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**Intracranial IL-17A overexpression decreases cerebral amyloid angiopathy by upregulation of ABCA1 in an animal model of Alzheimer's disease**

**Abbreviated title:** Role of cerebral IL-17A in Alzheimer's disease

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## **Abstract**

Neuroinflammation is a pervasive feature of Alzheimer's disease (AD) and characterized by activated microglia, increased proinflammatory cytokines and/or infiltrating immune cells. T helper 17 (Th17) cells are found in AD brain parenchyma and interleukin-17A (IL-17A) is identified around deposits of aggregated amyloid  $\beta$  protein ( $A\beta$ ). However, the role of IL-17A in AD pathogenesis remains elusive. We overexpressed IL-17A in an AD mouse model via recombinant adeno-associated virus serotype 5 (rAAV5)-mediated intracranial gene delivery. AD model mice subjected to injection of a vehicle (PBS) or rAAV5 carrying the lacZ gene served as controls. IL-17A did not exacerbate neuroinflammation in IL-17A-overexpressing mice. We found that IL-17A overexpression markedly improved glucose metabolism, decreased soluble  $A\beta$  levels in the hippocampus and cerebrospinal fluid, drastically reduced cerebral amyloid angiopathy, and modestly but significantly improved anxiety and learning deficits. Moreover, the ATP-binding cassette subfamily A member 1 (ABCA1), which can transport  $A\beta$  from the brain into the blood circulation, significantly increased in IL-17A-overexpressing mice. In vitro treatment of brain endothelial bEnd.3 cells with IL-17A induced a dose-dependent increase in protein expression of ABCA1 through ERK activation. Our study suggests that IL-17A may decrease  $A\beta$  levels in the brain by upregulating ABCA1 in blood-brain barrier endothelial cells.

## **Keywords:**

Alzheimer's disease; ATP-binding cassette transporters; blood-brain barrier; cerebral amyloid angiopathy;  $A\beta$ ; interleukin 17; mouse behavior; ERK signaling; endothelial

cells

## 1. Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia (Fratiglioni *et al.*, 2000; Kawas *et al.*, 2000; Kukull *et al.*, 2002), characterized by extracellular amyloid  $\beta$  (A $\beta$ ) deposition in the brain parenchyma and cerebral blood vessels. Additionally, neuroinflammation is a consistent feature of AD and thought to influence the disease pathogenesis (McManus *et al.*, 2014). Immune responses occurring in brains of AD patients can be driven by resident immune cells and/or brain infiltrating peripheral immunocompetent cells (Ghosh and Geahlen, 2015; Togo *et al.*, 2002). Microglia, the innate immune cells in the central nervous system (CNS), are activated by A $\beta$  deposits and secrete inflammatory cytokines and chemokines, contributing to AD progression (Hickman *et al.*, 2008; Ridolfi *et al.*, 2013; Rodriguez *et al.*, 2016).

Central-memory CD4<sup>+</sup> T lymphocytes account for more than 80% of the cells in the cerebrospinal fluid (CSF) and routinely penetrate the CNS. Lymphocytes in the CNS are replenished by newly immigrating cells approximately twice per day (Engelhardt and Ransohoff, 2005; Hickey, 2001; Smolders *et al.*, 2013). Numerous lines of evidence indicate the presence of T cells in the brains of AD patients (Ferretti *et al.*, 2016; Lombardi *et al.*, 1999; Monsonogo *et al.*, 2003; Shalit *et al.*, 1995; Togo *et al.*, 2002) although their possible roles in AD pathogenesis have been studied less than those of microglia. T cells in the brains of AD patients increased as compared with subjects with non-AD degenerative dementias and age-matched controls (Lueg *et al.*, 2015; Togo *et al.*, 2002). T cells breaching the brain vasculature and parenchyma can be either detrimental or beneficial for brain homeostasis (Kipnis *et al.*, 2004; Monsonogo *et al.*, 2006; Ziv *et al.*, 2006). Depletion of regulatory T (Treg) cells in an AD mouse model

accelerated the onset of cognitive deficits without altering brain A $\beta$  load (Dansokho *et al.*, 2016). Administration of A $\beta$ -specific type 1 T helper (Th1) cells into the brain lateral ventricle of an AD mouse model enhanced removal of cerebral A $\beta$  deposits and promoted neurogenesis without harmful effects (Fisher *et al.*, 2014). Thus, these T cells play beneficial roles in the AD pathophysiology. In contrast, intravenous administration of A $\beta$ -specific Th1 cells caused brain infiltration, increased microglial activation and A $\beta$  deposition, and impaired cognitive function in an AD mouse model but similarly administered A $\beta$ -specific IL-17-producing helper T (Th17) cells infiltrated into the brain without exacerbating microglia activation, A $\beta$  deposition and cognitive deficits in the same mice (Browne *et al.*, 2013).

IL-17A or IL-17 is a signature cytokine of Th17 cells and is involved in anti-microbial host defense and inflammation. A plethora of evidence supports the notion that Th17 cells and IL-17A play a pathogenetic role in certain autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis, and inflammatory bowel disease. Respiratory infection with *Bordetella pertussis* in an AD mouse model (McManus *et al.*, 2014) and intrahippocampal administration of A $\beta$ 42 into rats (Zhang *et al.*, 2013) induced infiltration of Th17 cells and expression of IL-17A in the brain, which were associated with exacerbation of an AD-like phenotype in these experimental animals. It is interesting to note that plasma IL-17A levels have been identified as one of the plasma biomarkers for AD diagnosis and neocortical A $\beta$  load (Burnham *et al.*, 2014; Doecke *et al.*, 2012). However, the role of IL-17A in AD pathophysiology is unclear. In this study, we investigated the effects of IL-17A overexpression via intracranial delivery of recombinant adeno-associated virus serotype 5 (rAAV5)-mediated IL-17A on A $\beta$  load,

microglia activation, glucose metabolism and behavioral function using a transgenic animal model of AD.

## 2. Material and Methods

### 2.1. Experimental animals and intracranial injection of rAAV-IL-17A

A rAAV vector, rAAV5-IL-17A, was prepared as described previously (Yang *et al.*, 2016). A control rAAV vector encoding LacZ, rAAV5-LacZ, was similarly prepared. B6.Cg-Tg (APP<sup>swe</sup>, PSEN1<sup>dE9</sup>) 85Dbo/J mice (TgAPP<sup>swe</sup>/PS1<sup>dE9</sup> mice) purchased from Jackson Laboratory, were used as an AD mouse model to study a potential role of IL-17A in the AD pathogenesis. For intracranial injection, 2-month-old TgAPP<sup>swe</sup>/PS1<sup>dE9</sup> mice were anesthetized by Nembutal® (pentobarbital) and placed on a stereotaxic instrument with a motorized stereotaxic injector (Stoelting, Wood Dale, IL). A midline incision was made to expose the bregma. A hole in the skull was made by a drill 0.5 mm posterior to the bregma and 1.0 mm right to the midline. rAAV5-IL-17A ( $1.5 \times 10^{10}$  vector genomes (vg) in 10  $\mu$ l PBS/mouse) was injected unilaterally into the right lateral brain ventricle at the depth of 2 mm at a rate of 1  $\mu$ l/min. Mice subjected to rAAV5-IL-17A injection are referred to as IL-17A-overexpressing mice (n=8). Mice similarly treated with 10  $\mu$ l PBS or rAAV5-LacZ ( $1.5 \times 10^{10}$  vg/mouse) served as controls and are referred to as control mice (n=9): Four PBS-injected and 5 rAAV5-LacZ-injected mice were combined together because no phenotypic differences between 2 controls were found (more information in Supplementary Figure 1). Due to the difference in brain amyloid content between male and female TgAPP/PS1 mice (Akhter *et al.*, 2015; Gallagher *et al.*, 2013; Sierksma *et al.*, 2013; Taniuchi *et al.*, 2007; Wang *et al.*, 2003), we used only male mice for this study. All animal protocols were prospectively approved

by the Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria.

## *2.2. Behavioral tests*

### *2.2.1. Behavioral schedule and statistics*

A battery of behavioral tests was performed on experimental mice at 12 months of age as previously described (Lim *et al.*, 2011). After measuring body weight and adapting mice to handling, the tests were conducted over a 16-day period as follows: spontaneous alternation (days 1–10), open-field (days 6–8), elevated plus-maze (days 9 and 10), and Morris water maze (days 11–16). In each test, whenever possible, the apparatus was wiped clean with a wet cloth and dried before the next mouse was introduced to minimize odor cues. For T-maze, Mann-Whitney U test was used to assess the chance of alternation compared with theory (50%) and unpaired t-test was used for the intergroup differences. For open-field, the intergroup differences were assessed by 2 x 3 analysis of variance (ANOVA) for 2 independent groups and 3 days of testing with repeated measures on the second factor. The unpaired t-test was used for the elevated plus-maze and the probe test of the Morris water maze and a 2 x 5 ANOVA for 2 independent groups and 5 days of testing with repeated measures on the second factor was used for 5-day acquisition of the Morris water maze. Results are expressed as mean  $\pm$  standard error of the mean (SEM). In all cases,  $P < 0.05$  was considered to be significant.

### *2.2.2. Exploration and anxiety*



Spontaneous alternation was measured in a T-maze, made of white acrylic and consisting of a central stem flanked on each side by 2 arms. The maze width was 9 cm, the wall height 20 cm, and each arm 30 cm in length. On the initial trial, the mice were placed in the stem with the right arm blocked by a plastic barrier (forced choice). After entering the available arm, the mice were kept in it for 1 min by closing the barrier behind them. The mice were then retrieved and after removing the barrier, placed back in the stem for a free-choice trial, either into the same arm or the opposite arm (4-paw criterion). On the following 10 days, the same 2-trial procedure was repeated, except that the blocked arm was switched from right on odd days to left on even days.

The number of alternations and the latencies before responding during the choice trial were measured. In the absence of any decision within 1 min, the mice were briefly prodded from behind, usually not more than once, far from the choice point so that a response could be recorded on every trial.

Motor activity was measured in the open-field chamber that was made of white acrylic with a 50 cm × 50 cm surface area. Each mouse was placed in a corner of the open-field. The activity in central (25 cm × 25 cm surface area) and peripheral zones was recorded in a 5-min session for 3 consecutive days and analyzed by video tracking software (SD Instruments, San Diego, CA). The distance traveled and the time spent resting (<2 cm/s), moving slow (2–5 cm/s), or moving fast (>5 cm/s) in each zone were measured, as well as the time spent in the periphery and center of the apparatus.

The elevated plus-maze apparatus consisted of 4 arms in a cross-shaped form 70 cm in length with a 10 cm × 10 cm central region. Two of the arms were enclosed on 3 sides by walls (10 cm in height) facing each other, while the other two were open,

except for a minimal border (0.5 cm in height) used to minimize falls. A mouse was placed in the central region and then the number of entries and the time spent inside enclosed and open arms were measured in a 5-min session on 2 consecutive days with the same video tracking system. The open/total arm entries and duration ratios were also calculated.

### *2.2.3. Spatial learning and memory*

The Morris water maze consisted of a pool of blue opaque plastic, 116 cm in diameter with 75-cm high walls, filled with water (20°C) at a height of 31 cm. Powdered milk was evenly spread over the water surface to camouflage the escape platform (8 x 8 cm) made of white plastic and covered with a wire mesh grid to ensure a firm grip. The watered milk was removed every day after a few hours of training and the pool rinsed with clean water. The pool was contained in a room with external visual cues such as light fixtures and a ladder. The mice were placed next to and facing the wall successively in north (N), east (E), south (S), and west (W) positions, with the escape platform hidden 1 cm below water level in the middle of the NW quadrant. The same video tracking equipment was used to estimate path length and escape latencies in 4-trial sessions for 5 days with a 15-min inter-trial interval. The mice remained on the platform for at least 5 s. When the mice failed to reach the escape platform within the 1 min cut-off period, they were retrieved from the pool and placed on the platform for 5 s. After swimming, the mice were kept dry in a plastic holding cage filled with paper towels. In the morning after the acquisition phase, a probe trial was conducted by removing the platform and placing the mouse next to and facing the N side. The time spent in the previously correct quadrant was measured for a single 1-min trial. In the afternoon, the

visible platform subtask was conducted, with the escape platform lifted 1 cm above water level and shifted to the SE quadrant. A 17-cm high pole was inserted on top of the escape platform as a viewing aid. The same procedure was adopted as with place learning except that the subtest was conducted on a single day.

### *2.3. Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT)*

Forty-two and forty-four weeks after rAAV injection, IPGTT and ITT were conducted, respectively, as previously described (Ize-Ludlow *et al.*, 2011). Animals were fasted for 16 h, weighed and intraperitoneally injected with D-glucose (1.5 g/kg) for IPGTT. Blood was obtained using the tail nick method before and at 30, 60, 90 and 120 min after the D-glucose injection for monitoring glucose levels using Glucometer (ReliOn® Prime, Arkray USA, Minneapolis MN). Plasma insulin levels were determined by an insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL). For ITT, mice were fasted for 4 h, weighed, and intraperitoneally injected with human insulin (0.75 units/kg; Novolin® R, Novo Nordisk, Princeton, NJ). Blood sugar was monitored before and at 15, 30, 60, and 90 min.

### *2.4. Murine CSF isolation*

Forty-four weeks after rAAV5-IL-17A intracranial injection, mice were deeply anesthetized with Nembutal® (pentobarbital) and CSF was isolated from the cisterna magna compartment using the method previously described (DeMattos *et al.*, 2002). Mice were anesthetized by Nembutal® (pentobarbital) and fixed face down on a narrow platform. An incision was made from the top of the skull to the dorsal thorax. The musculature from the base of the skull to the first vertebrae was carefully removed to expose the meninges overlying the cisterna magna. The surrounding area was gently

cleaned with PBS using cotton swabs to remove any residual blood or other interstitial fluid. The arachnoid membrane covering the cistern was punctured with a 29 gauge insulin syringe. A polypropylene narrow bore pipette was immediately placed in the hole to collect CSF. As the primary CSF exiting the compartment was collected, a second collection was performed after the cistern was refilled within 2 min. About 10 to 15  $\mu$ l CSF was collected from each mouse.

### *2.5. Immunohistochemical analyses*

Forty-four weeks after rAAV5-IL-17A intracranial injection, mice were deeply anesthetized with Nembutal® (pentobarbital) and cardinally perfused with cold PBS and their brains were quickly removed. The neocortices and hippocampi of the left hemispheres were separately dissected and stored at  $-80^{\circ}\text{C}$  for further studies. The right hemispheres were fixed in 4% paraformaldehyde for 48 h, stored overnight in 30% sucrose in 0.1 M PBS and frozen in Tissue-Tek optimal cutting temperature compound. Coronal sections (35  $\mu$ m) of the brains were immuno-stained with anti-IBA1 antibody (Wako, Osaka, Japan) for detection of activated microglia. Endogenous peroxidase was eliminated by 1%  $\text{H}_2\text{O}_2$  in 10% methanol Tris-buffered saline (TBS) treatment. After washing with 0.1 M Tris buffer (pH 7.5) and 0.1M TBS (pH 7.4), sections were blocked with 5% normal serum in 0.1 M TBS with 0.5% triton-X-100 (TBS-T) and then incubated with primary antibodies in 2% serum in TBS-T for 18-48 h at  $4^{\circ}\text{C}$ . For the negative controls, slides were processed without primary antibody. After rinsing, the sections were incubated with biotinylated secondary antibodies in 2% serum TBS-T for 2 h at room temperature. The avidin-biotin peroxidase method using 3, 3'-diaminobenzidine as a substrate (Vector, Burlingame, CA) was performed. Sections were counterstained with

hematoxylin. For the detection of fibrillar A $\beta$  plaque, tissue sections were stained in 1% thioflavin S (Sigma) and rinsed with 70% ethanol. After washing with H<sub>2</sub>O, the sections were mounted in 75% glycerol in H<sub>2</sub>O. Histomorphometry was performed for quantification of amyloid deposits and reactive/activated glial cells using an Olympus BX61 automated microscope, Olympus Fluoview system, and Image Pro Plus v4 image analysis software (Media Cybernetics, Silver Spring, MD, USA) capable of color segmentation and automation via programmable macros. Five coronal brain sections from each mouse were analyzed, each separated by an approximately 400- $\mu$ m interval, starting at 1.3 mm posterior to the bregma to caudal. Both neocortex and hippocampus were found in all the brain sections and analyzed separately. Stained areas were expressed as a percentage of total neocortex or hippocampus, respectively. For cerebral amyloid angiopathy (CAA), thioflavin S-positive blood vessels in 3 to 4 right hemisphere sections for each mouse were enumerated and expressed as a number of thioflavin S-positive blood vessels per square millimeter. Data were expressed as mean  $\pm$  SEM (Kou *et al.*, 2015).

#### *2.6. Quantification of plasma and CSF IL-17A by ELISA*

Approximately 100  $\mu$ l of blood/mouse was drawn from the tail vein at 3, 7, and 11 months after injection by using microhematocrit heparin tubes (Fisher Scientific, Pittsburgh, PA) and centrifuged for 20 min at 2,000 xg for plasma isolation. Levels of IL-17A in plasma and CSF were determined by ELISA (eBioscience, Inc. San Diego, CA).

#### *2.7. Quantification of brain A $\beta$ and CSF by ELISA, and ABCA1 by western blotting*

The neocortex and hippocampus were removed from -80°C, lysed using the Bio-Plex cell lysis kit (Bio-Rad Laboratories, Hercules, CA) and homogenized according to

the manufacturer's protocol, and centrifuged at 16,000 ×g for 30 min at 4°C. The supernatants containing buffer soluble A $\beta$  were collected and the protein concentrations in the supernatants were determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The pellets containing insoluble A $\beta$  were further dounce homogenized in guanidine hydrochloride (final concentration, 5 M) and then rock-shaken for 3 to 4 h at room temperature. Levels of buffer soluble and insoluble A $\beta$  in the neocortex and hippocampus, and A $\beta$  in the CSF were quantified by A $\beta$ 42 and A $\beta$ 40 ELISA kits (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

The protein lysates were used to determine ABCA1 levels by western blotting, also. After determining protein concentrations, the proteins were electrophoresed under reducing conditions in 10% SDS–PAGE gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C anti-ABCA1 antibody (1:1000 dilution) (Abcam, Cambridge, MA), or anti- $\beta$ -actin antibody (Sigma, St. Louis, MO) and specific bands were visualized by an enhanced chemiluminescence system (Amersham, Arlington Heights, IL). The optical densities of the protein bands were determined by densitometric scanning using an HP Scanjet G3010 Photo Scanner and Image J V1.40 (NIH, MD).

### *2.8. Cell culture and western blot*

The bEnd.3 cells (ATCC® CRL2299™) were grown in ATCC-formulated Dulbecco's modified eagle's medium containing 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) in a humidified 37°C incubator with a 5% CO<sub>2</sub> atmosphere. Cells were plated on culture dishes at 4 x 10<sup>4</sup>/ml cells. Forty-eight hours after seeding, bEnd.3 cells were treated with 0, 1, 10, 100 ng/ml of IL-17A (Cell

Signaling Technology, Danvers, MA) for 24 h to determine ABCA1 protein expression. Other sets of bEnd.3 cells were treated with 100 ng/ml of IL-17A for 0 (no IL-17A), 5, 15 and 30 min to detect the activation of p-ERK1/2. Different sets of bEnd.3 cells were pre-treated with U0126 (25  $\mu$ M) or DMSO (vehicle) for 1 h followed by treatment with or without IL-17A (100 ng/ml) for 30 min or 24 h to determine expression levels of ERK or ABCA1, respectively. The cell lysates were prepared by using radioimmune precipitation assay (RIPA) lysis buffer containing complete mini-protease-inhibitor and phosphatase inhibitor cocktail tablets (Roche Diagnostics Corporation, Indianapolis, IN) and centrifuged at 12,000 xg for 10 min at 4°C for collecting supernatants. After determining protein concentrations, the proteins were electrophoresed under reducing conditions in 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with anti-phospho-ERK1/2, anti-ERK1/2 antibody (1:1000 dilution) (Cell Signaling Technology, Danvers, MA), anti-ABCA1, or anti- $\beta$ -actin antibody and specific bands were visualized by an enhanced chemiluminescence system (Amersham, Arlington Heights, IL). The optical densities of the protein bands were determined by densitometric scanning using an HP Scanjet G3010 Photo Scanner and Image J V1.40 (NIH, MD). The optical density of p-ERK1/2 band was divided by that of total ERK1/2 band on the same lane from the same membrane for normalization.  $\beta$ -actin was used to normalize expression levels of ABCA1.

### *2.9. Statistical analysis*

Data were expressed as mean  $\pm$  SEM. The data were analyzed using SPSS version 22 software (IBM, Armonk, NY). One-way ANOVA followed by a least-significant-difference test was used for statistical comparisons among multiple groups

and t-test for statistical comparisons between two groups. P values less than 0.05 were considered statistically significant.

### 3. Results

#### 3.1. Overexpression of IL-17A via intracranial delivery of rAAV5 improves glucose metabolism

The bioactivity of IL-17A produced by the AAV-IL-17A vector was confirmed using 3T3 cells described previously (Yang *et al.*, 2016). To explore the potential role of IL-17A in AD, we intracranially delivered rAAV5-IL-17A into 2-month-old TgAPP<sup>swe</sup>/PS1<sup>dE9</sup> mice. Three, seven, and eleven months after injection, the average plasma IL-17A levels in IL-17A-overexpressing mice were  $14.88 \pm 1.95$ ,  $14.69 \pm 1.28$ , and  $9.31 \pm 0.95$  ng/ml respectively and the average CSF IL-17A level in IL-17A-overexpressing mice was  $270.73 \pm 48.83$  ng/ml 11 months after injection, while IL-17A was undetectable in control mice by ELISA (Figure 1A). The regional distribution of rAAV5 in brain was found mostly in hippocampal neuronal cells determined by beta-gal immunostaining (supplementary Figure 2A and B). Consistently, the copy number of rAAV5 in hippocampus is 2.5-fold and 12-fold higher than those in cortex and in cerebellum by qPCR method, respectively (supplementary Figure 2C) (more information in supplementary material and methods). And the distribution of rAAV5 in peripheral tissues, heart, lung, liver, spleen, kidney and intestine were undetectable by using the current method (data not shown).



Blood glucose and insulin levels in IL-17A-overexpressing mice were significantly lower than those in control mice throughout IPGTT (Figure 1B and C). There was no significant difference in ITT between groups (data not shown).

### *3.2. Overexpression of IL-17A improves anxiety and spatial learning but not long-term memory*

#### *3.2.1. Exploration and anxiety*

In the T-maze spontaneous alternation test, mice in each group alternated above chance ( $P < 0.05$ ) (Figure 2A) and choice latencies showed no difference between groups (Figure 2B). In the open-field, IL-17A-overexpressing mice had a higher center/total time ratio than control mice (Figures 3A and B) ( $P < 0.01$ ). Resting time and moving slow time increased over days in both groups, whereas moving fast time decreased over days (Figures 3C and D). There was a significant group x day interaction, because moving slow time increased and moving fast time decreased relatively more in IL-17A-overexpressing mice than controls (Figures 3D and 3E). In contrast, distance travelled declined equally in both groups (Figure 3F). In the elevated plus-maze test, open arm duration was higher in IL-17A-overexpressing mice on day 1 but open/total arm entries lower in that group on day 2 ( $P < 0.05$ ) (Table 1).

#### *3.2.2. Spatial learning and memory*

In the acquisition phase of the Morris water maze test, there were significant reductions in latencies and path lengths between days in both groups ( $P < 0.05$ ) (Figure 4A and B). Furthermore, escape latencies in IL-17A-overexpressing mice were quicker than control mice ( $P < 0.05$ ) which is an overall group effect irrespective of days (Figure

4A). In the probe trial, the percentages of time spent in the correct quadrant were  $27.40 \pm 3.09$  s and  $26.73 \pm 3.58$  s for IL-17A-overexpressing and control mice, respectively ( $P > 0.05$ ) (Figure 4C). There were no intergroup differences in the visible platform subtask, also (data not shown).

### *3.3. Overexpression of IL-17A in mouse brain does not increase microglia activation but reduces A $\beta$ in CSF and CAA*

Fibrillar A $\beta$  deposits are closely associated with activated microglia. Microglia can be activated by various endogenous and exogenous factors through their receptors including toll-like receptors, scavenger receptors, and numerous cytokine and chemokine receptors (Kierdorf and Prinz, 2013). IL-17A overexpression may aggravate microglia activation in an AD mouse model. **Activation of microglia is characterized by cell retraction and thickening and increased body size.** Microglial cell body to cell size ratio **has been shown to be positively correlated with microglial activation** using Iba1 immunostaining (Hovens IB, Nyakas C, Schoemaker RG. **A novel method for evaluating microglial activation using ionized calcium-binding adaptor protein-1 staining: cell body to cell size ratio.** *Neuroimmunol Neuroinflammation* 2014;1(2):82-8.). Therefore, the brain sections were subjected to **immunohistochemical analysis with anti-Iba1 antibody for detection of activated microglia.** Microglia stained with Iba1 **did not increase in IL-17A-overexpressing mice (Figure 5A).** Microglial cell body to cell size ratio (Figure 5B) and the total number of microglial cells **did not show significant differences between groups (Figure 5C).**

To determine the effect of IL-17A overexpression on A $\beta$  load in the brain, A $\beta$  deposits were visualized by thioflavin S fluorescence and quantified by morphometry

(Figure 6A and B). The areas positive for thioflavin S fluorescence in the neocortex and hippocampus showed no statistical differences between groups:  $0.36 \pm 0.03\%$  and  $0.27 \pm 0.03\%$  for IL-17A-overexpressing mice,  $0.37 \pm 0.03\%$  and  $0.21 \pm 0.22\%$  in control mice, respectively. However, both CSF A $\beta$ 40 and A $\beta$ 42 were dramatically decreased in IL-17A-overexpressing mice ( $1.88 \pm 0.37$  and  $0.50 \pm 0.09$  ng/ml, respectively) compared with that in control mice ( $7.35 \pm 1.58$  and  $2.05 \pm 0.35$  ng/ml, respectively) ( $P < 0.01$  and  $P < 0.001$ , respectively) (Figure 6C). Hippocampal soluble A $\beta$ 40 levels in IL-17A-overexpressing mice ( $0.20 \pm 0.05$  ng/mg protein) were also significantly decreased compared with that in control mice ( $0.81 \pm 0.39$  ng/mg protein) ( $P < 0.05$ ) while no differences were found in hippocampal soluble A $\beta$ 42 levels and in neocortical soluble A $\beta$ 40 and A $\beta$ 42 levels between groups (Figure 6D and E). No differences were found in insoluble A $\beta$ 40 and A $\beta$ 42 levels in the neocortex and hippocampus between groups (Figure 6F and G) ( $P > 0.05$ ).

Vascular A $\beta$  deposition (CAA) is associated with vascular dysfunction and thought to play an important role in the AD pathogenesis (de la Torre, 2004; Smith and Greenberg, 2009). We enumerated the number of blood vessels positive for thioflavin S fluorescence in the right neocortices. The number of thioflavin S stained blood vessels in IL-17A-overexpressing mice ( $0.05 \pm 0.02/\text{mm}^2$ ) considerably decreased compared with that in control mice ( $0.24 \pm 0.06$ ) (Figure 6H and I) ( $P < 0.01$ ).

### *3.4. IL-17A increases ABCA1 expression by ERK1/2 activation*

In order to investigate possible mechanisms by which IL-17A decreases A $\beta$  in CSF and CAA, protein levels of ABCA1 in IL-17A-overexpressing mice were determined

by immunoblot analysis. Hippocampal ABCA1 protein increased in IL-17A-overexpressing mice compared with control mice ( $P < 0.05$ ) (Figure 7 A and B), while there were no changes in cortex (data not shown). To further explore signal pathways of the increase in ABCA1 by IL-17A a mouse brain endothelial cell line, bEnd.3 cell, was used to determine the level of ERK1/2. Immunoblot analysis consistently revealed a dose-dependent increase in ABCA1 expression by IL-17A treatment (control vs 100 ng/ml IL-17A,  $P < 0.05$ ) (Figure 8A). Moreover, phospho-ERK1/2 (p-ERK1/2) levels were significantly increased in bEnd.3 cells ( $P < 0.05$ ) by 100 ng/ml IL-17A (Figure 8B). U0126 (25  $\mu$ M), an ERK1/2 kinase inhibitor, completely inhibited both the activation of ERK1/2 and the upregulation of ABCA1 expression by IL-17A (Figure 8C and D) ( $P < 0.05$ ).

#### 4. Discussion

IL-17A is produced by Th17 cells and other immune cells, such as neutrophils, macrophages, dendritic cells, and natural killer, lymphoid tissue inducer and  $\gamma\delta$ -T cells (Korn *et al.*, 2009; Zenaro *et al.*, 2015). Th17 cells infiltrate into the brain parenchyma in AD patients and mouse models, suggesting that Th17 cells may contribute to neuroinflammation by release of proinflammatory cytokines IL-17A and IL-22 (McManus *et al.*, 2014; Zhang *et al.*, 2013). IL-17A is released by neutrophils surrounding A $\beta$  deposits in two transgenic mouse models of AD (5XFAD and 3xTg-AD mice), which suggests that IL-17A may be involved in AD pathogenesis and cognitive impairment (Zenaro *et al.*, 2015). However, the role of the IL-17A in the AD brain has not been well studied. To the best of our knowledge, this is the first study to demonstrate the effects of

IL-17A overexpression on AD-like pathophysiology in an AD mouse model. The distribution of rAAV5 was mainly expressed in the brain **not in** peripheral tissues (such as heart, lung, liver, spleen, kidney and intestine). However, plasma IL-17A levels in IL-17A overexpressing mice were one twenty-ninth of the CSF IL-17A levels. There exist several possibilities. Despite of undetectable viral in peripheral tissue, we still cannot exclude the possibility that a few AAV5 leaked into brain vessels during intraventricular injection. On **the** other hand, because **the blood-brain barrier plays** an important role in regulating the rate of transport in ( $K_{in}$ ) and out ( $K_{out}$ ) of the brain (PMID: 24009582), the difference in  $K_{in}$  and/or  $K_{out}$  may be **the** main cause to distribution of IL-17A in CSF and plasma in IL-17A overexpressing mice.

Insulin signaling impairment/ insulin resistance (IR) is a common and important pathophysiologic alteration between type 2 diabetes mellitus (T2DM) and AD (Craft and Watson, 2004; Sebastiao *et al.*, 2014; Verdile *et al.*, 2015), although the cellular and molecular mechanism that associates T2DM with AD is unknown (Rios *et al.*, 2014). IR may be one of the causes of AD. Hyperinsulinemia/insulin resistance may lead to alterations of protein expression or posttranslational modification central to AD pathogenesis, including tau, A $\beta$  and the A $\beta$ -degrading protease neprilysin (Morales-Corraliza *et al.*, 2016). A longitudinal study found that peripheral IR is associated with a higher risk of AD (Schrijvers *et al.*, 2010). IR in the CNS is involved in AD progression (Biessels and Reagan, 2015) and intranasal insulin delivery improved cognitive function in a subset of patients with AD or mild cognitive impairment (Benedict *et al.*, 2011; Claxton *et al.*, 2015; Craft *et al.*, 2012). Inversely, AD may be one of the causes of IR. Fasting glucose levels and/or insulin sensitivity are altered in 81% of AD patients

suggesting that AD may lead to a diabetic phenotype (Janson *et al.*, 2004). A recent study demonstrated that intracerebroventricular infusion of A $\beta$  oligomers induces peripheral insulin resistance possibly by triggering neuroinflammation (Clarke *et al.*, 2015). Therefore, AD appears to be reciprocally associated with IR, suggesting that improving IR may alleviate AD and vice versa. In this study, IL-17A significantly improved glucose metabolism in IL-17A-overexpressing mice compared with that in control mice. Although the IL-17A level in CSF was about 29 times higher than that in plasma, it is unclear whether the effect of IL-17A on glucose metabolism is due to activation of IL-17A signal transduction pathways in CNS, peripheral tissues or both.

A $\beta$  accumulation occurs in the brain parenchyma and CAA and plays a causal role in AD pathogenesis. CAA is the deposition of A $\beta$  in the walls of cerebral blood vessels and identified at autopsy in 75-98% of AD patients (Jellinger and Attems, 2003). A $\beta$  deposition in CAA affects cerebral blood flow, alters BBB permeability, interferes with A $\beta$  clearance or efflux from the brain and triggers deleterious inflammatory responses, contributing to development of vascular fragility, hemorrhages and, ultimately, dementia (Cupino and Zabel, 2014; Ghiso *et al.*, 2014; Kalaria, 1992). According to the two-hit vascular hypothesis of AD, brain vascular damages (hit 1) initiate A $\beta$  accumulation (hit 2) in the brain, leading to neurodegeneration and memory decline in AD (Sagare *et al.*, 2012). Clinical imaging, epidemiological and pharmacotherapy studies support the notion that vascular changes play a central role in early AD pathogenesis (de la Torre, 2004; Iturria-Medina *et al.*, 2016). If not all, a subset of AD patients have disruptions of the BBB (Erickson and Banks, 2013), resulting in the reduction of cerebral blood flow (Spetsieris *et al.*, 2015) and A $\beta$  clearance in late-onset AD (Mawuenyega *et al.*, 2010). We found that

IL-17A-overexpressing mice show an approximately 4-fold decrease in A $\beta$  deposition in CAA compared with control mice. Soluble A $\beta$  levels in the hippocampus and CSF are similarly decreased in IL-17A-overexpressing mice. Contrary to this observation, a decrease in CSF A $\beta$ , especially A $\beta$ 42, is accompanied by an increase in brain A $\beta$  deposition in the early stages of AD patients (Kuhlmann *et al.*, 2016; Maia *et al.*, 2013). Therefore, CSF A $\beta$  levels do not always reflect A $\beta$  load in the brain. Altogether, IL-17A may protect the brain vasculature from A $\beta$  accumulation and prevent CAA. It is worth noting that IL-17A does not alter microglia activation in our experiments. Microglial cells are known to be important for amyloid plaque formation and clearance. However, eliminating microglia in AD mice failed to change amyloid plaque formation and modulate A $\beta$  pathology (Grathwohl *et al.*, 2009; Spangenberg *et al.*, 2016). Thus, microglia may not be involved in reduction of A $\beta$  deposits in IL-17A-overexpressing mice.

Cognitive impairment along with anxiety in AD animal models, which resembles that in AD patients, has been reported (Foidl *et al.*, 2016; Pietrzak *et al.*, 2015; Sil and Ghosh, 2016; Starkstein *et al.*, 2007). Protofibrillar A $\beta$  1-42 injection induced anxiety and AD-like pathology in rats (Sharma *et al.*, 2016), suggesting the deleterious effect of elevated A $\beta$  levels on behavior in AD. In our current study, IL-17A-overexpressing mice manifested behavioral alterations relative to controls. IL-17A-overexpressing mice were characterized by more rapid habituation in the open-field test, as indicated by more pronounced increases in moving slowly and more pronounced decreases in moving quickly across days of testing. Moreover, IL-17A-overexpressing mice had a higher center/total time ratio, indicating less anxiety in exploring the open-field. The lesser anxiety displayed by this group was also observed in the elevated plus-maze test, where

open arm duration was increased relative to controls on day 1 though not on day 2 of testing when exploration is usually minimal. Therefore, IL-17A overexpression may improve anxiety by reducing soluble A $\beta$  levels in the hippocampus. During acquisition of the Morris water maze task, IL-17A-overexpressing mice reached the escape platform faster than control mice. In contrast, there was no change in probe or visible platform subtasks, indicating cognitive improvement in the acquisition process but not long-term memory, visuomotor control, or swimming speed. Our results are consistent with the previous studies reporting the lack of association between A $\beta$  deposits in brain and behavioral changes (Heikkinen et al. 2004; Holcomb et al. 1999; Howlett et al. 2004; Montarolo et al. 2013; Morgan 2003). It is possible that spatial memory deficits in AD model mice correlate with the selective modulation of the levels of hippocampal A $\beta$  42 (Faucher et al. 2015; Puolivali et al. 2002), which are unchanged in IL-17A-overexpressing mice.

ABCA1 is one of the routes of A $\beta$  clearance from the brain across the BBB. The depletion and overexpression of ABCA1 increases and decreases brain A $\beta$  in AD mouse models, respectively (Wahrle *et al.*, 2005; Wahrle *et al.*, 2008). In human, a loss-of-function mutation in ABCA1 with occurrence of 0.2% is associated with a high risk of AD and cerebrovascular disease (Nordestgaard *et al.*, 2015). Rarer non-synonymous variants of ABCA1 increase the risk for late onset AD, suggesting its protective effect against AD (Lupton *et al.*, 2014). Interestingly, the level of hippocampal ABCA1 in IL-17A-overexpressing mice is increased compared with that in control mice. Because IL-17A can induce activation of ERK1/2 signal (Guo *et al.*, 2014; Rodgers *et al.*, 2015; Song *et al.*, 2015) and because ERK1/2 signaling regulates ABCA1 expression in different types of



cells (Chang *et al.*, 2013; Mulay *et al.*, 2013), we determined if IL-17A would increase ABCA1 expression via ERK1/2 activation in endothelial bEnd.3 cells. As expected, IL-17A increased phospho-ERK1/2 but not total ERK1/2. The upregulation of ABCA1 by IL-17A was blocked by U0126, an ERK inhibitor. The ERK1/2 signaling pathway also participates in the spatial memory (Gao *et al.*, 2016; Ghasemi *et al.*, 2014; Liu *et al.*, 2014). Thus, IL-17A overexpression may reduce CAA and soluble A $\beta$  in the CSF and hippocampus by upregulating ABCA1 through the ERK1/2 signaling pathway.

## **5. Conclusions**

Overexpression of IL-17A via intracranial delivery of rAAV5-IL-17A improved glucose metabolism, reduced A $\beta$  levels in the CSF, hippocampus and CAA, and alleviated the anxiety in AD mice. Furthermore, our study suggests that IL-17A may decrease A $\beta$  in the brain by upregulating expression of ABCA1 through the ERK1/2 signaling pathway. However, IL-17A neither improved parenchymal A $\beta$  deposits nor exacerbated neuroinflammation in an AD mouse model. Our findings suggest that IL-17A may play a protective role in the pathogenesis of AD.

## **Conflict of interest**

The authors declare no conflict of interest.

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## Figure Captions

### **Figure 1. Overexpression of IL-17A in AD mouse brain and its effect on IPGTT.**

(A) The levels of IL-17A in plasma and CSF were determined by ELISA at the indicated time points. (B and C) Forty-two weeks after injection, mice were fasted for 16 h and injected i.p. with 1.5 g glucose/kg body weight and blood glucose and insulin were measured before and after glucose injection at the indicated time points. (Control mice, n = 9; IL-17A-overexpressing mice, n = 8. \* P < 0.05, # P < 0.01 and ### P < 0.001)

### **Figure 2. Effects of IL-17A overexpression on T-maze.**

The number of alternations (A) and the latencies (B) before responding with 1 min cut-off are shown as mean  $\pm$  SEM. \* P < 0.05 relative to chance levels at 50% (Control mice, n = 9; IL-17A-overexpressing mice, n = 8)

### **Figure 3. Effects of IL-17A overexpression on mice in open-field.**

The percentages of peripheral time (A), central time (B), resting time (C), moving slow time (D) and moving fast time (E) together with total travelling distances (F) in a 5-min session are shown as mean  $\pm$  SEM for 3 days. (Control mice, n = 9; IL-17A-overexpressing mice, n = 8)

### **Figure 4. Effects of IL-17A overexpression on cognitive function in Morris water maze.**

(A) Total escape latencies (s), (B) total path lengths (cm) per day, and (C)

percentage of time in correct quad of probe test are shown as mean  $\pm$  SEM. (Control mice, n = 9; IL-17A-overexpressing mice, n = 8)

**Figure 5. Effect of IL-17A overexpression on microglia activation by IBA1 staining.**

(A) Forty-four weeks after injection, mice were euthanized at 52 weeks of age and brain sections were subjected to immunohistochemistry using anti-IBA1 antibody. Scale bars 500  $\mu$ m. (B) The ratio of microglial cell body to cell size in hippocampus and (C) the total number of microglial cells in hippocampus were determined. (Control mice, n = 9; IL-17A-overexpressing mice, n = 8)

**Figure 6. Effect of IL-17A overexpression on A $\beta$  load and cerebral amyloid angiopathy in the brain.**

(A) Fibrillar A $\beta$  deposits in the brain were visualized by thioflavin S staining. Scale bars 500  $\mu$ m. (B) Average percentages of areas showing thioflavin S positive staining measured by morphometry in the cortex and hippocampus are shown. (C) The levels of A $\beta$ 40 and A $\beta$ 42 in CSF were measured by ELISA. (D, E, F, and G) The levels of soluble and insoluble A $\beta$ 40 and A $\beta$ 42 in cortex and hippocampus were measured by ELISA. Data are shown as mean  $\pm$  SEM. (H) Cerebral amyloid angiopathy (CAA) in the right neocortices in control mice and IL-17A-overexpressing mice was visualized by thioflavin S fluorescence and blood vessels positive for thioflavin S fluorescence were enumerated. (I) The numbers of blood vessels positive for thioflavin S fluorescence per square millimeter are shown (mean  $\pm$  SEM). Scale bars 100  $\mu$ m. (Control mice, n = 9; IL-17A-overexpressing mice, n = 8. # P < 0.01)

### **Figure 7. Effect of IL-17A on ABCA1 expression in the hippocampus**

(A) Hippocampal lysates were analyzed by western blotting using ABCA1 antibody and (B) bar graph represents the results of densitometric analysis. (Control mice, n=9; IL-17A-overexpressing mice, n = 8, ## P < 0.001)

### **Figure 8. Effects of IL-17A on ERK1/2 and ABCA1 expression in bEnd.3 cells. (A)**

bEnd.3 cells were treated with 0 (Control), 1, 10 and 100 ng/ml IL-17A for 24 h. The cell lysates were analyzed by western blotting using ABCA1 antibody and bar graph represents the results of densitometric analysis. (B) bEnd.3 cells were treated with 100 ng/ml IL-17A for 0 (Control), 5 min, 15 min and 30 min. The cell lysates were analyzed by western blotting using phospho-ERK1/2 and total ERK1/2 antibodies and bar graph represents the results of densitometric analysis. (C) bEnd.3 cells were pretreated with DMSO (vehicle) or phospho-ERK1/2 inhibitor, U0126 (25  $\mu$ M), for 1 h followed by treatment with or without IL-17A (100 ng/ml) for 30 min. The cell lysates were analyzed by western blotting using phospho-ERK1/2 and total ERK1/2 antibodies and bar graph represents the results of densitometric analysis. (D) bEnd.3 cells were pretreated with DMSO or U0126 (25  $\mu$ M) for 1h followed by treatment with or without IL-17A (100 ng/ml) for 24 h. The cell lysates were analyzed by western blotting using ABCA1 antibody and bar graph represents the results of densitometric analysis. The statistical results, \* P<0.05 and ## P<0.001, are from three independent experiments as shown in A, B, C and D.