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Centella asiatica attenuates Aβ – induced neurodegenerative spine loss and dendritic simplification

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Abstract

The medicinal plant *Centella asiatica* has long been used to improve memory and cognitive function. We have previously shown that a water extract from the plant (CAW) is neuroprotective against the deleterious cognitive effects of amyloid-β (Aβ) exposure in a mouse model of Alzheimer's disease, and improves learning and memory in healthy aged mice as well. This study explores the physiological underpinnings of those effects by examining how CAW, as well as chemical compounds found within the extract, modulate synaptic health in Aβ-exposed neurons.

Hippocampal neurons from amyloid precursor protein over-expressing Tg2576 mice and their wild-type (WT) littermates were used to investigate the effect of CAW and various compounds found within the extract on Aβ-induced dendritic simplification and synaptic loss. CAW enhanced arborization and spine densities in WT neurons and prevented the diminished outgrowth of dendrites and loss of spines caused by Aβ exposure in Tg2576 neurons. Triterpene compounds present in CAW were found to similarly improve arborization although they did not affect spine density. In contrast caffeoylquinic acid (CQA) compounds from CAW were able to modulate both of these endpoints, although there was specificity as to which CQAs mediated which effect.

These data suggest that CAW, and several of the compounds found therein, can improve dendritic arborization and synaptic differentiation in the context of Aβ exposure which may underlie the cognitive improvement observed in response to the extract *in vivo*. Additionally, since CAW, and its constituent compounds, also improved these endpoints in WT neurons, these results may point to a broader therapeutic utility of the extract beyond Alzheimer's disease.

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Keywords

Synaptic health; amyloid-β; *Centella asiatica*; neuroprotection

Introduction

Alzheimer's disease (AD) affects more than 5 million people in the United States alone [1]. Amyloid-β (Aβ) peptide accumulates in the brain of AD patients [2] leading to the plaques that are the pathological hallmark of the disease. The amyloid hypothesis [3] suggests that this Aβ accumulation drives the neurodegeneration and synaptic loss that underlie cognitive decline. Decreased spine density, a reflection of synaptic loss, is widely reported in AD patients as well as animal models of AD [4, 5]. This loss of spines and dendritic complexity are prominent features in early-stage AD and correlate significantly with cognitive decline [6] supporting the idea that these endpoints represent the structural basis of cognitive dysfunction in AD.

The plant *Centella asiatica* (L) Urban (Apiaceae), also known as Gotu Kola, is used in traditional Chinese and Ayurvedic medicine to improve cognitive function and reverse cognitive impairments [7]. The neuroprotective and cognitive enhancing effects of *Centella asiatica* have been well-documented. Extracts of *Centella asiatica* have been shown to attenuate neurobehavioral and neurochemical effects of stroke [8], accelerate nerve regeneration [9], show antioxidant effects [10] and improve cognitive function in both human studies as well as in vivo models [11, 12]. Studies from our own lab have shown that a water extract of *Centella asiatica* (CAW) attenuates Aβ-induced cognitive impairments in the Tg2576 mouse model of AD [13]. These mice express a mutant form of human amyloid precursor protein (APP) leading to age-dependent Aβ accumulation in the hippocampus and cortex, and concomitant learning and memory deficits [14]. We found that two weeks of treatment with CAW in the drinking water normalized the behavioral deficits normally observed in aged Tg2576 animals [13]. Similarly we have also observed that CAW improves cognitive performance in aged wild-type (WT) animals as well, and this behavioral improvement was accompanied by increased synaptic gene expression in the brains of treated animals [15]. We have been able to recapitulate many of the beneficial effects of CAW using *in vitro* model systems.

We have seen that treatment with CAW protects against Aβ-induced cell death [16] as well as Aβ-induced mitochondrial dysfunction and oxidative stress in neuroblastoma cells [17]. However the effects of CAW on synaptic plasticity have yet to be examined. Additionally comparatively little is known about which compounds within the CAW extract mediate its beneficial effects. In this study we examine the effects of CAW, and several of the compounds contained therein, on dendritic morphology in hippocampal neurons isolated from WT and Tg2576 animals.

Materials and Methods

Aqueous extract of Centella asiatica

Dried *Centella asiatica* was purchased (Oregon's Wild Harvest, GOT-03193c-OHQ01) and its identity was confirmed by comparing its thin layer chromatographic profile with that reported in the literature [18] and the *Centella asiatica* samples used in our previous studies [13, 16]. The water extract of *Centella asiatica* (CAW) was prepared by refluxing *Centella asiatica* (160g) with water (2000mL) for 2 hours, filtering the solution and freeze drying to yield a powder (~16–21g). Isolated neurons were treated with CAW at a concentration of 50ug/mL for 7 days.

The percent content of constituent compounds in CAW was assessed by HPLC coupled to UV detection (LC-UV) as previously described [16]. Briefly analysis was performed using an Agilent HPLC system coupled to a Surveyor Photodiode detector. An Agilent Eclipse Plus C8 column (4.6 × 150mm, 3.5 μ) with Eclipse Plus C8 guard column (4.5 × 12.5mm, 5μ) was used with a column temperature of 35°C. The column was eluted with a gradient of acetonitrile in water containing 0.05% acetic acid (acetonitrile increasing from 5% to 20% over 6 minutes, maintained at 20% from 6–12 minutes, then raised to 40% by minute 13 and 90% by minute 14, maintained at 90% until 17 minutes and returned to 5 % by minute 17.2 for equilibration at starting conditions by minute 20. Detection wavelengths were 205nm for triterpenes and 330nm for CQAs.

Caffeoylquinic acid (CQA) and triterpene treatment of primary neurons

The purified forms of 1,5-dicaffeoylquinic acid (1,5dCQA), isochlorogenic acid A (IsoA also called 3,5-dicaffeoylquinic acid), chlorogenic acid (CHLA), asiatic acid (AA), asiaticoside (AS), madecassic acid (MA) and madecassoside (MS) (Chromadex), were used to treat primary neurons. We have previously shown that IsoA and 1,5dCQA are two of the most abundant and potent diCQAs in CAW and are protective against Aβ toxicity in neuroblastoma cells [16]. CHLA was chosen as a representative monoCQA. Although CHLA did not elicit protection against Aβ toxicity in neuroblastoma cells [16] it is a metabolite of the diCQAs and therefore may be relevant in explaining the *in vivo* effects of the extract. The triterpene asiatic acid also has neuroprotective properties [19, 20]. The concentrations used were similar to their percent abundance in 50ug/mL CAW (Table 1), with the exception of AA and MA which were tested at higher concentrations equivalent to the molar concentrations of their glycoside counterpart (AS and MS respectively) since *in vivo* the glycosides would be metabolized into the aglycone form [21]

Culture of primary hippocampal neurons

Embryonic Tg2576 mice and their WT littermates were used to generate primary neuronal cultures. The Tg2576 line expresses the human APPswe double mutation (K670N-M671L) [14], resulting in an accumulation of $A\beta_{1\text{-}42}$ in the brain and the development of age-dependent Aβ plaques. Previous studies have shown that after several weeks in culture, neurons isolated from these animals display a dystrophic phenotype that includes simplified dendritic arborization and reduced spine density [22].

Hippocampal neurons were isolated from embryonic mice as previously described by Kaech and Banker [23]. All procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Portland VA Healthcare System (ACORP #3581-15). Briefly, embryos were harvested at 18 days of gestation and hippocampi isolated. For Sholl analysis of dendritic complexity neurons were plated on poly-l-lysine coated glass at 130,000 per coverslip in MEM media (GIBCO), 5% FBS (Atlanta Biologicals) and 0.6% glucose (Sigma Aldrich). After 4h, the coverslips were flipped into 60 mm dishes containing neural stem cell-derived glial cells (provided by Dr. Gary Banker, Jungers Center, OHSU) and maintained in 6 ml Neurobasal media (Gibco) supplemented with GlutaMAX (Gibco) and GS21 (Global Stem). Each dish was fed every week by removing 1 ml of the culture medium and adding 1 ml fresh Neurobasal media containing GlutaMAX plus GS21, with the first feed (at 5 DIV) containing 6 μM cytosine β-D-arabinofuranoside hydrochloride (AraC; Sigma-Aldrich). The fourth feed (19 DIV) also contained CAW (50μg/mL) or one of the following isolated compounds: CHLA (0.5μM), IsoA (0.75μM), 1,5dCQA (0.5μM), AA (0.5μM), AS (0.5μM), MA (1.5μM), MS (1.5μM). At 26 DIV, each coverslip was fixed in 4% paraformaldehyde (PFA) in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.4). Coverslips were stained with Anti-MAP2B (Sigma-Aldrich #M4403; 3.3 μg/ml) and Goat anti-mouse IgG1-Cy3 (Jackson ImmunoResearch #115-165-205; 1.5μg/ml) and imaged with a Zeiss ApoTome2 microscope. Blinded Sholl analyses were performed using the Fiji platform [24] with the plug-in created by Ferreira et al. [25]. At least 180 cells were analyzed per treatment condition across 4–6 separate cultures.

For analysis of dendritic spines, 150,000 hippocampal neurons were electroporated with plasmids encoding Green Fluorescent Protein (eGFP) under the control of the CMV immediate-early enhancer and the chicken β-actin promoter [26] and plated onto dishes with poly-l-lysine coated coverslips containing 300,000 cortical neurons of the same genotype (plated in Neurobasal media containing GlutaMAX plus GS21 7 days prior to the addition of the hippocampal neurons). This strategy promoted robust synapse formation while maintaining the electroporated hippocampal neurons at a density that permitted the unambiguous visualization of non-intersecting dendritic segments. After 4 hr, coverslips were flipped into 60 mm dishes containing stem cell-derived glial cells as described above. Each dish was fed every week with 1 ml Neurobasal media plus GlutaMAX and GS21, with the first feed (at 5 DIV) containing AraC and the second feed (at 12 DIV) containing CAW or isolated compounds at the concentrations described above. Coverslips were then fixed in 4% PFA in PHEM buffer at 19 DIV and immunostained with anti-GFP (Life Technologies #A11122; 2μg/ml), detected with Alexa-488-conjugated goat anti-Rabbit secondary antibodies (Life Technologies #A11034; 2 μg/ml). The immunostained neurons were imaged using a Zeiss ApoTome2 microscope and blind quantification was performed using FIJI software. 17–20 images were collected from different neurons in each treatment group, and spines were quantified on at least 100 μm segments of dendrite length per image.

Statistics

Statistical significance was determined using one- and two-way analysis of variance. Bonferroni post-hoc tests were also conducted. Significance was defined as $p \leq 0.05$. Analyses were performed using Excel or GraphPad Prism 6.

Results

CAW reverses Aβ-induced impairments in dendritic morphology in hippocampal neurons

Due to their chronic production of $A\beta_{1\text{-}42}$ cultured neurons from Tg2756 mice progressively develop neurodegenerative phenotypes including reduced dendritic complexity and decreased spine density relative to WT control neurons [22]. Our results were consistent with these findings showing that hippocampal Tg2576 neurons did in fact have substantially reduced dendritic complexity after 26 days in culture as compared to neurons from WT littermates (Figure 1A). CAW treatment normalized the simplified arborization in Tg2576 neurons (Figure 1B). Interestingly the extract also increased arborization in WT neurons above that observed in control levels (Figure 1C).

CAW had a similar effect on the number of dendritic spines in hippocampal neurons. Relative to WT control neurons spine density was markedly reduced in Tg2576 neurons after three weeks in culture (Figure 2A). This is again consistent with previous reports [22]. CAW treatment rescued this spine deficit in Tg2576 neurons to the same levels observed in WT controls (Figure 2B). Additionally, CAW also significantly increased spine density in WT neurons (Figure 2B).

Individual compounds from the CAW extract also reverse Aβ-induced impairments in dendritic morphology in hippocampal neurons

We have previously shown that diCQAs found in CAW, but not monoCQAs, nor triterpenes, can protect against Aβ toxicity in neuroblastoma cells [16]. Here we tested the effects of the CQAs IsoA, 1,5dCQA and CHLA as well as the triterpenes AA, AS, MA and MS on dendritic morphology in hippocampal neurons. Each compound was evaluated at a concentration similar to its percent composition in the dose of CAW tested with the exception of AA and MA. These triterpenes were negligible or undetectable in the extract (Table 1) but were tested at the same concentration as their glycosidic counterparts because of the potential for metabolism of AS and MS into AA and MA *in vivo* [21].

Both MA and MS reversed the deficits in dendritic arborization in Tg2576 neurons while AA and AS had no effect (Figure 3A). 1,5dCQA and CHLA, but not IsoA, were similarly able to attenuate the decreased arborization in Tg2576 neurons (Figure 3B). Notably, MA, 1,5dCQA and CHLA increased arborization in WT neurons as well (Figure 3A, 3B). When evaluating spine density we found that only Iso A was able to rescue the Aβ induced deficits (Figure 4B), neither the triterpenes nor the other CQAs tested improved spine density in Tg2576 neurons (Figure 4A, 4B). IsoA also significantly increased density in WT neurons (Figure 4B).

Discussion

The plant *Centella asiatica* has been used for centuries to improve memory and cognitive function [7]. We have previously demonstrated that the water extract of *Centella asiatica* (CAW) improves cognitive performance in aged Tg2576 mice [13] as well as aged WT mice [15]. This cognitive enhancement was accompanied by increases the expression of synaptic genes in the brains of treated animals [15] suggesting possible effects of CAW on synaptic plasticity. Here we directly evaluated the effects of CAW as well as individual compounds found in the extract on spine density and dendritic arborization in Aβ-exposed hippocampal neurons.

CAW restored Aβ-induced deficits in dendritic complexity and spine density in neurons from Tg2576 animals. However, evaluation of individual CQAs and triterpenes from the extract yielded mixed results. The effects of the CQAs differed between arborization and spine density with CHLA and 1,5dCQA restoring Aβ-induced deficits in arborization and IsoA reversing the deleterious effects of Aβ on spine density. We, and other groups, have previously demonstrated that CQAs are neuroprotective in neuroblastoma cells [16, 27] which is consistent with the improvements in neuronal health observed in this study in Tg2576 hippocampal neurons.

When assessing the triterpenes found within CAW, both MA and MS attenuated the diminished arborization in Tg2576 neurons. Interestingly none of the triterpenes improved the Aβ-induced reduction in spine density in these neurons. These results were somewhat unexpected given that we did not previously observe a protective effect of any of these triterpenes from *Centella asiatica* against Aβ toxicity in our previous work in neuroblastoma cells [16], however they are in line with reports of other triterpene compounds protecting against Aβ toxicity in different *in vitro* models [28, 29].

Interestingly we also saw an effect of CAW and some of its constituent compounds on these synaptic endpoints in WT neurons. These results support previous findings that juice from *Centella asiatica* increases arborization in the hippocampus of rats [30]. However, given the tight regulation of synaptic homeostasis in proper neuronal and synaptic activity [31, 32] these results raise the question of whether this would be functionally beneficial *in vivo*. Although more work is necessary to confirm that increases in arborization and spine density are in fact observed in the brains of WT animals, our previous work has demonstrated both increased synaptic gene expression and a cognitive-enhancing effect of CAW in healthy older animals [15] suggesting that if the extract does in fact modulate those endpoints *in vivo* the effects would not be deleterious. This is further supported by previous research that has demonstrated that mice treated with a CQA-rich extract show improved spatial learning which was attributed to increased synaptic formation in the hippocampus [33].

It is also notable that we saw effects of MA and MS but not of AA and AS since the compounds are structurally so similar. The same is true of IsoA and 1,5dCQA which we expected to yield similar results given that they are positional isomers. This could be because MA and MS were used at a higher concentration than AA and AS, and IsoA at a higher concentration than the 1,5dCQA, reflecting their relative composition in the entire

CAW extract. It is possible similar effects would have been observed if higher concentrations of AA, AS and 1,5dCQA were tested. However, the fact that 1,5 dCQA but not IsoA increased arborization suggests that there may be a specificity of signaling interactions that does depend on chemical conformation. The work presented here, along with our previous studies, suggests that individual compounds from CAW elicit distinct biological effects both in the context of Aβ exposure as well as in healthy neurons (Table 2). While further research is needed to determine what accounts for these differential effects, these results underscore the benefit of using the complete extract rather than individual components in isolation.

CAW is a complex mixture containing various other CQAs as well as many classes of compounds beyond triterpenes and CQAs evaluated in this study. The plant *Centella asiatica,* has been shown to contain other saponins, quinic or benzoic acid derivatives, flavonoids and several acetylenic compounds [34] and many of these types of compounds have been associated with increased spine density and improved cognitive function [35, 36]. Further research is necessary to determine if these compounds also participate in the effects of CAW on synaptic plasticity.

The exact mechanism by which CAW, or its chemical constituents, improves these dendritic endpoints also remains to be elucidated. The complex regulation of spine formation and arborization potentially implicates a variety of biological pathways and processes including histone modification, transcription factors, microRNAs, kinases, hormones and cell surface receptors [37, 38]. Although details on its molecular mechanisms are limited, *Centella asiatica* has been shown to activate ERK1/2 and AKT in neuroblastoma cells [39, 40], two pathways that have been shown to affect dendritic morphology as well [41, 42]. Studies are ongoing in our lab to evaluate signal transduction pathways affected by CAW and the role they play in the neuroprotective effects of the extract.

These findings demonstrate that CAW, and several of its constituent compounds, can increase synaptogenesis and arborization in isolated hippocampal neurons, changes that could underlie the cognitive enhancing effects of CAW observed in both aged Tg2576 and WT mice [13, 15]. Studies in our lab are underway to confirm whether oral treatment with CAW, or compounds found within the extract, increases spine density or arborization in the brains of Tg2576 or WT animals and whether these effects are related our previous findings regarding the mitochondrial effects of the extract [15, 17]. These future studies along with the observations presented here will help elucidate the therapeutic potential of CAW, which may extend beyond AD to conditions associated with cognitive impairment.

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Abbreviations

CAW	water extract of *Centella asiatica*
AD	Alzheimer's Disease
CQA	caffeoylquinic acid
IsoA	isocholorogenic acid A
1	5diCQA, 1,5-dicaffeoylquinic acid
CHLA	chlorogenic acid
AA	asiatic acid
AS	asiaticoside
MA	madecassic acid
MS	madecasssoside

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Highlights

- *Centella asiatica* attenuates Aβ-induced neuronal dystrophy in hippocampal neurons.
- Individual compounds from the plant improve Aβ-induced abnormalities as well.
- *Centella asiatica* also enhances arborization and spine density in healthy neurons.

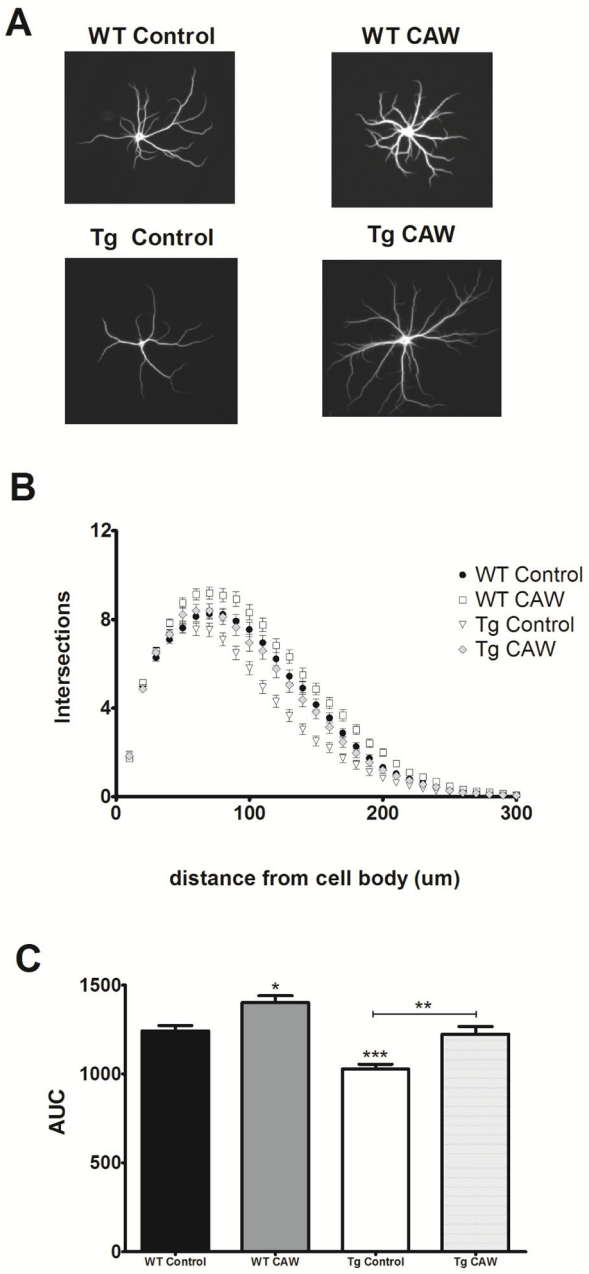

Figure 1. CAW increases dendritic arborization in Tg2576 and WT hippocampal neurons

A) Representative images from each treatment group. B) Sholl analysis of the total number of dendritic branches of from Tg2576 and WT hippocampal neurons (n = 200–250 neurons per treatment condition). CAW treatment (50 ug/mL) increased dendritic complexity in WT control neurons, and restored the extent of dendritic arborization of Tg2576 neurons to control levels. C) CAW increased the cumulative arborization (as quantified by area under the curve (AUC)) in WT neurons and restored the cumulative arborization of Tg2576 neurons to control levels. *p<0.05; **p<0.01; ***p<0.001.

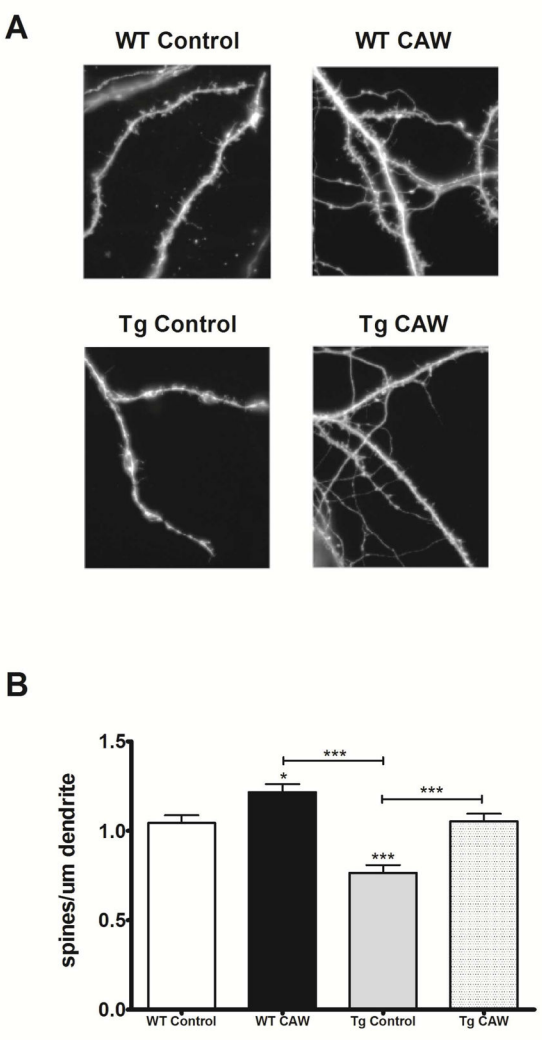

Figure 2. CAW increases dendritic spine density in WT and Tg2576 hippocampal neurons

A) Representative images from each treatment condition; B) CAW (50 ug/mL) increased the number of dendritic spines in WT neurons and restored spine density of Tg2576 neurons to control levels (n = 17–20 dendritic segments per treatment condition). *p<0.05; ***p<0.001.

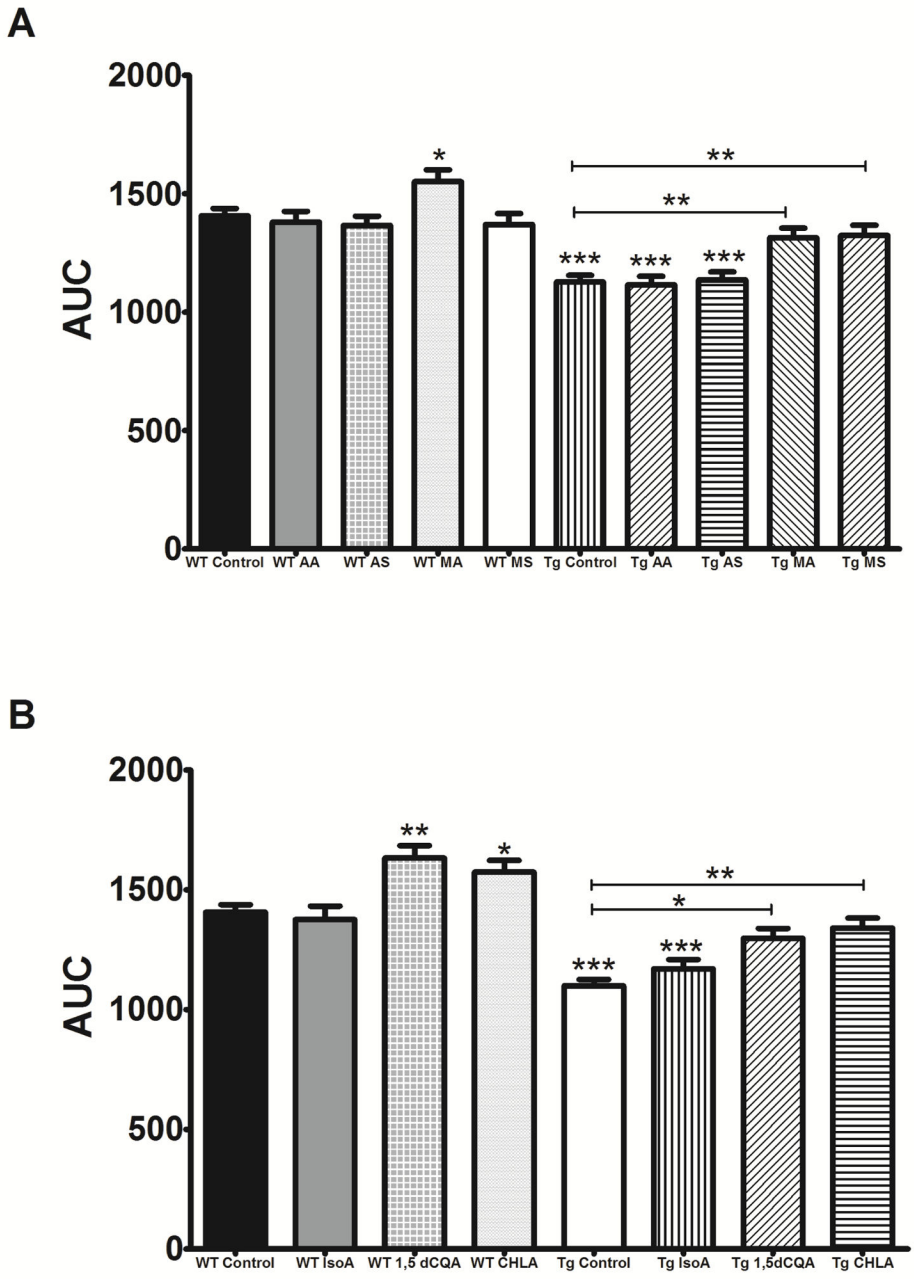

Figure 3. Individual compounds from CAW increase dendritic arborization in WT and Tg2576 hippocampal neurons

A) MA (1μM) and MS (1μM) restored the cumulative arborization of Tg2576 hippocampal neurons to control levels and MA increased the cumulative arborization in WT neurons as well while AA (0.5uM) and AS (0.5uM) had no effect. (n = 180–250 neurons per treatment condition). *p<0.05; **p<0.01; ***p<0.001. B) 1,5dCQA (0.5μM) and CHLA (0.5μM) but not IsoA (0.75μM) increased cumulative dendritic arborization in WT control neurons, and restored the extent of dendritic arborization of Tg2576 neurons to control levels (n = 180–250 neurons per treatment condition). *p<0.05; **p<0.01; ***p<0.001.

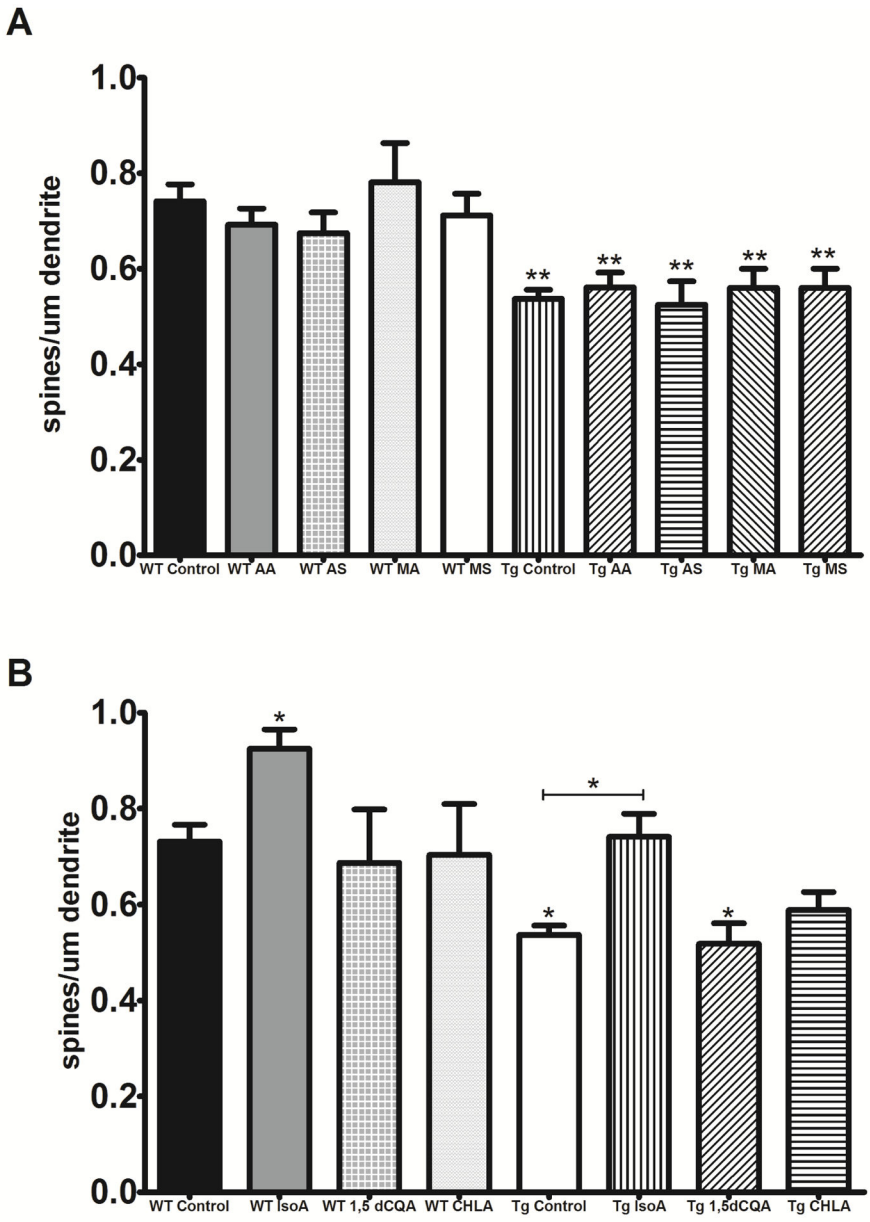

Figure 4. IsoA increases dendritic spine density in WT and Tg2576 hippocampal neurons

A) None of the triterpenes in CAW (AA [0.5μM], AS [0.5μM], MA [1μM] or MS [1μM]) had any effect on the spine density in either WT or Tg2576 neurons. (n = 17–20 dendritic segments per treatment condition). **p<0.01. B) IsoA (0.75 μM)) increased the number of dendritic spines in WT hippocampal neurons and restored spine density of Tg2576 neurons to control levels however neither dCQA1,5 (0.5μM) nor CHLA (0.5μM) had any effect on spine density in either genotype. (n = 17–20 dendritic segments per treatment condition). *p<0.05.

Table 1

Composition of CAW

The weight by weight (w/w) % composition of each triterpene and three of the CQAs in the CAW mixture was determined from LC-UV analysis at 330nm.

	w/w % Composition in CAW
Chlorogenic acid	0.43%
Isochlorogenic acid A	0.76%
1,5 dicaffeoylquinic acid	0.41%
Asiatic acid	0.02%
Asiaticoside	0.53%
Madecassic acid	Not detectable
Madecassoside	0.84%

Table 2

Biological effects of CAW and some of its chemical components

A summary of the effects of CAW, as well as the triterpenes and CQAs tested, on Aβ toxicity in neuroblastoma cells and arborization and spine density in Tg2576 and WT neurons.

	Protects against Aβ toxicity in neuroblastoma cells*	Improves dendritic arborization in Tg2576 neurons	Improves dendritic spine density in Tg2576 neurons	Improves dendritic arborization in WT neurons	Improves dendritic spine density in WT neurons
CAW	+	+	+	+	+
Chlorogenic Acid	−	+	−	+	−
Isochlorogenic Acid A	+	−	+	−	+
1,5 dicaffeoylquinic acid	+	+	−	+	−
Asiatic acid	−	−	−	−	−
Asiaticoside	−	−	−	−	−
Madecassic acid	−	+	−	+	−
Madecassoside	−	+	−	−	−

*Gray et al. Journal of Alzheimer's Disease, 2014