

phosphorylated tau. However, I could not find any difference in the level of phosphorylated tau in young adult 11-12 mo mice with depression. As revealed in previous studies, phosphorylated tau levels increase with age in the cerebrospinal fluid [81]. Furthermore, misfolding and spreading of tau were found in the hippocampus and the adjacent cortical areas in old mice [82]. Therefore, it is thought that the level of phosphorylated tau in the hippocampus of 11-12 mo mice in this study could be saturated, resulting in a ceiling effect, and made it hard to see the phosphorylated tau level difference between the groups.

Studies have found that tau is expressed in the gut [57], and increased phosphorylation of tau is found in the colon of Crohn's disease patients [83]. In this study, increased tau phosphorylation was found in the ileum and colon of 11-12 mo WT-SUCMS and TG-CTL mice compared to WT-CTL. Additional studies are needed to identify whether it is an enteric nerve-restricted AD pathological phenotype or is a brain-related phenomena. In addition, if the phosphorylated tau in the gut is transferred from the brain, it should be confirmed whether it migrated from the brain to the gut through the vagus nerve or circulation.

In this study, altered gut microbiota composition by depression in young adult mice (young adult depression) induced through SUCMS

were identified. Although it is considered that depression induced by SUCMS caused changes in the microbiota composition in this study, previous reports have revealed that a “bidirectional” communication exists between the brain and gut microbiota [45–47]. Therefore, there is a possibility that the depressive condition induced by SUCMS affected dietary factors and caused the alteration of gut microbiota composition, which subsequently led to young adult depression. However, in the SUCMS pilot study, control and SUCMS mice showed no difference in food consumption, when the same amount of food and water were provided for both groups (data not shown), indicating that dietary factors were not altered by SUCMS. The young adult depression induced by SUCMS resulted in increased serum corticosterone concentration (Fig. 2C), which is presumed to eventually cause the change in gut microbiota composition [46]. Although young adult depression comes prior to the alteration of gut microbiota composition, the altered gut microbiota composition changed by young adult depression may have affected the brain, working as positive feedback. Therefore, further study is needed to confirm the effect of gut microbiota alteration in young adult depression.

The present study focuses on the role of gut microbiota in the depression–induced early onset AD. *Clostridiaceae species incertae*

sedis and *Lactobacillus species incertae sedis* are commonly regulated by both aging and depression. The alteration of these gut microbiota species may have played an important role in young adulthood depression-induced dementia in later life. *Muribaculum intestinale*, the gut microbiota decreased by both AD and depression can be one of the causes of depression-induced early onset AD. Furthermore, *Alistipes finegoldii, Bacteroides* sp., *Bacteroides uniformis, and Bacteroides dorei vulgatus* were commonly found and increased by SUCMS-induced depression in 5 mo and 11-20 mo as shown in Table 1, indicating that these four microbiota species can be considered as an important depression-associated microbiota species. In addition, the amount of *Candidatus Arthromitus* sp., *Lachnospiraceae bacterium 3.2*, *Bacteroidales bacterium M6, Azospirillum species incertae sedis, Bacteroidales bacterium M12, Bacteroidales bacterium M1, Bacteroides caecimuris*, and *Lactobacillus animalis murinus* were increased by SUCMS-induced depression only at 5 mo, which indicates that these gut microbiota species change acutely after depression, but the increase does not last until the later stage.

Interestingly, the gut microbiota altered by young adult depression identified in this study match with depression and AD associated gut microbiota identified in previous studies. Aizawa et al. reported that

the individuals with lower genus *Bifidobacterium* and *Lactobacillus* are more common in patients with depression compared to healthy control subjects [84], while *Bifidobacterium pseudolongum* (included in genus *Bifidobacterium*), *Lactobacillus animalis murinus* (included in genus *Lactobacillus*), and *Lactobacillus species incertae sedis* (included in genus *Lactobacillus*) were found to be decreased by SUCMS-induced young adult depression in this study. Furthermore, Naseribafrouei et al. and Jiang et al. found that the family *Lachnospiraceae*, genus *Prevotella* and *Ruminococcus* were downregulated while the genus *Alistipes* was upregulated in the fecal sample of depressive patients compared to the healthy control subjects [36, 37]. This study also revealed that *Lachnospiraceae bacterium 3.2* and *Lachnoclostridium species incertae sedis* (included in family *Lachnospiraceae*), *Prevotella species incertae sedis* (included in genus *Prevotella*), and *Ruminococcus species incertae sedis* (included in genus *Ruminococcus*) are decreased, while *Alistipes finegoldii* (included in genus *Alistipes*) is increased by SUCMS-induced young adult depression. Also, Zhuang et al. reported that the genus *Lachnoclostridium* was significantly decreased in the fecal sample of AD patients [35], and this study identified that *Lachnoclostridium species incertaesedis* is associated with SUCMS-induced young adult depression.

Intriguingly, several animal model studies have discovered that FMT of normal mice feces, depletion of gut microbiota with antibiotics, and transplantation of certain bacteria can either ameliorate cognition deficit or reduce the Aβ deposit in AD model mice [85–89]. Furthermore, transferring feces of depressive patients to naïve Sprague–Dawley rats induced depressive symptoms [90], and injection of certain bacteria into depression animal model ameliorated depressive behaviors [91, 92]. These studies imply the possibility of alleviating AD and depression symptoms through gut microbiota transplantation. In this study, several gut microbiota species that are associated with depression and AD have been identified. *Muribaculum intestinale* was decreased by both AD and depression, while *Bacteroides dorei vulgatus, Bacteroides uniformis, Bacteroides* sp., and *Alistipes finegoldii* were increased by young adult depression and maintained this status until later stages. Although further studies are needed to confirm the effect of these microbiota, these gut microbiota can be novel therapeutic targets for the treatment and prevention of AD.

Although some studies regarding altered gut microbiota composition in AD patients or animal model have performed taxonomy profiling, their results are limited to genus level as they used 16S rRNA sequencing for the characterization of gut microbiota [35, 38].

However, the present study showed the taxonomy profiling of gut microbiota involved in depression-induced early onset AD at the species level through shotgun metagenomic sequencing. Therefore, specific, and accurate identification of putative therapeutic gut microbiota was available.

This study unveiled the changes in gut microbiota composition and AD pathological characteristics in the brain and gut of depression-induced early onset AD mice. Although this study encompasses high novelty in this field, the findings demand additional research to clarify the mechanism and causal relationship at the molecular level and *in vivo*.

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Health Initiative Scientific Advisory Board, DZNE International Scientific Review Panel (Humboldt), Scientific Advisory Board for UKPRP Air Pollution and Cognitive Health Consortium, and InSPIRE; leadership or fiduciary role in other board, society, committee, or advocacy group, paid or unpaid, with Faculty of Public Health Academic & Research Committee as Chair, Faculty of Public Health Board as a trustee, Public Health England–University of Cambridge Academic Liaison Committee meeting as Chair, and East of England Public Health England Research and Evaluation Hub as co–Chair; all outside the submitted work. I Filip reports payment or honoraria for lectures, presentations, speakers' bureaus, manuscript writing, or educational events from Avicenna Medical and Clinical Research Institute in the form of financial support, outside the submitted work. B J Hall reports consulting fees from WHO, and holds a US S&P index fund and a US Bond Index fund, all outside the submitted work. C Herteliu reports grants from the Romanian National Authority for Scientific Research and Innovation (CNDS–UEFISCDI, project number PN–III–P4–ID–PCCF–2016–0084), outside the submitted work. A Kandel reports grants from the University at Buffalo Clinical and Translational Institute. M Kivimaki reports support for the present manuscript from the MRC (S011676) and the Wellcome Trust (221854/Z/20/Z) as a grant paid to their institution. S

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국문초록

알츠하이머병은 치매 증상을 나타나게 하는 많은 질환 중 유병률 1위를 차지하는 가장 보편적인 질환이다. 알츠하이머병의 주요 신경병리학적 특징은 신경세포 외부에 침착되는 아밀로이드 베타 펩티드가 주성분인 신경반과 신경세포 내부의 신경섬유덩어리이다. 알츠하이머병의 병인기전에 대하여는 수많은 과학자들이 수십년에 걸쳐서 연구해왔으나 다중적 요인들이 작용하고 있는 측면과 더불어 현재 임상 4상에서 임상적으로 사용되는 아두카누맙 이외에는 알츠하이머병의 진행을 늦추거나 막을 수 있는 근본적 약물적 치료 방법은 존재하지 않는 상황이다.

알츠하이머병 환자에 있어서 우울증 및 우울증 이력이 발병 위험성을 높인다는 것이 여러 연구를 통해 밝혀진 바 있다. 또한, 최근 연구들에서 장내미생물과 뇌가 양방향으로 상호작용하며, 여러 뇌 질병에 있어 장내미생물의 변화 등이 중요한 역할을 수행할 수 있음이 보고되었다.

본 연구에서는 알츠하이머병 동물 모델인 APP/PS1 생쥐에서 청년 시기의 만성적 스트레스에 의한 우울증이 알츠하이머병의 발병 시기에 미치는 영향과 장내미생물 및 장 내 변화를 조사/분석하였다. 4개월령 APP/PS1 생쥐와 야생형 생쥐를 사회적 패배를 기반으로 한 만성적인 스트레스에 노출시켜 우울증 증상을 유도하였다. 만성적 스트레스에 의한 우울증이 유도된 APP/PS1 생쥐는 스트레스를 받지 않은 APP/PS1 생쥐에 비교하여 5-6개월령의 시기에 이른 인지기능 저하를 보였으며, 야생형 생쥐는 11-12개월령에 청년 시기의 만성적 스트레스에 의한 우

울증으로 인한 인지기능 저하를 나타내었다. 또한, 우울증이 유도된 5-6개월령 APP/PS1 생쥐의 뇌 내에서 아밀로이드 베타 펩티드 응집의 증가는 나타나지 않았으나 인산화 된 타우가 뇌 해마 부위에서 증가됨을 확인하였다. 뿐만 아니라 11-12개월령 생쥐의 소장과 대장에서도 우울증이 유도된 야생형 생쥐와 APP/PS1 생쥐에서 타우 인산화 증가가 확인되었다. 또한, 샷건 메타지놈 시퀀싱을 통해 장내미생물총의 변화를 종 수준에서 확인하였고 우울증에 의해 여러 장내미생물종이 변화되어 있음을 확인하였다. 특히 *Muribaculum intestinale*는 APP/PS1 동물 모델에서 알츠하이머병과 만성적 스트레스로 인한 우울증이 발현된 생쥐 그룹 모두에서 감소되었다. 이를 통해 본 연구에서는 동물 실험을 통하여 청년 시기의 우울증 경력이 중년기 이후 나타나는 알츠하이머병의 주요한 위험 요인으로 작용할 수 있다는 것을 증명하였으며, 이에는 장내미생물총의 변화가 동반되었다. 이 연구를 통하여 장내미생물총의 변화와 장 내의 타우 병증이 알츠하이머병의 새로운 치료 타겟이 될 수 있다는 점을 시사하는 연구결과를 제시하였다.

주요어 : 알츠하이머병, 우울증, 장내미생물, 사회적 패배를 기반으로 한 만성적인 스트레스, 타우병증

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