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Stress Accelerates Depressive-Like Behavior through Increase of SPNS2 Expression in Tg2576 Mice

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Abstract

To investigate the relationship between depression and AD, water avoidance stress (WAS) was induced for 10 days in both Tg2576 mice and wild-type (WT) mice. After WAS, memory function and depressive-like behavior were investigated in Tg2576 mice. Tg2576 WAS mice exhibited more depressive-like behaviors than WT WAS and Tg2576 control (CON) mice. Strikingly, Tg2576 CON mice showed more depressive-like behaviors than WT mice. Moreover, corticosterone and phospho-glucocorticoid receptor (p-GR) levels were also higher in Tg2576 WAS mice in comparison to Tg2576 CON mice. Spinster homologue 2 (SPNS2) is a member of non-ATP-dependent transporter. The role of SPNS2 was widely known as a sphingosine-1-phosphate (S1P) transporter, which export intracellular S1P from cells. Using GEO database to analyze SPNS2 gene expression changes in patients with AD and depression, we show that SPNS2 gene expression correlates with AD and depression. Interestingly, Tg2576 WAS mice displayed significantly increased levels of SPNS2 w1hen compared to Tg2576 CON counterparts. SPNS2 levels were also higher in Tg2576 CON mice in comparison with WT CON mice. Remarkably, we found a decrease in S1P brain levels and an increase in S1P serum levels of Tg2576 WAS mice in comparison with Tg2576 CON mice. Accordingly, WAS induced group further decreased S1P levels in the brains. However, the level in the serum further increased in comparison with non-induced group. Therefore, these results suggest that AD and depression could be associated, and that Tg2576 transgenic mice are more susceptible to stress-induced depression through the release of S1P by SPNS2 up-regulation.

Key Words: Alzheimer's disease (AD), Depression, Tg2576, SPNS2, S1P

INTRODUCTION

Alzheimer's disease (AD) is characterized by a very slow onset and a gradual progression (Lyketsos *et al*., 2011). In the early stages of the disease, AD patients mainly show memory problems for remembering recent events and as AD progresses, symptoms such as loss of functional abilities, cognitive impairment and behavioral changes begin to emerge (Atri, 2019). Although there is no current cure for AD, there are currently six drugs approved by the United States Food and Drug Administration (FDA) for its treatment: rivastigmine, galantamine, donepezil, and aducanumab which can temporarily reduce symptoms (Orini *et al*., 2022). Many hypotheses about AD have been developed, including extracellular amyloid beta (Aβ) deposition that leads to the formation of senile plaques, accumulation of intracellular tau-containing neurofibrillary tangles (NFTs), neuroinflammation, oxidative stress and cholinergic neuronal damage (Du *et al*., 2018). Also, mutations in genes for the amyloid precursor protein (APP), presenilin-1(PS1), and presenilin-2 (PS2) may lead to autosomal dominant forms of AD (Liu *et al*., 2022). However, the exact pathogenesis and causes of AD are not precisely known

Depression is known as major depressive disorder (MDD) or clinical depression (Otte *et al*., 2016). MDD is characterized by depressed mood (dysphoria) and loss of interest (anhedonia), changes in appetite, sleeping problems, difficulty thinking and thoughts of death for a duration of at least two weeks (Fekadu *et al*., 2017). Stressful life events, adverse childhood

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experiences, certain personality traits and genetic factors may all be causing factors of MDD (Burke *et al*., 2019). Globally, MDD is a serious public health problem with very high prevalence (Jacob, 2012). Studies addressing the pathophysiology of depression found changes in monoaminergic neurotransmission, imbalances in excitatory and inhibitory signaling in the brain, hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, decreased levels of brain 5-hydroxytryptamine (5-HT) and abnormalities in normal neurogenesis (Krishnan and Nestler, 2008). Moreover, clinical research indicates that MDD patients display a 19% smaller left hippocampal volume when compared to control subjects. However, there were no differences between the two groups in other regions of whole brain (Bremner *et al*., 2000).

Depression is widely known as one of the most common comorbid psychiatric disorders in AD (Rovner *et al*., 1989). Many studies have reported that, in fact, depression acts as a risk factor for AD, with a common pathophysiological mechanism between the two diseases having been elucidated (Caraci *et al*., 2010). Chronic inflammation, down regulation of neurotrophic signaling, and chronic activation of microglia and astroglia are some of the most common pathophysiological elements of AD and depression (Charney and Manji, 2004; Caraci *et al*., 2010; Dafsari and Jessen, 2020). Moreover, many studies indicate that exposure to chronic stress leads to progression of AD and depression (Charney and Manji, 2004; De Kloet *et al*., 2005; Butterfield *et al*., 2007). Chronic stress induces the secretion of adrenocorticotrophic hormone releasing factor (CRF) and vasopressin (AVP) from the hypothalamus, which in turn activates the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary, stimulating the secretion of glucocorticoids (GCs) from the adrenal cortex. High level of GCs activates GR (Pariante and Lightman, 2008), reduce neurogenesis in the hippocampal dentate gyrus, and cause neuronal death in hippocampal neurons (Sapolsky, 1986; Krishnan and Nestler, 2008; Frodl and O'Keane, 2013).

The role of sphingolipid transporter 2 (SPNS2) was first studied in zebrafish (Osborne *et al*., 2008). Based on mutagenesis studies in zebrafish, SPNS2 in the yolk syncytial layer (YSL) was found to function as a sphingosine-1-phosphate (S1P) transporter (Kawahara *et al*., 2009). Moreover, the role of SPNS2 in S1P transporter was studied in SPNS2 knockout (KO) mice, showing that restoration of lymphocytes from the spleen to the lymph nodes and loss of circulating lymphocytes were the same as blocking exit from lymph nodes directed by lymph S1P and plasma S1P (Mendoza *et al*., 2012). SPNS2 deficiency in SPNS2KO mice leads to reduced circulating lymphocytes, early-onset hearing loss, and cataracts (Nijnik *et al*., 2012; Nagahashi *et al*., 2013; Chen *et al*., 2014). Also, the downregulation of SPNS2 shows an anti-fibrotic and anti-inflammatory effect in proximal tubular epithelial cells as well as enhanced proliferation, migration, invasion, and metastasis of colorectal cancer (CRC) cells through activation of AKT and ERK signaling pathway (Blanchard *et al*., 2018; Lv *et al*., 2021). Moreover, lipopolysaccharide (LPS) or amyloid beta peptide 1-42 oligomers (Aβ42) treatment in primary cultures of microglia derived from SPNS2 KO mice reduced the levels of pro-inflammatory cytokines when compared with control cultures (Zhong *et al*., 2019). However, little is known about the function of SPNS2 in AD and depression pathogenesis.

Sphingosine-1-phosphate (S1P) is widely known for regulating survival and differentiation as well as for its involvement in cell migration, proliferation, and autophagy (Hla, 2003). The neuroprotective levels of S1P were found to be reduced in the brains of neurodegenerative disorders such as AD (van Echten-Deckert *et al*., 2014). In line with this, it was also discovered that S1P stimulates cortisol secretion in zona fasciculata cells of bovine adrenal glands through G protein-coupled receptors, protein kinase C (PKC), and extracellular calcium (Rábano *et al*., 2003). Moreover, repeated stress in rats was shown to increase the levels of S1P in serum and promote changes in iNOS, GFAP and NR1 expression in various brain regions (Jang *et al*., 2008). Interestingly, it was observed that knockdown of SPNS2 leads to increased extracellular levels of S1P in A549 lung cancer cells (Bradley *et al*., 2014). Moreover, cultured microglia from SPNS2 KO mice showed reduced S1P levels in the media and lower level of pro-inflammatory cytokines compared to littermate normal microglia (Zhong *et al*., 2019).

The Relationship between AD and depression remains undiscovered. However, SPNS2 transports S1P out of the cell, allowing S1P to bind with S1P receptors (S1PR) (Lucaciu *et al*., 2020). The binding of S1P to S1PR can stimulate the production of cytokines that promote inflammation, leading to the persistence or amplification of the inflammatory response (Obinata and Hla, 2019). This can exacerbate the symptoms of depression and dementia (Leonard, 2007). Thus, SPNS2 and S1P may play a potential role in both depressive-like behavioral and AD.

In the present study, we investigated whether stress-induced depression could be more severe in Tg2576 transgenic mice, a widely used AD mouse model, and the comorbidity mechanisms between AD and depression through SPNS2 up-regulation.

MATERIALS AND METHODS

Ethical statement

The experimental protocols were carried out according to the guidelines for animal experiments of the Faculty of Disease Animal Model Research Center, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea) as well as Institutional Animal Care and Use Committee (IACUC) of Laboratory Animal Research Center at Chungbuk National University, Cheongju, Korea (ethics approval No. CB-NUA-1634-21-01). All efforts were made to minimize animal suffering and to reduce the number of animals used. All mice were housed in cages that were automatically maintained at 21-25°C and relative humidity of 45-65% with controlled 12 h light-dark cycle illuminating from 6 a.m. to 6 p.m. Food and water were provided ad libitum. They were fed a pellet diet consisting of crude protein 20.5%, crude fat 3.5%, crude fiber 8.0%, crude ash 8.0%, calcium 0.5%, phosphorus 0.5% per 100 g of the diet (obtained from Daehan Biolink, Eumseong, Korea).

Animal

Twelve month old Tg2576 mice were maintained and handled in accordance with the humane animal care and use guidelines of the Ministry of Food and Drug Safety. Tg2576 mice harboring human APP695 with Swedish double mutation (hAPP; HuAPP695; K670N/M671L) were purchased from Taconic Farms (Germantown, NY, USA), and the strain was

maintained in the animal laboratory at Chungbuk National University. The mice were randomly divided into four groups with 6 mice in each group (n=6, ♂3, ♀3): (I) the wild type control group; (II) the wild type stress induced group; (III) the Tg2576 control group; (IV) the Tg2576 stress induced group. Each group Before proceeding behavioral tests, water avoidance stress (WAS) was induced to mice for 10 days. The behavioral tests of learning and memory capacity were assessed using the water maze, probe, and object location memory tests. And depression related behavioral tests of Novelty-suppressed feeding test, tail suspension test, forced swimming test, sucrose preference test was assessed. Mice were sacrificed after behavioral tests by CO2 asphyxiation.

Water avoidance stress (WAS)

All stress sessions were performed at the same period of the day (between 10:00 am and 12:00 pm) to minimize the influence of circadian rhythms. Mice were placed on a 3×3-cm platform fixed in the center of a 40×40-cm pool filled with cold water for 1 h for 10 consecutive days. Control mice groups were submitted to a similar procedure using an empty pool. All the mice were kept in pairs in their home cage and placed individually in their WAS cage. This well characterized test represents a potent psychological stressor with large elevations of ACTH and corticosterone within 30 min (Million *et al*., 1999).

Morris water maze

The water maze test is a commonly accepted method for memory test, and I performed this test as described by (Morris, 1984). Maze testing was carried out by the SMART-CS (Panlab, Barcelona, Spain) program and equipment. A circular plastic pool (height: 35 cm, diameter: 100 cm) was filled with water made opaque with skim milk kept at 22-25°C. An escape platform (height: 14.5 cm, diameter: 4.5 cm) was submerged 1-1.5 cm below the surface of the water in position. Testing trials were performed on a single platform and at two rotational starting positions. After testing trial, the mice were allowed to remain on the platform for 120 s and were then returned to their cage. Escape latency and escape distance of each mouse was monitored by a camera above the center of the pool connected to a SMART-LD program (Panlab).

Probe test

To assess memory retention, a probe test was performed 24 h after the water maze test. The platform was removed from the pool which was used in the water maze test, and the mice were allowed to swim freely. The swimming pattern of each mouse was monitored and recorded for 60 s using the SMART-LD program (Panlab). Retained spatial memory was estimated by the time spent in the target quadrant area.

Passive avoidance test

The passive avoidance test is generally accepted as a simple method for testing memory. The passive avoidance response was determined using a "step-through" apparatus (Med Associates Inc., Vermont, USA) that is divided into an illuminated compartment and a dark compartment (each 20.3×15.9×21.3 cm) adjoining each other through a small gate with a grid floor, 3.175 mm stainless steel rods set 8 mm apart. On the first day, the mice were placed in the illuminated compartment facing away from the dark compartment for the training trial. When the mice moved completely into the dark compartment, it received an electric shock (0.45 mA, 3 s duration). Then the mice were returned to their cage. One day after training trial, the mice were placed in the illuminated compartment and the latency period to enter the dark compartment defined as "retention" was measured. The time when the mice entered into the dark compartment was recorded and described as step-through latency. The retention trials were set at a cutoff time limit of 3 min.

Novelty-suppressed feeding

The novelty-suppressed feeding test (NSF) is often used as a measure of depression-like behaviors. Like the open field test, the NSF test is based on rodents' innate fear of novel spaces. However, the NSF test introduces an additional component of motivation, as the food-deprived animal's drive to eat conflicts with its fear of novel open spaces. Mice were food-deprived 24 h prior to the test, with free access to water, and were moved to the dimly lit testing room one to two hours before the test. Mice were placed into one corner of an open field apparatus (17 in.×17 in.×12 in.) with clear acrylic walls and an opaque white acrylic floor. A food pellet was placed in the center of the open field and animals were placed in one corner. Latencies to approach and to begin eating were recorded with a limit of 15 min. As soon as the mouse was observed to eat, or the 15-min time limit was reached, the mouse was removed from the open field and placed in the home cage and observed until it began to eat in the home cage.

Sucrose preference test

For the Sucrose preference test (SPT), the mice were simultaneously exposed to tap water and 1% sucrose solution for 48 h, followed by 24 h of water and food deprivation. They were then exposed to two identical bottles for 1 h, one filled with the 1% sucrose solution and the other filled with tap water. The sucrose and water consumption were determined by measuring the volume of liquid consumed. The sucrose preference is defined as the ratio of the volume of sucrose solution consumed to the total volume of sucrose solution and water consumed over an hour.

Tail suspension test

TST was performed as described in the references (Ruan *et al*., 2015). Briefly, animals were suspended above the floor and recorded by a video camera for 6 min. The duration of immobile behavior was manually measured blinding to the treatment. The increase of immobility indicated the depression-like behavior.

Forced swim test

FST was performed as described in the references (Ruan *et al*., 2015). Briefly, animals were placed in a cylinder containing water and recorded by a video camera for6 min. The duration of climbing and immobile behaviors was manually measured in the first 2 min and last 4 min respectively blinding to the treatment. The increase of immobility and decrease of climbing both indicated the depression-like behaviors.

Collection and preservation of brain tissues

After behavioral tests, mice were perfused with PBS with heparin under inhaled CO2 anesthetization. The brains were immediately removed from the skulls, after that, only the hippocampus region was isolated and stored at –80°C until bio-

chemical analysis.

Western blot analysis

Western blotting was performed as described. To detect target proteins, specific antibodies against BACE1 and APP (1:1000; Abcam, Inc., Cambridge, UK), SPNS2, P-GR, PSEN2 (1:500; Cell Signaling Technology, Inc., Danvers, MA, USA), GR, and β-actin (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used. The blots were then incubated with the corresponding conjugated secondary antibodies such as anti-mouse, anti-rabbit, and anti-goat purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Immunoreactive proteins were detected with an enhanced chemiluminescence Western blotting detection system.

Immunohistochemistry

The brains were collected from mice following perfusion and immediately fixed in 4% paraformaldehyde for 24 h. The brains were transferred successively to 10%, 20% and 30% sucrose solutions. Subsequently, brains were frozen on a cold stage and sectioned in a cryostate (20 μm-thick). Sections were treated with endogenous peroxidase (3% H2O2 in PBS), followed by an additional two washes in PBS for 10 min each. The brain sections were blocked for 1 h in 3% bovine serum albumin (BSA) solution and incubated overnight at 4°C with S1P transporter 2 (SPNS2; 1:200; Cell Signaling Technology, Inc.), glucocorticoid receptor phosphorylation at Serine 211 (1:500; Santa Cruz Biotechnology Inc.). After incubation with the primary antibodies, brain sections were washed three times in PBS for 10 min each. After washing, brain sections were incubated for 1-2 h at room temperature with the biotinylated goat anti-rabbit, goat anti-mouse, or donkey anti-goat IgG-horseradish peroxidase (HRP) secondary antibodies (1:500; Santa Cruz Biotechnology, Inc.). Brain sections were washed three times in PBS for 10 min each and visualized by a chromogen diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) reaction for up to 10 min. Finally, brain sections were dehydrated in ethanol, cleared in xylene, mounted with Permount (Fisher Scientific, Hampton, NH, USA), and evaluated on a light microscope (Microscope Axio Imager. A2; Carl Zeiss, Oberkochen, Germany; ×50 and ×200).

Measurement of SPNS2 levels

Lysates of brain tissue were obtained through a protein extraction buffer containing protease inhibitor. S1P and SPNS2 levels were assessed utilizing a commercially available enzyme-linked immune-sorbent assay (ELISA) kit obtained from Koma Biotech Inc. (Camarillo, CA, USA, total tau: #KMB7011), MyBioSource Inc. (Sandiego, CA, USA, S1P: #MBS7269992, SPNS2: #MBS9363777), Protein was extracted from brain tissues using a protein extraction buffer (PRO-PREP; Intron Biotechnology, Seongnam, Korea), incubated on ice for 1 h, and then centrifuged at 13,000×g for 15 min at 4°C. In brief, 50 μL of sample was added into a pre-coated plate and incubated for 2 h at 37°C. After removing any unbound substances, a biotin-conjugated antibody specific for DVL3 was added to the wells. After washing, avidin-conjugated horseradish peroxidase (HRP) was then added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount tau bound in the initial step. Following the addition of stop-solution the optical density was measured at 450 nm in a Molecular Devices VersaMax (Molecular Devices, San Jose, CA, USA).

Serum collection and serum ELISA assay

Whole blood collected from mice was processed within 2 h of collection using the following protocol. Collected blood was centrifuged at 1000×g for 15 min at room temperature, after which the serum supernatant was aspirated and transferred to a new tube. This was subsequently aliquot into Eppendorf tubes and stored at –80°C until analyzed. An ELISA was used to measure cortisol level in the serum, according to the manufacturer's instructions.

Selection of GEO dataset

Gene expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) is a database archives and freely distributes microarray, next-generation sequencing (NGS) and other forms of high-throughput functional genomic data (Barrett *et al*., 2012). The following keywords were used in the GEO database: (Dementia) AND (Alzheimer's disease) AND (Depression) And (Whole brain). The gene expression profile of GSE117589 and GSE101521 were obtained from GEO database. On GSE117589 dataset, I used sporadic AD (SAD) and control induced pluripotent stem cells (iPSCs) for analysis of AD related genes. Also, GSE101521 was selected to analysis MDD related gene expression compared with non-psychiatric controls and DSM-IV non-suicides MDD patients.

Gene ontology enrichment and target prediction analysis

GSE117589 was analyzed on the GPL25371 Platform Affymetrix Human Genome U133 Plus 2.0 by using GEO2R web application (http://www.ncbi.nlm.nih.gov/geo/geo2r/). Moreover, GSE101521 database was analyzed by using platform GEO RNA-seq Experiments Interactive Navigator (GREIN, http://www.ilincs.org/apps/grein/). I analyzed this data as described in the reference (Mahi *et al*., 2019).

Statistical analysis

The data were analyzed using the GraphPad Prism software (Version 4.03; GraphPad software, Inc., San Diego, CA, USA). Data are presented as mean ± SEM. The differences in all data were assessed by two-way analysis of variance (ANOVA). When the p value in the Student's t-test indicated statistical significance, the differences were assessed by the Dunnett's test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Depression-related behaviors caused by chronic stress in Tg2576 mice

It is widely known that chronic stress is used to induce a depression experimental model. In this study, we induced water avoidance stress to the Tg2576 AD model mice for 1 h for 10 consecutive days. After 10 days of stress, a series of behavioral tests were performed to validate depressive-like behavior, learning ability, and memory in these mice (Fig. 1A). Depression in Tg2576 mice was evaluated by the novelty-suppressed feed test (NSF). Compared to WT WAS mice and WT CON mice, latency increased by approximately 13.9%, and in Tg2576 WAS mice, latency was increased by 14.2%

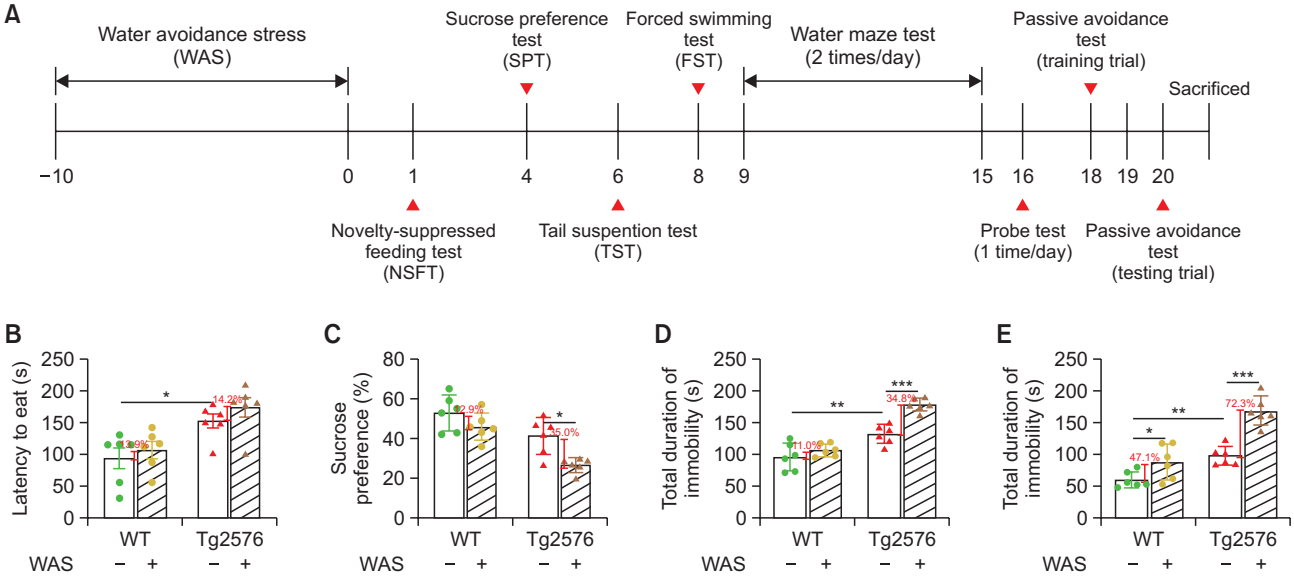

Fig. 1. Depression-related behavior caused by chronic stress in Tg2576 mice. The timeline shows the experimental schedule for water avoidance, depressive-related behavior tests and AD-related behavior tests (A). Validation of depressive-related behavior in Tg2576 AD mouse model, through novelty-suppressed feeding test (NSF) (B), sucrose preference test (SPT) (C), tail suspension test (TST) (D), and forced swimming test (FST) (E). Depressive-like behavior in WT CON mice, WT WAS mice, Tg2576 mice, Tg2576 WAS mice was evaluated by the total duration of latency to feed time in the NSF (B, sec), percentage of sucrose preference in the SPT (C, %). Total duration of immobility in the TST (D, s) and latency to first immobility time (E, s). Data is presented as mean ± S.E.M. from n=6. Statistical significance was set at **p*<0.05, ***p*<0.01, ****p*<0.001 in comparison to WT group.

compared to Tg2576 CON mice (Fig. 1B) (n=5, ns). In order to evaluate anhedonia, a sucrose preference test (SPT) was conducted. In WT WAS mice, sucrose preference (%) was decreased by 12.9% compared to WT CON mice, and in Tg2576 WAS mice, preference was reduced by 35% compared to Tg2576 CON mice (Fig. 1C) (n=5, *p*=0.0199, respectively). Next, tail suspension (TST) and forced swimming (FST) tests were conducted to measure the coping ability of Tg2576 WAS mice. In the TST, immobility was increased by 11% in WT WAS mice when compared to WT CON mice, and by 34.8% in Tg2576 WAS mice when compared to Tg2576 CON mice (Fig. 1D) (n=5, *p*<0.001, respectively). Similarly, in the FST, immobility was increased by 47.1% in WT WAS mice compared to WT CON mice, and by 72.3% in Tg2576 WAS mice compared to Tg2576 CON mice (Fig. 1E) (n=5, *p*<0.001, respectively).

Corticosterone serum level of Tg2576 WAS mice

Stress triggers the hypersecretion of GCs, which is considered a major biological risk factor of depression (Chojnowska *et al*., 2021) Furthermore, phosphorylation of GRs needs to occur at one of its multiple serine residues to bind with GCs (Ismaili and Garabedian, 2004). Therefore, in the case of depression, GR phosphorylation is increased by high levels of GCs (Rogatsky *et al*., 1998; Pariante and Miller, 2001). An ELISA assay was performed to measure the corticosterone levels in the serum of Tg2576 mice. Corticosterone levels of WT WAS mice increased by 23.1% compared to WT CON mice, whereas it increased by 37.8% in Tg2576 WAS mice compared to Tg2576 CON mice (Fig. 2A) (n=6, *p*=0.03, respectively). The hippocampus is a brain region deeply involved not only in learning and memory, but also in the regulation of depression and stress responses. In the context of depression, research often focuses on the hippocampus because it plays a crucial role in the body's response to stress. Thus, Western blot was performed to measure the levels of phosphorylated GR (p-GR) and GR in mouse hippocampal area. Tg2576 WAS mice had increased levels of p-GR compared to the Tg2576 CON mice and WT WAS mice (Fig. 2B) (n=3 ns). To confirm the protein expression level of p-GR, immunohistochemistry was performed in the mouse hippocampus. Tg2576 WAS mice showed a higher expression level of p-GR compared with Tg2576 CON mice and WT WAS mice (Fig. 2C).

AD-related behavior in Tg2576 WAS mice

To investigate spatial learning ability and memory loss in Tg2576 WAS mice, the Morris water maze test, probe test and passive avoidance test were performed sequentially (Fig. 3). During the training period, Tg2576 WAS mice exhibited longer escape latency but there was no significant difference in the distance traveled. On the final day, Tg2576 WAS mice showed an escape latency and swimming distance of approximately 51.5 s and 1949.3 cm, respectively, whereas WT WAS mice displayed 28 s and 1583 cm in the same parameters (Fig. 3A, 3B) (n=5, *p*=0.028, respectively). The day after water maze test (day 8), a probe test was performed to measure the time spent in the target of quadrant zone to assess memory function. The time spent in the target of quadrant zone decreased in Tg2576 WAS mice (16.44%) compared with WT WAS mice (24.75%) (Fig. 3C) (n=5, *p*=0.0067, respectively), suggesting that Tg2576 WAS mice spend more time finding the platform than WT WAS mice. Moreover, Tg2576 WAS mice showed more immobility during their performance, which led to shorter swimming distances compared with Tg2576 CON mice and WT WAS mice. Next, the passive avoidance test was conducted to validate short-term memory and memory retention ability of Tg2576 WAS mice. There were no significant changes

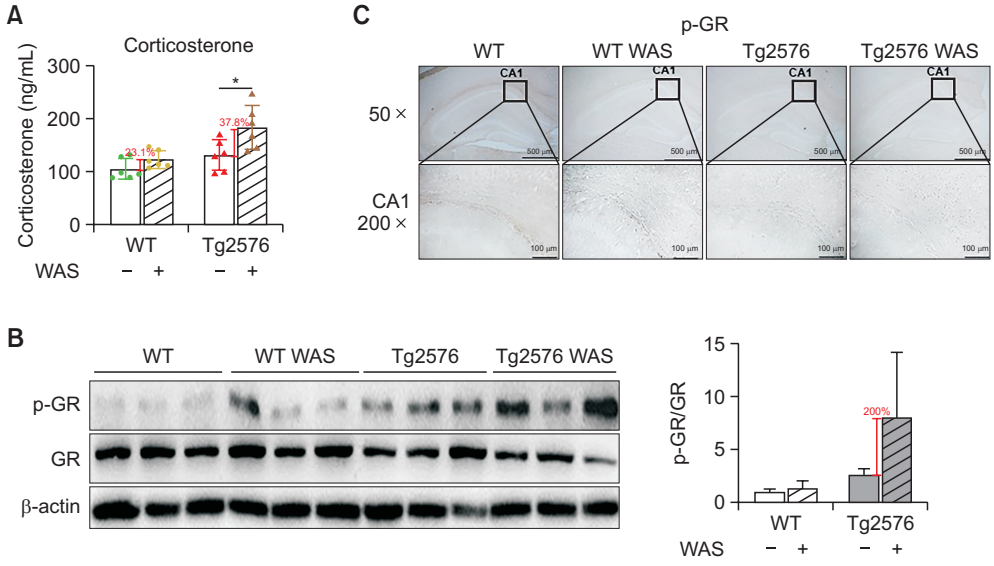

Fig. 2. Corticosterone serum level of Tg2576 WAS mice. ELISA was performed to determine corticosterone levels (A). Western blotting was performed to ascertain depression-related protein expression levels (B). Immunostaining of p-GR in the hippocampus was performed in 10 μm-thick sections of mice brain (C). Data is presented as mean ± S.E.M. from n=6. Statistical significance was set at **p*<0.05 in comparison to WT group.

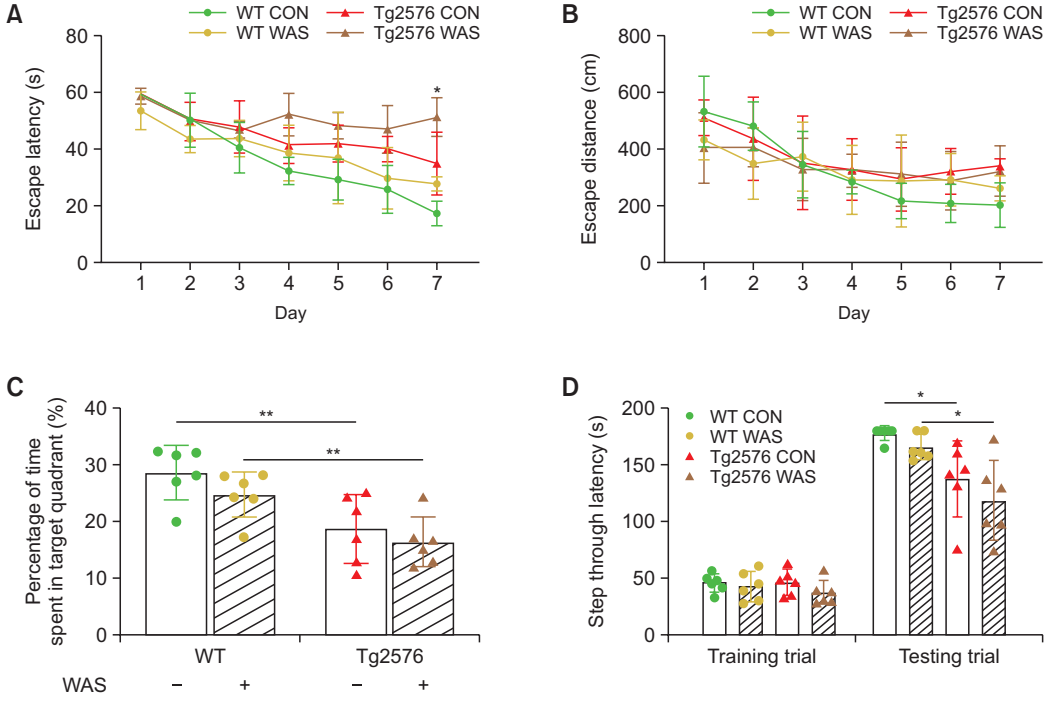

Fig. 3. AD-related behavioral alterations in Tg2576 WAS mice. To assess spatial learning ability and memory loss in the Tg2576 WAS mice, Morris water maze test (A, B), probe test (C) and passive avoidance test (C) were performed. Memory and spatial learning ability in Tg2576 WAS mice and WT WAS mice were evaluated using escape latencies (A, sec) and escape distance (B, cm) for 7 days; In both Morris water maze test (A, B), the X-axis represents the number of days, indicating the progression of time throughout the experiment. After the water maze test, measured time spent in target quadrant (C, %) by probe test for 1 day. In the passive avoidance test, latency was considered for analysis (D, sec). Data is presented as mean ± S.E.M. from n=6. Statistical significance was set at **p*<0.05, ***p*<0.01 in comparison to WT group.

regarding latency. However, in the test trial, Tg2576 WAS mice showed a decreased latency (116.43 s) compared with WT WAS mice (167.3 s) (Fig. 3D) (n=5, p=0.0231, respectively).

Aβ protein levels in the brains of Tg2576 WAS mice

The Aβ peptide, derived from amyloid precursor protein (APP), forms senile plaques, which are considered biomarkers of AD. To measure the protein expression levels of Aβ, ELISA assay was performed in the Tg2576 WAS mice brains. The expression levels of $Aβ_{(1-40)}$ in Tg2576 WAS mice had no significant difference compared with Tg2576 CON mice. However, WT WAS mice showed an increased expression of $Aβ_{(1-42)}$ by 14.2% when compared to WT CON mice, while Tg2576 WAS mice displayed a 27%-increased compared to Tg2576 CON mice (Fig. 4A) (n=5, p=0.0238, respectively). Next, western blot was performed to measure the expression levels of AD-related proteins, having found no significant difference between Tg2576 WAS mice and Tg2576 CON mice (Fig. 4B) (n=5, ns).

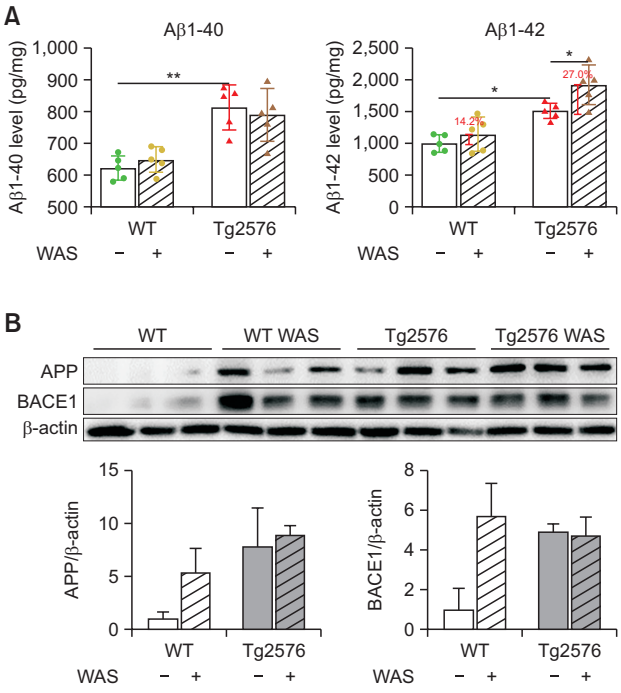

Fig. 4. Aβ protein levels in the brains of Tg2576 WAS mice. ELISA was performed to measure the expression levels of Aβ in mice brains (A). Western blot was performed to measure Aβ-related proteins in mouse brains (B). Data is presented as mean ± S.E.M. from n=5. Statistical significance was set at *p<0.05, **p<0.01 in comparison to WT group.

SPNS2 expression correlates with AD and depression

Based on the GEO database, SPNS2 expression in both AD and depression was analyzed using gene expression profile of GSE117589 (Supplementary Table 1) and GSE101521 (Supplementary Table 2). On the GSE117589 dataset, sporadic AD (SAD) and control iPSCs were used to identify SPNS2 gene expression in AD. Date from subjects between the ages of 60 and 80 were considered, regardless of gender. Moreover, AD patients showed an increase in the expression levels of SPNS2 when compared with controls (Fig. 5A). Next, on the GSE101521 dataset, non-psychiatric controls and DSM-IV non-suicides MDD patients were considered. Data from subjects older than 19 years of age were considered, regardless of gender. Similarly, and as described above, SPNS2 expression matched with statistical significance. It was confirmed that SPNS2 was increased DSM-IV non-suicides MDD patients compared with non-psychiatric controls (Fig. 5B). To confirm the relationship between SPNS2, depression and AD, we used the Comparative Toxicogenomics Database (http://ctdbase.org). The inference scores of SPNS2 related to cognition disorders and learning disabilities were 87.08 and 82.71, respectively, and depressive disorder has scored 66.28 (Fig. 5C).

Brain and serum expression levels of SPNS2 and S1P of Tg2576 WAS mice

The role of SPNS2 in depression and AD is not well known. However, the correlation analysis of SPNS2 expression in AD and MDD patients above mentioned shows higher expression levels of SPNS2 in samples of these patients when compared with controls. Comparably, western blot analysis also showed the Tg2576 WAS mice brain samples display much higher expression levels of SPNS2 than those of Tg2576 CON, WT CON and WT WAS mice (Fig. 6A). Accordingly, the brains of the Tg2576 WAS mice showed higher numbers of SPNS2-

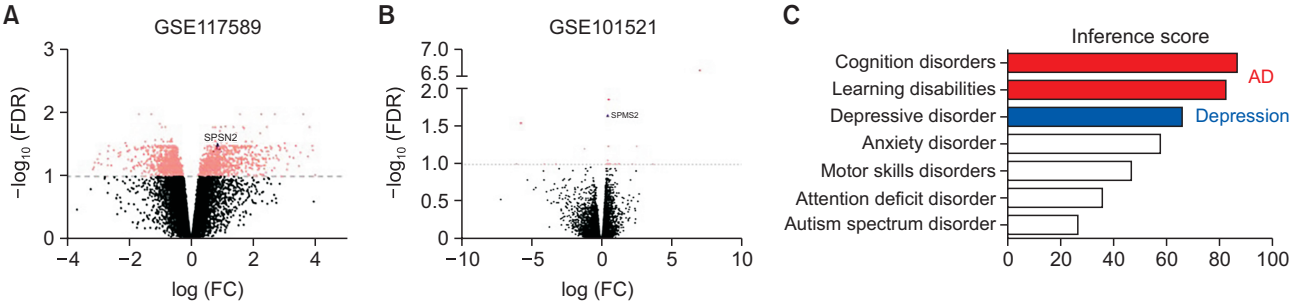

Fig. 5. SPNS2 expression correlates with AD and depression. The gene expression profile of GSE117589 and GSE101521 datasets were obtained from GEO database. GSE117589 was used to identify SPNS2 gene expression levels of AD patients and corresponding controls (A). GSE10521 was used to identify SPNS2 gene expression levels of DSM-IV MDD patients and corresponding controls (B). The CTD database was used to determine the relationship between SPNS2, depression and AD (C). SPNS2 matched with statistical significance by Benjamini-Hochberg FDR <0.1 and CPM <0 in both datasets.

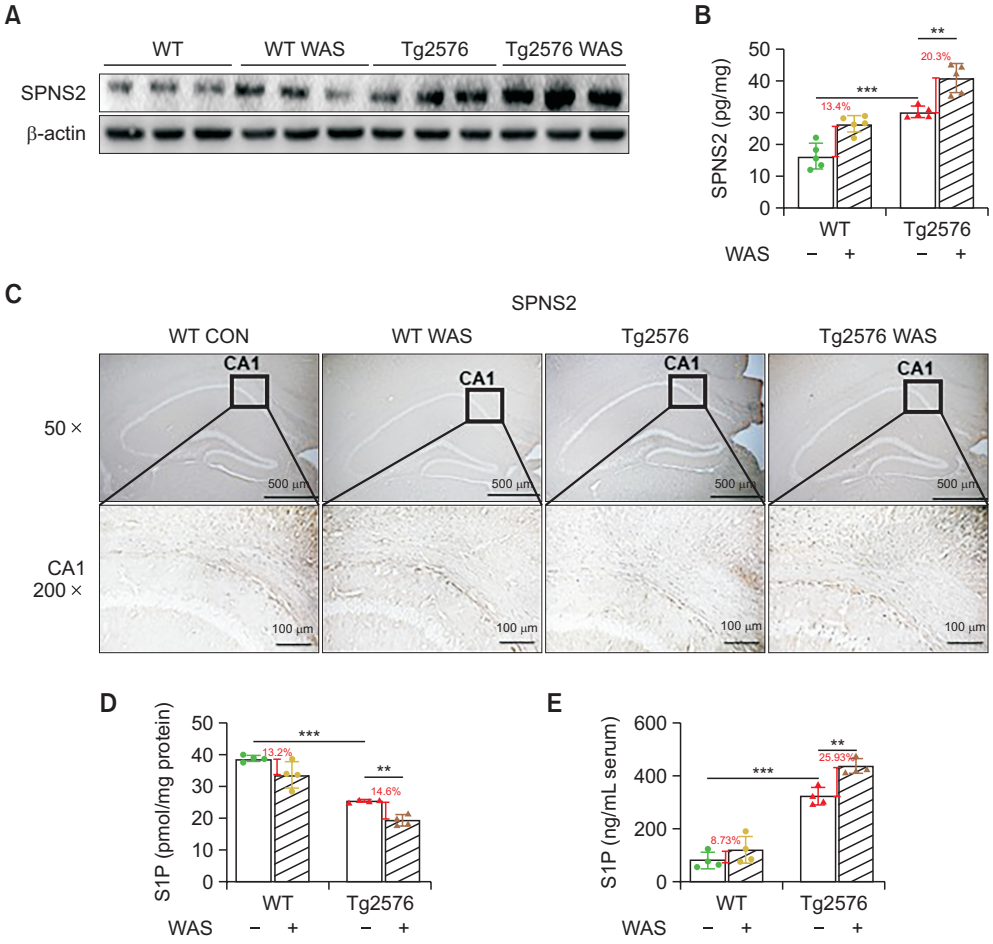

Fig. 6. Brain and serum expression levels of SPNS2 and S1P of Tg2576 WAS mice. Western blot was performed to determine SPNS2 protein levels (A). ELISA was performed to assess SPNS2 expression levels (B). Data is presented as mean ± S.E.M. from n=5. Statistical significance was set at **p*<0.05, ***p*<0.01, ****p*<0.001 in comparison to WT group. Immunostaining of SPNS2 was conducted in 10 μm-thick hippocampal sections of mice brains (C). ELISA was conducted to ascertain S1P in brain (D) and serum (E) levels of Tg2576 WAS mice. Data is presented as mean ± S.E.M. from n=4. Statistical significance was set at ***p*<0.01, ****p*<0.001 in comparison to WT group.

immuno reactive cells compared to those of Tg2576 CON, WT CON and WT WAS mice (Fig. 6B) (n=5, *p*=0.0015, respectively). In line with this, ELISA experiments showed that Tg2576 WAS mice brains had increased SPNS2 expression levels compared to Tg2576 CON, WT CON and WT WAS mice brains (Fig. 6C). Interestingly, analysis of post-mortem brain tissue of AD patients showed a reduced level of S1P (He *et al*., 2010). On the other hand, analysis of cerebrospinal fluid (CSF) in mild cognitive impairment patients showed elevated S1P levels (Ibáñez *et al*., 2013). However, the role of S1P is not known in depression. An ELISA assay was used to measure S1P levels in Tg2576 WAS mice brains and serum. In the brains of Tg2576 WAS mice, the levels of S1P were decreased by 14.6% compared to Tg2576 CON mice (Fig. 6D) (n=3, *p*=0.0063, respectively). Contrastingly, the levels of S1P were increased in serum samples of Tg2576 WAS mice compared to Tg2576 CON mice by (25.93%) (Fig. 6E) (n=4, *p*=0.0019, respectively). In Tg2576 mice without WAS, depressive-like behavior increased by approximately 50%, whereas the expression of SPNS2 and S1P was significantly elevated by 170%. Thus, the protein expression levels were approximately three times higher than the depressive-like behavior. It is believed that SPNS2 and S1P levels may partially contribute to the development of depressive-like behavior.

Expression levels of SPNS2 and S1P in primary cell cultures from mice.

Primary cell cultures were used to address SPNS2 mechanisms of action in depression and AD. Cells were treated with $A\beta_{(1-42)}$ (2 μM) and corticosterone (100 μM) to recapitulate AD and depressive-like phenotypes, respectively. Western blot was performed to determine the expression levels of SPNS2, APP, and p-GR. Accordingly, co-incubation with both drugs led to an increased expression of SPNS2 and APP when compared to treated individually (Fig. 7A). Moreover, co-incubation with both drugs also led to an increased expression of SPNS2 in ELISA (Fig. 7B) (n=3, *p*=0.0119, respectively). To confirm that SPNS2 mediates the export of S1P from endothelial cells, an ELISA assay was used to assess S1P levels in both cell lysates and culture media. S1P levels were decreased in cell lysates co-treated with $A\beta_{(1-42)}$ and corticosterone comparison to individual treatment of $A\beta_{(1-42)}$ and corticosterone (n=5, ns)

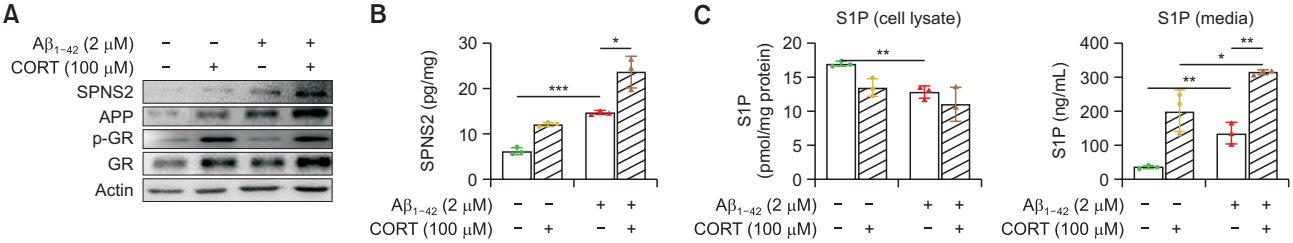

Fig. 7. Expression levels of SPNS2 and S1P in primary cell cultures from mice. Western blot was performed to validate expression levels of SPNS2, APP, and p-GR (A). ELISA was performed to assess the expression levels of SPNS2 (B). Data is presented as mean ± S.E.M. from n=4. Statistical significance was set at **p*<0.05, ***p*<0.01, ****p*<0.001 in comparison to WT group. ELISA was conducted to determine S1P levels in cell lysate (C) and culture media (D). Data is presented as mean ± S.E.M. from n=3. Statistical significance was set at **p*<0.05, ***p*<0.01, ****p*<0.001 in comparison to WT group.

(Fig. 7C). However, S1P levels in the culture media from cells co-treated with $A\beta_{(1–42)}$ and corticosterone were much higher compared to individual treatments with $A\beta_{(1–42)}$ (n=3, *p*=0.0049) and corticosterone (n=3, *p*=0.0322) (Fig. 7D).

DISCUSSION

Depression is a common psychiatric disorder in people diagnosed with AD (Gutzmann and Qazi, 2015). As such, several studies indicate that depression is one of the most frequent comorbid disorders in AD (Moretti *et al*., 2002; Verkaik *et al*., 2007). Moreover, depression symptoms are known as risk factors for development of AD (Jorm, 2000). These reports suggest the existence of common neuropathological mechanisms between depression and AD. In the present study, experiment was conducted to address how depression may have an effect in an AD mouse model. Water avoidance stress (WAS) was given to Tg2576 mice. WAS is a well-known animal model of psychological stress in rodents which accelerates depressive-like phenotype (Hassan *et al*., 2014). Our results show that Tg2576 WAS mice were more prone to depressive-like behaviors than Tg2576 CON mice and WT WAS mice. Furthermore, Tg2576 WAS mice appear to have much more severe memory dysfunction than Tg2576 CON mice. Moreover, Aβ protein in the brains of Tg2576 WAS mice was higher compared with Tg2576 CON counterparts. These results suggest that stress enhances depressive-like and AD behavioral and molecular hallmarks much more severely in Tg2576 mice.

Increasing evidence has shown that stress triggers depressive episodes and plays a critical role as a risk factor in AD by the high secretion of cortisol and activation of GRs, which eventually may lead to neurodegeneration in AD (Aznar and Knudsen, 2011; Ouanes and Popp, 2019). Chronic stress activates the secretion of CRF and AVP from the hypothalamus. The secretion of adrenocorticotrophic hormone (ACTH) from the pituitary, finally stimulates the secretion of the GCs from the adrenal cortex and high level of GCs activates GR (Pariante and Lightman, 2008), reduces neurogenesis in the hippocampal dentate gyrus, and cause neuronal death in hippocampal neurons (Sapolsky, 1986; Krishnan and Nestler, 2008; Frodl and O'Keane, 2013). For this reason, GCs and GR are used as a fingerprint of stress (Chojnowska *et al*., 2021). In the present study, Tg2576 WAS mice showed higher corticosterone levels in serum and expression levels of GR and p-GR in brains than those in Tg2576 CON and WT WAS mice. Moreover, *in vitro* results confirmed that p-GR expression levels were increased in response to corticosterone treatment. These results suggest that the stress-mediated severe depression could be associated with increased corticosteroid levels and effect more pronounced in Tg2576 AD mice than WT mice.

The mechanistic connection between SPNS2 and either depression or AD has not yet been elucidated, but several studies have suggested that SPNS2 is a critical factor in the development of depression (Hasan *et al*., 2022; Martín-Hernández *et al*., 2023). In the GEO database was analyzed to investigate gene expression profiles of both AD and depression samples. Using GSE117589 (Supplementary Table 1) and GSE101521 (Supplementary Table 2) datasets, we observed that SPNS2 matched with statistical significance by Benjamini-Hochberg FDR <0.1 and CPM <0 in both AD and depression. It was also reported that SPNS2 gene expression levels were higher in AD and depressed patients compared to normal people. In fact, we show that SPNS2 presents higher expression levels in Tg2576 WAS mice brains compared to Tg2576 and WT WAS mice brains. To confirm the possible pathway connecting the two diseases, we used the Comparative Toxicogenomics Database (CTD). Using the CTD database, we found that SPNS2 was closely related to both AD and depression. Moreover, it was confirmed that the expression levels of SPNS2 were also increased in primary cell cultures treated with $A\beta_{(1–42)}$ and corticosterone. These results indicate that SPNS2 may be crucial biomarker of both AD and depression phenotypes. SPNS2 is a transporter which is essential for S1P export from endothelial cells in brain (Wang *et al*., 2020). Data using SPNS2 deficient mice has demonstrated that SPNS2 regulates plasma S1P levels and is critical for thymocyte egress from lymphoid organs (Hisano *et al*., 2012). Treatment with the SPNS2 inhibitor 16d (SLF1081851) also inhibited S1P release in both mice and rat (Fritzemeier *et al*., 2022). S1P is a bioactive lipid which plays role in neural development and survival (Herr and Chun, 2007). Current research suggests that reduction of S1P levels in the brain is associated with neurodegeneration (Ceccom *et al*., 2014; Couttas *et al*., 2014). A decrease in the S1P / sphingosine ratio in the hippocampus was found to be correlated with AD pathogenesis in human brain samples (Ceccom *et al*., 2014). Moreover, loss of S1P and sphingosine kinase (SphK) activity in the hippocampus and temporal cortex can sensitize these areas to synaptic loss and neuronal cell death in response to amyloid accumulation in both human and APPswe/PS1ΔE9 mice brain samples (Couttas *et al*., 2014). Hence,

higher expression of SPNS2 could be associated an accelerated depressive-like phenotype induced by stress in Tg2576 AD mice.

The accumulated extracellular S1P binds to S1P receptors (S1PRs) through SPNS2, which induces proliferation and synthesis of pro-inflammatory cytokines in both neural S1P-lyase (SGPL1) ablated mice and BV2 cells (Hla and Brinkmann, 2011; Karunakaran *et al*., 2019). To confirm the correlation between SPNS2 and S1P, the levels of S1P were measured in the Tg2576 WAS mice brains and serum. Previous evidence shows that cellular stress promotes inflammation via S1P signaling (Pei *et al*., 2021), with S1P inducing autophagy in PC-3 cells (Huang *et al*., 2014). Comparing Tg2576 CON and WT WAS mice, intracellular S1P levels decreased in the brains of Tg2576 mice, but increased in their serum. Remarkably, WAS treatment further decreased S1P levels in the brains of Tg2576 mice and further increased in their serum. Previous evidence shows that extracellular release of S1P from primary cells in the central nervous system (CNS) supports a role of S1P as an autocrine / paracrine physiological messenger in the cerebellum (Anelli *et al*., 2005). Furthermore, abnormal S1P signaling in the CNS leads to neurodegeneration, brain tumor, and stroke (Karunakaran and van Echten-Deckert, 2017). These data indicate that stress-mediated depression could affect more Tg2576 transgenic mice through SPNS2 upregulation mediated by S1P signaling, with SPNS2 serving as a critical target for development of both AD and depression through the release of S1P. Altogether, our data suggests that stress elevates depression in an AD animal model through the S1P-dependent release of SPNS2.

CONFLICT OF INTEREST

The authors have no competing of interest to report.

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AUTHOR CONTRIBUTIONS

SSY: Conceptualization, Data curation, YRK: Formal analysis, Validation, Roles/Writing - original draft, Writing - review & editing. DWL: Conceptualization, Validation. HJH: Conceptualization, Writing - review & editing. IJY: Conceptualization, Writing - review & editing. JYC: Conceptualization, Validation. JY: Conceptualization, Validation, Writing - review & editing. DJS: Formal analysis, Conceptualization. SBH: Writing - review & editing. JTH: Resources; Conceptualization; Supervision; Funding acquisition; Writing - Original Draft; Writing - Review & Editing. All authors contributed to Writing - Review & Editing.

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