The effects of astaxanthin treatment on a rat model of Alzheimer’s disease

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ABSTRACT

Alzheimer’s disease (AD), a progressive neurodegenerative disorder characterized by memory loss and dementia, could be a consequence of the abnormalities of cortical milieu, such as oxidative stress, inflammation, and/or accompanied with the aggregation of β-amyloid. The majority of AD patients are sporadic, late-onset AD, which predominantly occurs over 65 years of age. Our results revealed that the ferrous amyloid buthionine (FAB)-infused sporadic AD-like model showed deficits in spatial learning and memory and with apparent loss of choline acetyltransferase (ChAT) expression in medial septal (MS) nucleus. In hippocampal CA1 region, the loss of pyramidal neurons was accompanied with cholinergic fiber loss and neuroinflammatory responses including glial reaction and enhanced expression of inducible nitric oxide synthase (iNOS). Surviving hippocampal CA1 pyramidal neurons showed the reduction of dendritic spines as well. Astaxanthin (ATX), a potent antioxidant, reported to improve the outcome of oxidative-stress-related diseases. The ATX treatment in FAB-infused rats decreased neuroinflammation and restored the ChAT+ fibers in hippocampal CA1 region and the ChAT expression in MS nucleus. It also partly recovered the spine loss on hippocampal CA1 pyramidal neurons and ameliorated the behavioral deficits in AD-like rats. From these data, we believed that the ATX can be a potential option for slowing the progression of Alzheimer’s disease.

1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease with symptoms including mood change, severe memory loss, and progressive cognitive disorder (Blair et al., 2007; Jahn, 2013). Pathological studies showed the presence of β-amyloid (Aβ) plaques, neurofibillary tangles, microglial and astrocytic activation, neuronal loss, and the alteration of synapses in the brains of AD patients (González-Reyes et al., 2017; Metaxas and Kempf, 2016; Murphy and LeVine, 2010; Rajendran and Paolicelli, 2018). Epidemiological studies showed that 95% of AD patients are sporadic, late-onset AD, where the causes are rather poorly known, but the risk is modulated by multiple factors including environmental exposure, genetic risk factors, age, sex, mitochondrial damage, and oxidative stress (Tramutola et al., 2017; Wainaina et al., 2014). Lecanu and Papadopoulos (2013) created a non-transgenic animal model, namely the ferrous amyloid buthionine (FAB) rat model, to simulate the progression of late-onset AD. This model comprehensively and reproducibly simulated the pathology of late-onset AD, including memory loss, glial activation, deposition of Aβ plaques, increased cerebrospinal fluid hyperphosphorylated tau protein, and neuronal losses in CA1-3 hippocampal regions, dentate gyrus, and temporal cortex (Lecanu et al., 2006; Lecanu and Papadopoulos, 2013).

**Abbreviations:** AD, Alzheimer’s disease; ANOVA, analysis of variance; ATX, astaxanthin; Aβ, beta-amyloid; BDNF, brain-derived neurotrophic factor; CREB, cyclic AMP regulated element binding protein; ChAT, choline acetyltransferase; FAB, ferrous amyloid buthionine; GFAP, glial fibrillary acidic protein; H2O2, hydrogen peroxide; H&E, hematoxylin and eosin; Iba1, ionized calcium binding adaptor molecule 1; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LY, Lucifer yellow; MS, medial septal; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NGF, nerve growth factor; Nrf2, nuclear translocation of nuclear factor erythroid 2-related factor 2; PB, phosphate buffer; PBS, phosphate-buffer saline; PSD95, postsynaptic density protein 95; SD, Sprague-Dawley; STZ, streptozotocin; SNAP25, synaptosomal-associated protein 25; TNF-α, tumor necrosis factor-α.

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The effects of oxidative stress had been recognized as the main contributing factor in late-onset AD. Some studies demonstrated that mitochondrial damage resulting in increased oxidative stress in early stages of AD before the appearance of the deposition of Aβ plaques and the onset of clinical symptoms of AD (Prasansuklab and Tencomnnoo, 2013). In AD, the level of antioxidant enzymes was decreased, therefore the balance was destroyed and the oxidative stress accumulated (Tönnes and Trushina, 2017). In addition, in vivo and in vitro studies indicated abnormal homeostasis of metal ions could also influence Aβ aggregation (Wang and Wang, 2017). Combined metal ions with Aβ fibril stabilized the latter and made it harder to degrade. The redox cycling of metal ions in the presence of Aβ could generate hydroperoxide (H₂O₂) and degraded to form unstable reactive oxygen species, leading to oxidative cellular damage and neuronal dysfunction or loss (Greenough et al., 2013).

Recently, antioxidant supplementations have been used to ameliorate oxidative stress-related diseases, including diabetes mellitus, rheumatoid arthritis, and AD. In this regard, supplementing vitamin E was found to attenuate the toxic effects of Aβ and improve the cognitive performance of Aβ-induced AD rats and extended the survival rate in AD patients (Pavlik et al., 2009). Curcumin treatment was reported to reduce carbonyls and facilitated the disaggregation of Aβ in APP Tg2576 transgenic mice (Lim et al., 2001). Thus, antioxidant supplementation seems to have great potential in treating AD. Astaxantin (ATX) is a more potent antioxidant than other carotenoids and believed to be the strongest antioxidant. The chemical structures of ATX enable it to chelate divalent metal ions and possess antioxidant capacity to quench singlet oxygen and eliminate free radicals (Choï and Koo, 2005; Hernández-Marin et al., 2012; Lobos et al., 2016; Shimizu et al., 1996). Besides these, ATX also has anti-inflammatory capacities, including inhibiting neuroinflammation and reducing the release of pro-inflammatory cytokines (Kurashige et al., 1990). In light of these, we explored the potentials of ATX in treating AD by investigating the effectiveness of ATX in FAB-induced AD rats.

2. Material and methods

2.1. Animals

Sixty male, 3–4 month-old, Sprague-Dawley (SD) rats (BioLASCO, Taiwan) were used in this study. They were housed and cared under the supervision of the Animal Care and Use Committee of the National Chung-Hsing University under guidelines of the National Science Council of Taiwan. Animals were caged individually in temperature (24 ± 1 °C), humidity (60–65%) and light-controlled room (12/12 h light-dark-cycle) with food and water ad libitum. FAB was infused intraventricularly (30 rats; please see below) to induce the experimental AD. Ten intact rats were used as normal group (Nor). Ten normal rats were treated with high-dose of ATX (Nor + hATX) to find out whether ATX alone had an effect on animals. Rats, infused intraventricularly and administered intraperitoneally with equal amount of solvent (please see below), were sham-operated control group (Sham, n = 10). FAB-infused rats were divided into three groups: FAB-infusion group (FAB, n = 13), FAB infusion and low-dose ATX treatment group (FAB + lATX, n = 7) and FAB infusion and high-dose ATX treatment group (FAB + hATX, n = 10). ATX treatment was described section below.

2.2. Ferrous amyloid buthionine (FAB) rat model

The FAB rat model followed the protocol developed by Lecanu et al. (2006). For continuous intraventricular infusion of FAB, rats were first anesthetized with ketamine and xylazine (8 mg ketamine and 1 mg xylazine/100 g body weight) and placed on a stereotactic device. With a micromanipulator, the cannula of a brain infusion kit (Alzet, Cupertino, CA, USA) was implanted into the lateral ventricle of the right brain at the following coordinates: ant/post = 1.0 mm, lat = 0.9 mm and dep = 4.5 mm to bregma. The osmotic pump (Alzet, 2ML4) of the infusion kit was filled with 2 ml FAB solution containing 15 μM Aβ1–42, 1 mM ferrous sulfate and 12 mM buthionine sulfoximine in saline. The osmotic pump was implanted subcutaneously on the back. The osmotic pump had a delivery rate of 2.5 μl/hr for 4 weeks. All animals after continuous intraventricular infusion of FAB could survive until sacrifice.

2.3. ATX treatment and related protocols

50 (high dose) or 5 (low dose) mg ATX (SIGMA-ALDRICH, St. Louis, MO, USA) was dissolved in 5 ml saline with 0.5% DMSO, and stored at 4 °C in dark. High dose (10 mg/kg body weight/day) and low dose (1 mg/kg body weight/day) solutions were used for the treatment of each corresponding animal, respectively. ATX (1 ml/kg body weight/day) was intraperitoneally injected at 9 a.m. for the last 7 days before the animal was scheduled to be sacrificed. Morris water maze task was conducted 1 h after ATX injection.

2.4. Morris water maze

A modified Morris water maze task was used to assess the learning and memory of the animals (Chen et al., 2017). The maze consisted of a black circular pool 145 cm in diameter and 52 cm deep. The pool was filled with water to a height of 23 cm. A round transparent platform 24 cm in diameter was placed 2 cm below the surface of the water. Some visual cues (triangle, round, square and star cardboards) were located at the edge of the pool. The task consisted of two stages, acquisition test and probe test. In the first stage, we measured escape latency and swimming distance. In the second stage, swimming distance and time spent in the target quadrant was calculated. The whole process was recorded with a video camera and analyzed with the SMART video tracking system (SMART 3.0 V, Panlab, Havard Apparatus, Cambridge, UK).

Acquisition testing: Rats were tested with two trials per day for 3 consecutive days. Before the beginning of the test, rats were placed on the platform for 60 s to familiar with the environment and the location of the platform. To run the test, rats were randomly placed into different quadrant of the pool in each trial. Rats were allowed to remain on the platform for 60 s after succeed to escape or failed to locate the under-water platform within 180 s. The escape latencies of the two trials each day were averaged for subsequent analyses.

Probe testing: On the 3rd day of the experiment, the platform in the pool was removed and the pool was planned with a virtual target quadrant and a virtual platform according to the previous location of the platform. After the final escape latency task and a 10 min break, the rat was placed into the diagonal area of the target quadrant. The rat was allowed to swim for 90 s and the swimming path analyzed.

2.5. Tissue preparation

To sacrifice, rats were deeply anesthetized with ketamine and xylazine (8 mg ketamine and 1 mg xylazine/100 g body weight). We followed previously published protocols to prepare tissues for intracellular dye injection and immunohistochemical and histochemical stainings (Chen et al., 2017, 2014). Briefly, rats were transcardially perfused with 2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3, for 30 min. Brains were sectioned with vibratome (Technical Products International, St. Louis, MO) into three parts: (1) two pieces of 350-μm-thick coronal slices contain the hippocampus for intracellular dye injection; (2) a 1000-μm-thick coronal slice contained the medial septal (MS) nucleus and a 2000-μm-thick coronal slice contained dorsal hippocampus for immunohistochemical staining; (3) the remaining tissue slices about 2000-μm thickness contained dorsal hippocampus for histochemical staining. The thick slices for immunohistochemical and histochemical stainings were immediately postfixed with 4% paraformaldehyde in 0.1 M PB for 1 day. The slices for intracellular dye
injection were soaked in $10^{-7}$ M 4', 6-diamidino-2-phenyl-indole in 0.1 M PB for 30 min at 4 °C to reveal cell nuclei for dye injection.

### 2.6. Intracellular dye injection and immunoclonation

We followed our earlier protocols for intracellular dye injection in brain slices (Chen et al., 2017, 2014). The slice was placed in a chamber containing 0.1 M PB on the stage of a fixed-stage fluorescence microscope (Olympus BX51). For dye injection, a glass micropipette filled with 4% Lucifer yellow (LY; Sigma-Aldrich) solution (4% in water) was mounted with the three-axial hydraulic micromanipulator (Narishige, Tokyo, Japan) for dye injection. An intracellular amplifier (Axoclamp-III; Axon, Foster city, CA, USA) was used to release LY into selected neuron. Following injection, the slice was postfixed in 4% paraformaldehyde in 0.1 M PB for 1 day. The slice was then cryo-protected with 30% sucrose in 0.1 M PB and cryosectioned into 60-μm-thick serial sections with a LEICA cryostat (CM1850, Nussloch, Germany) for subsequent immunoclonation.

The sets of serial sections from each injected slice were first incubated with 1% H$_2$O$_2$ and 1% Triton X-100 in 0.1 M PB for 30 min to remove endogenous peroxidase activity. They were then incubated with biotinylated rabbit anti-LY (1:200, Molecular Probes, Eugene, OR) in phosphate-buffer saline (PBS) containing 2% Bovine Serum Albumin (BSA) for 1 h at room temperature. The slices were then reacted with 0.05% 3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.01% H$_2$O$_2$ in 0.05 M Tris buffer (Sigma-Aldrich). Reacted sections were mounted on slides, air-dried, and coverslipped with Permount for subsequent analyses.

### 2.7. Histochemical staining

The postfixed thick slice was commissioned by the Animal Disease Diagnostic Center (ADDC of National Chung-Hsing University, Taichung, Taiwan). The slices were embedded in paraffin, sectioned into 2 μm thickness with a microtome and mounted sections onto slides. They were then processed for hematoxylin and eosin (H&E) staining. Sections were then air-dried, and coverslipped with Permount for subsequent analyses.

### 2.8. Immunohistochemical staining

After postfixation, the designated thick slices were cryo-protected with 30% sucrose in 0.1 M PB and sectioned into 30-μm-thick sections with a LEICA cryostat. Sixteen serial cryosections containing the dorsal hippocampus were collected and grouped orderly into 4 sets, with one in every 4 sections of the series in each set. The 4 sets of sections were processed for immunohistochemical stainings with ionized calcium binding adaptor molecule 1 (Iba1), glial fibrillary acidic protein (GFAP), iNOS and ChAT antibodies to reveal microglia, astrocyte, iNOS and ChAT immunoreactivities. From the thick slice containing the MS nucleus, the sections were reacted with goat anti-Iba1 (1:1000, EMD Millipore, USA), respectively, for 18 h at room temperature, and then reacted with 0.05% 3'-3'-diaminobenzidine tetrahydrochloride and 0.01% H$_2$O$_2$ in 0.05 M Tris buffer. Reacted sections were mounted on slides, air-dried, and coverslipped with Permount for subsequent analyses.

### 2.9. Statistical analysis

Cholinergic neurons in the MS nucleus were counted from 3 of the set of sections processed for the purpose from each rat. Number of CA1 pyramidal neurons in the dorsal hippocampus was counted from 500-μm-thick serial sections with a LEICA cryostat (CM1850, Nussloch, Germany) for subsequent immunoclonation. The sets of serial sections from each injected slice were first incubated with 1% H$_2$O$_2$ and 1% Triton X-100 in 0.1 M PB for 30 min to remove endogenous peroxidase activity. They were then incubated with biotinylated rabbit anti-LY (1:200, Molecular Probes, Eugene, OR) in phosphate-buffer saline (PBS) containing 2% Bovine Serum Albumin (BSA) for 1 h at room temperature. The sections were subsequently reacted with 0.05% 3'-3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.01% H$_2$O$_2$ in 0.05 M Tris buffer (Sigma-Aldrich). Reacted sections were mounted on slides, air-dried, and coverslipped with Permount for subsequent analyses.

### 3. Results

#### 3.1. The spatial learning and memory performance in FAB rats and ATX treatment

For hippocampus-related functions, we assessed the spatial learning and memory of the animals with Morris water maze task (Figs. 1 and 2). In acquisition test, the escape latencies of normal rats were 37.7 ± 7.6, 8.7 ± 3.5 and 6.0 ± 1.1 s (Fig. 1A, Nor) for the 1st, 2nd and 3rd day of the 3 consecutive days. The escape latencies of normal rats treated with high dose of ATX were 34.7 ± 8.2, 6.2 ± 1.4 and 5.1 ± 0.9 s (Fig. 1A, Nor + hATX). The escape latencies of sham-operated rats were 38.5 ± 8.5, 8.6 ± 3.0 and 6.4 ± 0.3 s (Fig. 1A, Sham). Those of FAB-infused rats were 36.5 ± 10.4, 22.4 ± 4.2 and 19.1 ± 6.2 s, respectively (Fig. 1A, FAB). FAB rats treated with low dose ATX spent 32.2 ± 12.0, 18.1 ± 2.3 and 18.6 ± 5.3 s to find the underwater platform (Fig. 1A, FAB + lATX). While FAB rats treated with high dose of ATX used 37.8 ± 17.3, 5.0 ± 0.7 and 3.9 ± 0.4 s to escape (Fig. 1A, FAB + hATX). Two-way ANOVA showed significant differences between different days of testing (F = 29.79, p < 0.001). Rats of all 5 groups spent for shorter time to find the underwater platform starting on the 2nd day of the 3 consecutive days of trials (Fig. 1A), but there were no significant difference among groups (F = 1.13, p = 0.349) and the day of testing and groups (F = 0.46, p = 0.910). Results of the escape time on the 3rd day (Fig. 1C) indicated that FAB rats spent more time to find the platform than sham control rats (p = 0.008, FAB vs Sham). Low dose ATX treatment in FAB animals failed to reduce it (p = 0.999, FAB + lATX vs FAB). However, High-dose ATX treatment in FAB animals effectively decreased search time (p = 0.003, FAB + hATX vs FAB).

The swimming distance in normal rats (Fig. 1B, Nor) respectively used 8.2 ± 1.9, 2.0 ± 0.8 and 1.3 ± 0.2 m to find the platform for the 1st, 2nd and 3rd day of the 3 consecutive days of tests. Normal animals treated with high-dose ATX were 6.2 ± 1.8, 1.3 ± 0.3 and 1.0 ± 0.1 m, respectively (Fig. 1B, Nor + hATX). Sham-operated rats were 8.5 ± 2.0, 2.0 ± 0.7 and 1.4 ± 0.2 m, respectively (Fig. 1B, Sham). FAB animals used 7.3 ± 2.1, 5.2 ± 1.0 and 3.6 ± 0.9 m for the 3 consecutive days (Fig. 1B, FAB), respectively. FAB rats treated with low dose ATX spent 6.9 ± 2.9, 4.0 ± 0.5 and 4.6 ± 1.1 m to find the underwater platform (Fig. 1B, FAB + lATX). While FAB rats treated with high dose of ATX used 7.8 ± 3.7, 1.2 ± 0.2 and 0.9 ± 0.1 m respectively to escape (Fig. 1B, FAB + hATX). Two-way ANOVA showed significant differences between...
different days of testing ($F = 24.29, p < 0.001$). Rats of all 5 groups swam for shorter distance to find the underwater platform starting on the 2nd day of the 3 consecutive days of trials (Fig. 1B). There were no significant difference between among groups ($F = 1.31, p = 0.263$) and the day of testing and groups ($F = 0.54, p = 0.856$). Results of the swimming distance on the 3rd day (Fig. 1D) indicated that FAB rats need more swimming distance to find the platform than sham control rats ($p = 0.007$, FAB vs Sham). Low dose ATX treatment in FAB animals failed to reduce it ($p = 0.742$, FAB + hATX vs FAB). However, High-dose ATX treatment in FAB animals effectively decreased swimming distance ($p = 0.001$, FAB + hATX vs FAB).

The spatial probe test at the end of the Morris water maze task was conducted to check animals’ performance in spatial memory (Fig. 2). The ratio of swimming distance in target quadrant (Fig. 2B) was $35.9 \pm 1.5\%$ in Nor, $35.0 \pm 1.2\%$ in Nor + hATX, $34.5 \pm 1.2\%$ in Sham, $25.4 \pm 2.4\%$ in FAB, $25.6 \pm 1.9\%$ in FAB + hATX and $35.5 \pm 3.0\%$ in FAB + hATX group, respectively ($F = 5.94, p < 0.001$). The ratio of swimming time in the target quadrant (Fig. 2B) was $36.9 \pm 1.8\%$ in Nor, $36.3 \pm 1.3\%$ in Nor + hATX, $35.4 \pm 1.7\%$ in Sham, $26.5 \pm 2.0\%$ in FAB, $25.2 \pm 1.9\%$ in FAB + hATX and $37.2 \pm 3.4\%$ in FAB + hATX group, respectively ($F = 5.48, p < 0.001$). The number of platform crossings (Fig. 2C) was $6.8 \pm 0.5$ in Nor, $7.5 \pm 0.6$ in Nor + hATX, $6.5 \pm 0.4$ in Sham, $2.4 \pm 0.5$ in FAB, $3.0 \pm 0.3$ in FAB + hATX and $6.0 \pm 1.2$ in FAB + hATX group, respectively ($F = 10.42, p < 0.001$). These indicated that FAB and FAB + hATX groups performed poorer while FAB + hATX group performed as good or close to sham control animals.

3.2 Neuroinflammatory responses in FAB rats and ATX treatment

To determine the extent of the neuroinflammatory responses in the
hippocampus, antibodies to Iba1 and GFAP were used to label microglia (Figs. 3 and 7A) and astrocytes (Figs. 4 and 7B). In normal animals, CA1 hippocampal region had a density of microglia of 12.1 ± 0.4 (Fig. 3A). High dose ATX treatment in normal animals and Sham-operated animals didn’t alter the microglial density (Fig. 3B and C; 12.3 ± 0.4 in Nor + hATX and 12.4 ± 0.4 in Sham). FAB rats significantly increased it to 38.9 ± 6.7 (Fig. 3D; p < 0.001, FAB vs Sham). Low dose ATX treatment in FAB animals (Fig. 3E; 36.8 ± 7.1) failed to reduce it (p = 0.999, FAB + lATX vs FAB). High-dose ATX treatment in FAB animals (Fig. 3F; 17.9 ± 1.6) effectively reduced microglial density (p = 0.005, FAB + hATX vs Sham) to close to sham control level (Fig. 7A; F = 12.91, p < 0.001).

The density of astrocytes in the CA1 field of hippocampus in normal animals was 23.7 ± 0.4 (Fig. 4A). It remained unchanged in the Nor + hATX and Sham group (Fig. 4B and 4C; 22.8 ± 0.3 in Nor + hATX and 23.8 ± 0.3 in Sham). FAB rats increased to 47.3 ± 4.2 (Fig. 4D; p < 0.001, FAB vs Sham). Low dose ATX treatment (Fig. 4E; 37.3 ± 1.2) moderately decreased it (p = 0.004, FAB + lATX vs FAB). High dose ATX treatment (Fig. 4F; 27.5 ± 1.4) reduced astrocyte density further to close to sham control level (Fig. 7B; F = 35.01, p < 0.001; p < 0.001, FAB + hATX vs Sham).

For the expression of iNOS, the density of iNOS+ neuron was low in normal animals (Fig. 5A; 2.9 ± 0.1). It remained low in Nor + hATX and Sham animals (Fig. 5B and C; 2.3 ± 0.4 in Nor + hATX and 2.5 ± 0.2 in Sham). It significantly increased to 5.6 ± 0.4 with FAB infusion (Fig. 5D; p < 0.001, FAB vs Sham). Low dose ATX treatment (Fig. 5E; 5.0 ± 0.2) failed to reduce it (p = 0.911, FAB + lATX vs FAB). High dose ATX treatment (Fig. 5F; 2.3 ± 0.7) significantly reduced it to close to sham control level (Fig. 7C; F = 14.07, p < 0.001; p < 0.001, FAB + hATX vs Sham).

### 3.3. The changes of cholinergic innervation in FAB rats and ATX treatment

The cholinergic fibers projections of hippocampus were mainly from cholinergic neurons in MS nucleus. We then explored the effects of FAB and/or ATX treatment on the distribution pattern of cholinergic fibers in CA1 field of hippocampus (Fig. 6) and cholinergic neurons in MS nucleus (Fig. 8). Nor + hATX and Sham groups didn’t alter either the densities of cholinergic fibers in dorsal hippocampal (Fig. 6B and C; 1.00 ± 0.02 in Nor + hATX and 1.02 ± 0.02 in Sham) or the relative IOD ratio of cholinergic neuron in MS nucleus (Fig. 8). Nor + hATX and Sham groups didn’t alter either the densities of cholinergic fibers in dorsal hippocampal (Fig. 6B and C; 1.00 ± 0.02 in Nor + hATX and 1.02 ± 0.02 in Sham) or the relative IOD ratio of cholinergic neuron in MS nucleus (Fig. 8B and C; 1.01 ± 0.02 in Nor + hATX and 1.00 ± 0.01 in Sham). Intraventricular FAB infusion reduced the densities of ChAT-immunoreactive nerve fibers in the dorsal hippocampus (Figs. 6C, D and 7D; 0.55 ± 0.04 in FAB, p < 0.001, Sham vs FAB) as well as the relative IOD ratio of ChAT + neurons in the MS nucleus (Figs. 8C, D and G; 0.76 ± 0.03 in FAB group; p < 0.001, FAB vs...
Sham). High dose, but not low dose ATX treatment restored the cholinergic fiber in the dorsal hippocampus (Figs. 6E, F and 7D; \(0.60 \pm 0.002\) in FAB + IATX and \(1.02 \pm 0.04\) in FAB + hATX; \(p = 0.836\), FAB + IATX vs FAB; \(p < 0.001\), FAB + hATX vs FAB) as well as the relative IOD ratio of cholinergic neurons in the MS nucleus (Fig. 8E–G; \(0.76 \pm 0.03\) in FAB + IATX and \(0.92 \pm 0.04\) in FAB + hATX; \(p = 0.999\), FAB + IATX vs FAB; \(p = 0.001\), FAB + hATX vs FAB).

### 3.4. The Effects of FAB-infusion and ATX treatment on hippocampal CA1 pyramidal neurons

First, H&E staining was used to evaluate the apparent changes in the CA1 pyramidal cells of the dorsal hippocampus. In normal animals, CA1 pyramidal neuron had relatively large cell body and readily identifiable nucleus (Fig. 9A, arrows; 98.6 ± 1.9 in Nor). Normal animals treated with high-dose ATX and sham-operated animals had no effect on CA1 pyramidal neurons as well (Fig. 9B, C and G; 99.2 ± 1.2 in Nor + hATX and 101.2 ± 1.8 in Sham). Four weeks of FAB infusion resulted in many karyopyknotic cells in the pyramidal cell layer (Fig. 9D, arrowheads) and the number of CA1 pyramidal neurons per 500 \(\mu\)m length was reduced as well (Fig. 9C, D, and G; 59.0 ± 7.3 in FAB group; \(p < 0.001\), FAB vs Sham). Unlike the effects on all cholinergic innervations, neither high dose nor low dose ATX treatment restored the densities of CA1 pyramidal neurons to sham control level (Fig. 9E–G; 57.4 ± 12.2 in FAB + IATX and 51.8 ± 10.2 in FAB + hATX, \(p = 0.999\), FAB + IATX vs FAB; \(p = 0.991\), FAB + hATX vs FAB).

To investigate the morphological correlates of spatial learning and memory changes, we looked into the densities of dendritic spines on hippocampal CA1 pyramidal neurons revealed with intracellular dye injection. The dendritic spine, a dynamic structure, is the mainly site of excitatory synapse on pyramidal neurons. Spine density on distal apical dendrites (Fig. 10A and D) was 18.0 ± 0.1 in Nor, 17.8 ± 0.1 in Nor + hATX, 17.6 ± 0.2 in Sham, 10.7 ± 0.5 in FAB, 11.5 ± 0.5 in FAB + IATX and 14.0 ± 0.3 in FAB + hATX, respectively (\(F = 101.42\), \(p < 0.001\)). Spine density on proximal apical dendrites (Fig. 10B and D) was 18.3 ± 0.1 in Nor, 18.3 ± 0.2 in Nor + hATX, 18.6 ± 0.2 in Sham, 13.1 ± 1.0 in FAB, 12.7 ± 0.4 in FAB + IATX and 15.3 ± 0.3 in FAB + hATX, respectively (\(F = 32.07\), \(p < 0.001\)). Whereas spine density on distal basal dendrites (Fig. 10C and D) was 18.0 ± 0.2 in Nor, 17.7 ± 0.2 in Nor + hATX, 18.3 ± 0.2 in Sham, 13.5 ± 0.8 in FAB + IATX and 16.6 ± 0.3 in FAB + hATX, respectively (\(F = 17.21\), \(p < 0.001\)). Thus, as compared to sham control animals, spine densities of CA1 pyramidal neurons on the distal and proximal segments of the apical dendrites and distal basal dendrites were significantly reduced, 40%, 29% and 22% respectively following 4 weeks of FAB infusion. The low dose ATX treatment couldn’t markedly change spine density on each
segment. The spine densities on CA1 pyramidal neurons were significantly restored, by 32%, 17% and 16% following high dose ATX treatment (Fig. 10 D).

4. Discussion

4.1. The effects of intraventricular FAB infusion

Due to the many possible factors of sporadic AD, such as eating habits, environment and chronic disease etc, the performance of the animal model can only be as close as possible to the symptoms of AD. Recently published sporadic AD animal models, such as FAB-induced and streptozotocin (STZ)-induced AD animal models, had been shown to result in pathology similar brain to AD including the presence of aggregated amyloid, hyper-phosphorylated tau protein, neuronal loss, reactive glia and behavioral disorders (Bittner et al., 2010; Che et al., 2018; Fronza et al., 2021; Lecanu et al., 2006; Majkutewicz et al., 2018; Paladugu et al., 2021; Pierzynowska et al., 2019; Taksima et al., 2019; Tiwari et al., 2021; Yang et al., 2020). In consistence with our findings following intraventricular FAB infusion, the continuous FAB infusion into lateral ventricle for 4 weeks had been shown to result in increased neuroinflammatory indicators (Bittner et al., 2010; Che et al., 2018; Lecanu et al., 2006; Tiwari et al., 2021; Yang et al., 2020). Aβ derivatives might bind to toll-like receptor 4 and activate p38 mitogen-activated protein kinases and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways to promote microglia and astrocyte activation (Reed-Geaghan et al., 2009; Tiwari et al., 2021). Activated microglia and astrocyte could in turn increase the expression of inflammatory cytokines (Munoz and Ammit, 2010), including IL-1β, IL-6, and TNF-α, which might worsen inflammation and increase oxidative stress. TNF-α increased intracellular expression of iNOS and peroxynitrite to lead to neuronal apoptosis or alternation of synaptic plasticity (Combs et al., 2001). These could be some of the plausible causes for the neuronal loss that we observed in the CA1 field of hippocampus following 4 weeks of intraventricular FAB infusion.

Our study firstly demonstrated the cholinergic dysfunction and the dendritic spine loss of CA1 hippocampal pyramidal neurons in FAB-
infused rat model. These two characteristics were widely seen in the pathology of AD patients and its animal models (Manczak et al., 2018; Moolman et al., 2004; Petrasek et al., 2016; Rajendran and Paolicelli, 2018; Xu et al., 2014). This study directly observed that CA1 hippocampal pyramidal neurons surviving 4 weeks of FAB treatment lost 22–40% of the dendritic spines on all representative segments of their basal and apical dendrites. Previously published study showed that the spine loss was about 50% in the hippocampus of patients who died of AD (Moolman et al., 2004). In the studies of APP gene transgenic mice and STD-induced AD rats, the dendritic spines on the CA1 hippocampal pyramidal neurons was decreased by about 45% (Manczak et al., 2018) and 47% (Xu et al., 2014), respectively. Disturbance of dendritic spine homeostasis are believed to underlie a number of neurodegenerative disorders. In STZ-induced model, the levels of pre- (synaptosomal-associated protein 25, SNAP25) and post-synaptic protein (postsynaptic density protein 95, PSD95) were also significantly reduced (Ding et al., 2021). Moreover, studies in human AD brain and transgenic mice showed that extracellular Aβ oligomers could lead to activation of the calcium-dependent phosphatase calcineurin, which in turn activates the transcriptional factor nuclear factor of activated T cells (NFAT). Activation of these signaling pathways was sufficient to produce dendritic simplification and dendritic spine loss in culture and in adult mouse brain (Dorostkar et al., 2015; Lobos et al., 2016; Wu et al., 2016).

In STZ-induced AD model, the brain-derived neurotrophic factor (BDNF)/Cyclic AMP regulated element binding protein (CREB) signaling axis related to synapse production was reduced (Tiwari et al., 2021). In addition, activated microglia might release IL-1β to antagonize the action of BDNF and TNF-α, which leads to phosphorylation and upregulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors resulting in excitotoxicity, thereby leading to spine loss (Ferguson et al., 2008; Leonoudakis et al., 2008; Tong et al., 2012). On the other hand, activated microglia may also play an important role in regulating dendritic spines to remove excess or damaged spines via phagocytosis (Chung and Barres, 2012). Alternatively, number of dendritic spines could also be modulated by alterations of presynaptic input. In the present study, we found the reduction of cholinergic terminals in the CA1 field of hippocampus following FAB treatment. Earlier studies had shown that cholinergic lesion rats had altered hippocampal glutamatergic synaptic transmission required for learning and memory (Fréchette et al., 2009; Kanju et al., 2012). Forebrain cholinergic lesion had also been shown to reduce the complexity and spine density of CA1 pyramidal cells (Fréchette et al., 2009). Another study using the same FAB rat model showed spatial learning and memory deficits, as well as alterations of cortical glutamatergic and hippocampal cholinergic systems (Petrasek et al., 2016). Thus, in addition to the increase of oxidative stress and the activation of...
microglia, the reduction of cholinergic fibers following FAB infusion might be an additional factor contributing to the trimming of dendritic spines on CA1 pyramidal neurons.

4.2. The effects of ATX treatment on FAB rats

In sporadic AD models, many published treatments could reduce inflammation or oxidative stress to improve behavioral deficits. The famous effect of astaxanthin was its strong anti-oxidant and anti-inflammatory ability. In this study, low-dose ATX decreased activation of microglia whereas only high-dose ATX could restore activation of microglia and astrocyte, iNOS expression, spine loss on hippocampal CA1 pyramidal neurons, the distribution pattern of cholinergic fibers in CA1 field of hippocampus and cholinergic neurons in MS nucleus, and cognitive disorder in FAB animal models. Some reports indicated that treatment of sulforaphene or liraglutide could reduce the inflammatory responses caused by STZ-infusion in the brain (Paladugu et al., 2021; Yang et al., 2020); treatment of rapamycin, sulfonamides derived from carvacrol or multi-target compound (QTC-4-MeOBnE) could decrease the oxidative stress caused by STZ-infusion (de Souza et al., 2020d; Ding et al., 2020c).
Fig. 9. Effects of ATX treatment on survival of CA1 hippocampal pyramidal neurons. Representative H&E stained from each group are illustrated (A-F). Arrows indicate surviving pyramidal neurons. FAB treatment resulted in cell shrinkage and karyopyknosis (arrowheads). The number of the surviving pyramidal neurons (arrows) in each group were counted and analyzed in G (F = 14.90, p < 0.001). *, p < 0.05 between the marked and Nor; #, p < 0.05 between the marked and Nor+ hATX; $, p < 0.05 between the marked and Sham. Bar =50 μm for all micrographs.
Representative micrographs of the distal (A) and proximal apical (B) and distal basal dendrites (C) of the hippocampal CA1 pyramidal neurons from each group were illustrated. Spine density were analyzed and plotted in D (F = 101.42, p < 0.001 in distal apical dendrite; F = 32.07, p < 0.001 in proximal apical dendrite; F = 17.21, p < 0.001 in distal basal dendrite), respectively, *, p < 0.05 between the marked and Nor; @, p < 0.05 between the marked and Sham; $, p < 0.05 between the marked and hATX; #, p < 0.05 between the marked and Nor + hATX; &$, p < 0.05 between the marked and FAB; &*, p < 0.05 between the marked and FAB + hATX. Bar =10 μm for A–C.

Fig. 10. Effects of ATX treatment on the dendritic spine loss of hippocampal CA1 pyramidal neuron.

In conclusion, we tested the effects of a potent antioxidant ATX on an intraventricular FAB infusion model of sporadic AD rats. Besides the presence of aggregated amyloid, hyper-phosphorylated tau protein, neuronal loss, reactive glia and behavioral disorders that showed in...
Lecanu’s reports, we found the withdrawal of cholinergic fibers and the dendritic spine loss of CA1 hippocampal pyramidal neurons in FAB-infused rats. ATX at sufficient concentration was found to reverse the behavioral disorder, neuroinflammatory responses, cholinergic fiber loss and spine loss in hippocampus. In addition, we believed that ATX treatment ameliorated the spatial learning and memory in FAB infused rats might directly through the modulation of dendritic spine of pyramidal neurons, or indirectly through the decreased oxidative stress, neuroinflammatory response or rebound cholinergic functions. In short, ATX has a great potential in ameliorating AD syndromes and perhaps in slowing oxidative stress-related neurodegeneration.

Author contributions

- Mu-Hsuan Chen: Conceptualization; Methodology; Validation; Investigation; Data Curation; Writing - Original Draft; Visualization.
- Tsyr-Juan Wang: Resources; Writing - Review & Editing.
- Li-Jin Chen: Resources; Methodology.
- Ming-Ying Jiang: Methodology.
- Yuet-Jan Wang: Methodology.
- Gui-Fang Tseng: Resources; Writing - Review & Editing.
- Jeng-Rung Chen: Methodology; Resources; Writing - Review & Editing; Supervision; Project administration; Funding acquisition.

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Declaration of Competing Interest

All authors declare no conflict of interest with the organizations that sponsored the research.

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