Huperzine A in treatment of amyloid-β-associated neuropathology in a mouse model of Alzheimer disease: abridged secondary publication

Q Yuan *, ZX Lin, W Wu, WN Albert, BCY Zee

KEY MESSAGES

1. Huperzine A treatment resulted in a reduction of acetylcholinesterase activity in TgCRND8 Alzheimer disease mice.
2. Huperzine A, an acetylcholinesterase inhibitor for Alzheimer disease, could not inhibit GSK3β activity, and therefore did not facilitate prevention of amyloid precursor protein processing/amyloid-β generation in TgCRND8 mice.

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[1] **Q Yuan**, [1] **ZX Lin**, [2] **W Wu**, [1] **WN Albert**, [3] **BCY Zee**

[1] *School of Chinese Medicine, Faculty of Medicine, The Chinese University of Hong Kong*
[2] *School of Biomedical Sciences, The University of Hong Kong*
[3] *Division of Biostatistics JC School of Public Health and Primary Care, The Chinese University of Hong Kong*

* Principal applicant and corresponding author: yuanqj@cuhk.edu.hk

Introduction

Huperzine A is a novel *Lycopodium* alkaloid extracted from traditional Chinese herb *Huperzia serrata* (Thunb) Trev (Qian Ceng Ta). It is a potent selective reversible acetylcholinesterase (AChE) inhibitor and has been used in China for the treatment of Alzheimer disease (AD) since 1996.[1-3] Large, randomised, placebo-controlled, double-blinded clinical trials revealed that huperzine A can enhance memory, cognitive skills, and daily life abilities of patients with AD.[1] Clinical trials of huperzine A for treatment of age-related memory deficiency have been conducted in the United States.[3] New mechanisms of action for huperzine A have been discovered. In addition to its AChE inhibitory effect, huperzine A influences amyloid precursor protein (APP) processing to reduce the formation of amyloid-β (Aβ) peptides.[3] However, there has been no sufficient experimental evidence from AD animal models to elucidate the precise molecular mechanisms of huperzine A on APP processing. This study aimed to investigate whether huperzine A inhibited GSK3β, thereby facilitated prevention of APP processing/Aβ generation in TgCRND8 mice.

Methods

This study was conducted from June 2015 to November 2017. Male TgCRND8 mice aged 5 months were randomly assigned to the treated or untreated group. The treated group received huperzine A dissolved in a vehicle of normal saline daily by intraperitoneal injection (0.80 μmol/kg). The untreated group received an equal volume of normal saline as a vehicle control. Treatment continued for 8 weeks, and then the mice were sacrificed by decapitation, and one hemisphere of each brain was processed for Aβ, IBA-1, and GFAP immunohistochemistry. The other hemispheres of the brains were processed for GSK3β, BACE1, CTF-APP, and B-actin Western blotting analysis.

Cross-sections of the brain were treated according to standard procedures. Briefly, the sections were incubated overnight at room temperature with the primary antibodies against Aβ (mouse, 1:3000, Sigma-Aldrich), IBA-1 (rabbit, 1:2000, Wako), and GFAP (mouse, 1:3000, Sigma-Aldrich) in 0.1 M PBS (pH 7.4) containing 10% normal goat serum and 0.2% Triton X-100. Then, antigens were visualised using Alexa 568-conjugated secondary antibody (1:800; Invitrogen). Finally, the sections were visualised under a fluorescence microscope (Zeiss).

To determine the brain cortex expression of GSK3β and its phosphorylation at serine 9, BACE1, and CTF-APP, tissue samples were homogenised in 0.1 ml lysis buffer reagent and serine protease inhibitor PMSF (both from Sigma-Aldrich) and centrifuged at 14,000 ×g for 30 min at 4°C. The supernatant was collected and total protein was measured using a protein assay (Bio-Rad). An equal volume of 2× sample buffer (100 mM Tris–HCl pH 6.8, 2.5% SDS, 20% glycerol, 0.006% bromophenol blue and 10% β-mercaptoethanol) was added to 30 μg total proteins. The samples were boiled and then electrophoresed in a 10%-15% SDS-polyacrylamide gel (Sigma-Aldrich) and transferred to a Hybond-P membrane (Amersham Bioscience).The blotted membrane was then incubated overnight with 5% skim milk in T-TBS (containing 0.1% v/v Tween 20). All antibody applications were done in T-TBS.

After the membranes were washed with T-TBS, they were incubated overnight at room temperature with GSK3β (rabbit, 1:3000, Cell Signaling Technology), GSK3β phosphorylated at Ser9 (rabbit, 1:3000, Cell Signaling Technology), BACE-1 (rabbit, 1:1000; Abcam), the C-terminal anti-APP antibody CT15 for full-length APP and CTF-β (rabbit, 1:2000, Cell Signaling Technology). The membranes were extensively washed with T-TBS and incubated for 1 hour with the secondary antibody (anti-mouse or anti-rabbit IgG peroxidase-conjugated antibody, 1:5000) [Sigma-Aldrich]. After washing, the proteins were detected using an ECL-Plus Western blotting detection system (GE Healthcare).

In accordance with methods described in our previous study,[4] brains were sectioned in 30 μm thickness using a microtome. Plaque deposition levels were examined in cortex. Images of 100× magnification were captured using a Zeiss microscope equipped with a SPOT camera and SPOT software (RT Color Diagnostic Instrument) on four sections per animal. By using ImageJ software, pictures were binarised to 8-bit black and white pictures and a fixed intensity threshold was applied to define the immunofluorescence staining. Measurements were performed for a percentage area covered by Bam-10, IBA-1, or GFAP immunostaining.

Results

Huperzine A treatment resulted in a reduction of AChE activity in the TgCRND8 mice

We examined whether huperzine A could inhibit AChE activity in TgCRND8 mice brain. To measure AChE activity, the brain cortex was added into 1% Tris-HCl buffer and homogenised. Homogenates were centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatant was used as AChE enzyme source and stored at -80°C. AChE activity was measured using an Amplite Colorimetric AChE Assay Kit (AAT Bioquest). The AChE inhibitory activity observed in the control was considered to be 100%. Huperzine A inhibited AChE activity (Fig. 1a).

Huperzine A treatment did not reduce Aβ plaque burden in the brain cortex of TgCRND8 mice

Aβ plaque immunostaining with the bam10 antibody and thioflavin S staining in TgCRND8 mice showed marked Aβ deposits in the cortex of TgCRND8 mice. Quantification of the Aβ immunoreactivity showed no significant reduction of plaque burden in huperzine A–treated animals compared with controls (Fig. 1b).

Huperzine A treatment did not ameliorate Aβ-associated reactive gliosis or astrocytosis in the brain cortex of TgCRND8 mice

Microgliosis and astrocytosis in TgCRND8 mice were elevated phenotypically as a consequence of amyloid deposition. The degree of microgliosis as evaluated by IBA-1 load in the brain cortex was significantly amplified in vehicle-treated TgCRND8 mice relative to wildtype mice (data not shown), whereas it was not significantly reduced in huperzine A–treated TgCRND8 mice relative to vehicle-treated TgCRND8 mice (Fig. 2). Likewise, the magnitude of astrocytosis as assessed by clusters of GFAP-immunoreactive astrocytes (GFAP burden) was not significantly reduced in huperzine A–treated TgCRND8 mice, relative to vehicle-treated TgCRND8 mice (Fig. 2).

Huperzine A treatment did not alter GSK3β activity or APP processing in TgCRND8 mice

We examined the levels of phosphorylated GSK3β, BACE-1, CTF-beta in the mice cortex. It is known that GSK3β is inhibited when Ser9 is phosphorylated.[5] However, we found no significant alteration of the inactive form of GSK3β phosphorylated at Ser9. The data suggest that huperzine A treatment did not alter GSK3β activity. Likewise, no remarkable alterations of BACE1 and CTF-β were observed in the brains of the huperzine A–treated TgCRND8 mice compared with vehicle-treated TgCRND8 controls (Fig. 3).

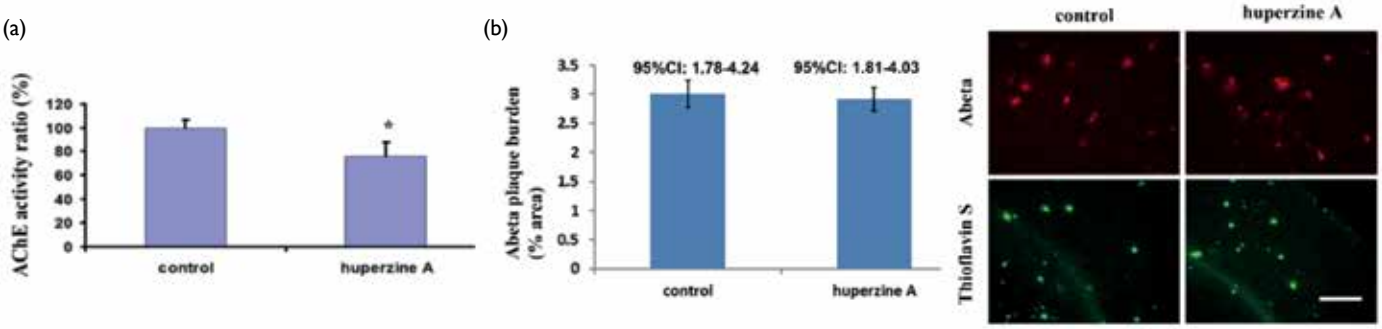

FIG 1. Effects of huperzine A treatment in the cortex of TgCRND8 mice on (a) acetylcholinesterase (AChE) activity and (b) amyloid-β plaque burden (as revealed by Thioflavin S staining and amyloid-β immunohistochemical staining).

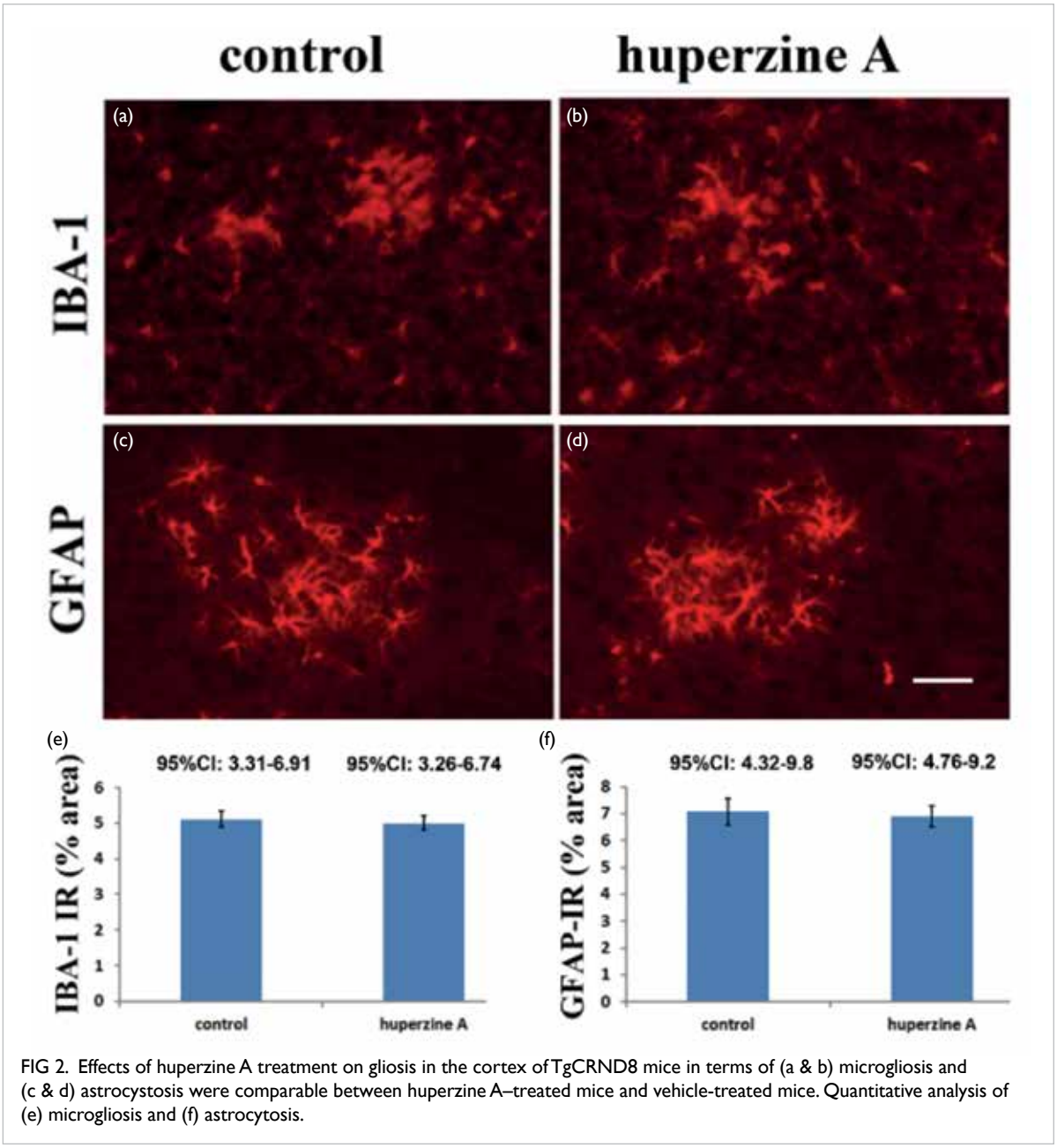

FIG 2. Effects of huperzine A treatment on gliosis in the cortex of TgCRND8 mice in terms of (a & b) microgliosis and (c & d) astrocystosis were comparable between huperzine A–treated mice and vehicle-treated mice. Quantitative analysis of (e) microgliosis and (f) astrocytosis.

Discussion

Although huperzine A is a therapeutic drug for AD by inhibiting AchE activity in patients with AD, the actual therapeutic role of huperzine A in Aβ neuropathology has not been fully evaluated. Data from this study suggest that regular administration of huperzine A may not involve the mechanisms targeting amyloidogenic APP cleavage pathway observed in TgCRND8 mice with an early-onset AD-like pathology. Our results suggest that huperzine A has no beneficial effects in Aβ neuropathology of AD. First, no significant effects on brain Aβ plaque burden and associated gliosis were found in the TgCRND8 mouse model of AD. Second, Huperzine A did not significantly reduce CTFs and BACE-1, a key enzyme for APP cleavage. Third, huperzine A did not significantly inhibit GSK3B activity in the brain of TgCRND8 mice. It has been shown that GSK3β activity increases in cells expressing Swedish APP mutation and in AD presenilin-1 and presenilin-2 mutation lymphoblast cells via inactive Ser9 phosphorylated GSK3β. Studies have shown that GSK3β affects APP processing by modulating BACE-1 activity, thereby facilitating Aβ production, reinforcing that GSK3β plays a key role in APP processing/Aβ generation. Our findings that huperzine A did not block GSK3β activity in the brain of TgCRND8 mice may explain

its effect on Aβ plaque burden and associated gliosis. Huperzine did not inhibit GSK3β activity and modulate BACE-1 activity, thereby failed to facilitate APP processing/Aβ production. To exclude the possibility that huperzine A we used may be invalid, we assessed its effect on AchE activity. Huperzine A used in this study inhibited AchE activity in the brain of TgCRND8 mice. These findings suggest that the effect of huperzine A in preventing Aβ neuropathology needs further studies to confirm.

Conclusions

Huperzine A, an AChE inhibitor for AD, could not inhibit GSK3β activity, and therefore did not facilitate prevention of APP processing/Aβ generation in TgCRND8 mice. Furthermore, huperzine A treatment did not inhibit Aβ-associated gliosis in TgCRND8 mice. The neuroprotective effect of huperzine A may need more studies to investigate the mechanisms involving targeting amyloidogenic APP cleavage pathway in AD treatment.

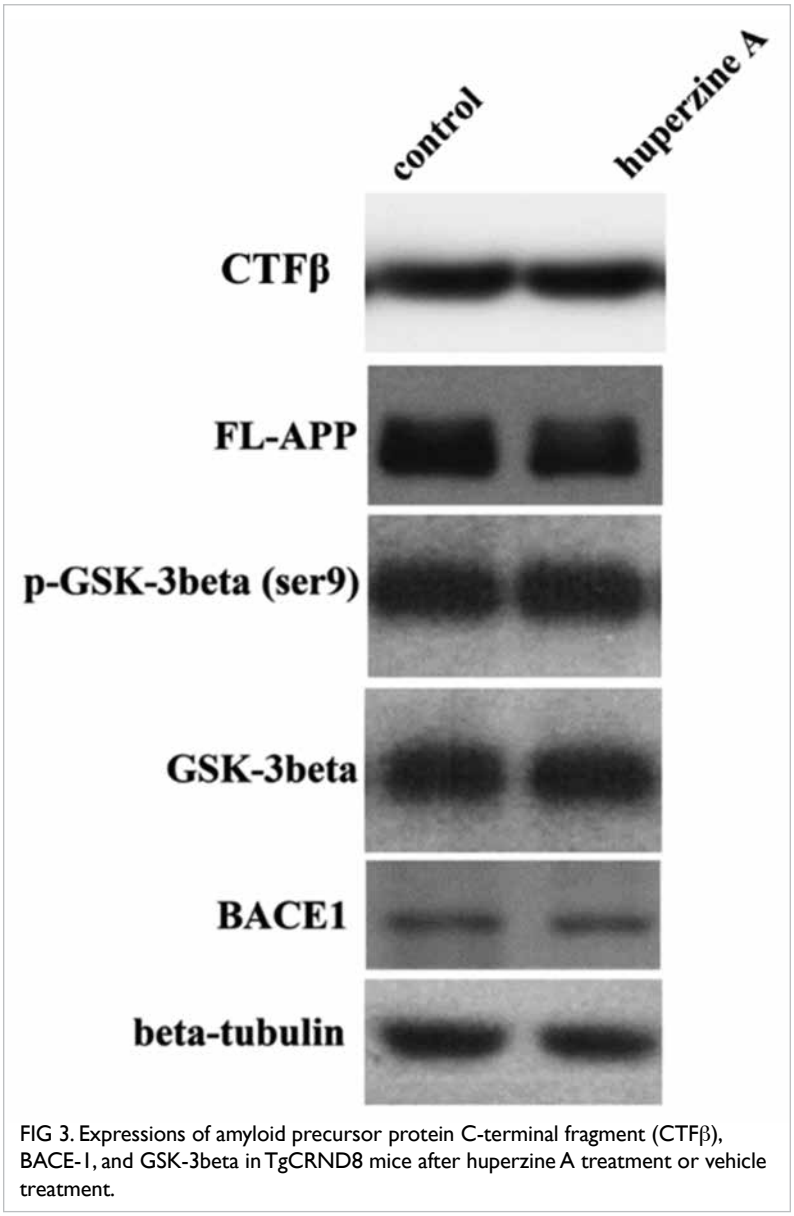

FIG 3. Expressions of amyloid precursor protein C-terminal fragment (CTFβ), BACE-1, and GSK-3beta in TgCRND8 mice after huperzine A treatment or vehicle treatment.

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