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Centella asiatica modulates antioxidant and mitochondrial pathways and improves cognitive function in mice

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Abstract

Ethnopharmacological relevance—This study investigates the cognitive enhancing effects of the plant *Centella asiatica* which is widely used Ayurvedic and traditional Chinese medicine.

Aim of the study—The goal of this study was to determine the effects of a water extract of the medicinal plant *Centella asiatica* (CAW) on cognitive ability as well as mitochondrial and antioxidant response pathways *in vivo*.

Materials and methods—Old and young C57BL/6 mice were treated with CAW (2mg/mL) in their drinking water. Learning and memory was assessed using Morris Water Maze (MWM) and then tissue was collected and gene expression analyzed.

Results—CAW improved performance in the MWM in aged animals and had a modest effect on the performance of young animals. CAW also increased the expression of mitochondrial and antioxidant response genes in the brain and liver of both young and old animals. Expression of synaptic markers was also increased in the hippocampus and frontal cortex, but not in the cerebellum of CAW-treated animals.

Conclusions—These data indicate a cognitive enhancing effect of CAW in healthy mice. The gene expression changes caused by CAW suggest a possible effect on mitochondrial biogenesis, which in conjunction with activation of antioxidant response genes could contribute to cognitive improvement.

Keywords

Aging; cognition; *Centella asiatica*; mitochondrial dysfunction; reactive oxygen species

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Introduction

Neuronal changes that occur during aging can in some cases result in impaired cognitive ability (Leal, 2013; Oberman, 2013). As the population of the US grows increasingly older, age-related cognitive impairment is becoming a more pressing concern both financially and societally (Costa, 2013).

The plant *Centella asiatica* (L) Urban, (Apiaceae), known in the United States as Gotu Kola, is used in traditional Chinese and Ayurvedic medicine to improve cognitive function (Shinomol, 2011). These effects of the plant have been demonstrated in a handful of small human studies both in healthy middle aged and older subjects (Dev, 2009; Wattanathorn, 2008) as well as those with mild cognitive impairment (Tiwari, 2008). Preclinical studies have also demonstrated the neuroprotective and cognitive enhancing effects of *Centella asiatica* in multiple rodent models (Gupta, 2003; Kumar, 2002; Veerendra Kumar, 2003). Our lab has previously shown that a water extract of *Centella asiatica* (CAW) added to the drinking water can attenuate cognitive impairments in the Tg2576 mouse model of Aβ accumulation without altering plaque burden (Soumyanath, 2012). We have also demonstrated that CAW can protect against Aβ toxicity *in vitro* (Gray, 2014). We have recently shown that, in neuroblastoma cells, CAW increases mitochondrial respiration and induces the expression of mitochondrial and antioxidant genes both in the presence and absence of Aβ exposure (Gray, 2015). This suggests a mechanism of action that may be relevant beyond Alzheimer's disease, to other conditions where mitochondrial dysfunction and oxidative stress are observed.

The healthy aging brain displays mitochondrial abnormalities, like decreased mitochondrial content and reduced electron transport chain (ETC) activity along with increased levels of reactive oxygen species (ROS) and markers of oxidative damage (Haider, 2014; Liu, 2002). Studies have also demonstrated a relationship between mitochondrial function, antioxidant capacity, and memory (Forster, 1996; Masiero, 2010; Olsen, 2013; Perrig, 1997) sparking an interest in identifying agents that target mitochondria and antioxidant pathways for the improvement of cognitive function.

In this study we explore the cognitive enhancing effects of CAW on both old and young C57BL/6 mice as well as its effects on expression of mitochondrial and antioxidant response genes *in vivo*.

Methods

CAW

Dried *Centella asiatica* was purchased from StarWest Botanicals, Sacramento, CA (Lot no. 45158). The identity of the plant was confirmed by visual examination and by comparing its thin layer chromatographic profile with that reported in the literature (Wagner, 1996) and the *Centella asiatica* sample used in our previous studies (Gray, 2014, 2015; Soumyanath, 2012). The dried water extract of *Centella asiatica* (CAW) was prepared by refluxing *Centella asiatica* (60g) with water (750mL) for 1.5 hours, filtering the solution to remove

plant debris and freeze drying to yield a powder (6g). A voucher specimen of the plant material (CA/2012/SW) is deposited in our laboratory.

Animals

18 month old C57Bl/6 mice were obtained from the NIA aged rodent colony and 6 week old C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed 3-5 animals per cage in a climate-controlled environment with a 12-hr light/12-hr dark cycle, and fed AIN-93M Purified Rodent Diet (Dyets Inc., Bethlehem, PA). Diet and water were supplied ad libitum. The average weight for the male mice in the aged cohort was 36g and was 28g for female mice in the aged cohort. In the young mouse cohort the average male weight was 26g and the average female weight was 19g. When the cohorts reached 20 months and 2 months of age, mice were randomly assigned to a control group or a CAW group which was exposed to CAW in their drinking water (2g/L) yielding 13 male CAW-treated mice, 12 male control mice, 13 female CAW-treated mice and 12 female control mice for the aged cohort and 9 male CAW-treated mice, 9 male control mice, 9 female CAW-treated mice and 9 female control mice for the young cohort. CAW exposure continued for 2 weeks prior to the beginning of behavioral testing and throughout the test period. Following 3 weeks of behavioral testing animals were sacrificed and tissue harvested as outline in the timeline below (Figure 1). 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 Medical Center.

Behavioral testing – Morris Water Maze

Hippocampal-dependent spatial learning and memory was assessed using a standard Morris Water Maze (MWM) task as described in previous studies from our lab (Harris, 2014; Quinn, 2010; Soumyanath, 2012). Briefly mice were placed in a cylindrical tank of opaque water (through the addition of non-toxic Tempera paint) in a testing room with various distinct visual cues on the walls. The first phase of testing was visual platform training to verify that performance was not impaired by vision or locomotor problems. In the visual platform phase mice were trained to swim to a plexiglass escape platform positioned above the water for 12 trials (6/day). At 24h after visual platform training the hidden platform phase began. Mice were trained for 30 trials (6/day) to learn the location of the submerged platform. During a given trial the mouse was placed in the tank at one of four pseudorandomly-chosen start points (N, S, E,W) and allowed 60s to find the platform. If the mouse failed to locate the platform within 60s, it was placed on the platform. The platform position remained fixed throughout the hidden platform phase. Escape latency (in seconds) and distance traveled before finding the platform (in meters) was acquired by ANYmaze video tracking software. Memory retention was assessed in the probe phase of the MWM which occurred at the end of each hidden platform test day as well as 24 and 72h after the fifth and final hidden platform trial. During the probe phase the platform was removed from the tank and time the mice spent in each quadrant was recorded. The percentage of time spent in the target quadrant, which previously held the hidden platform, was calculated.

Gene expression

Hippocampal, cortical, cerebellar and liver tissue was homogenized and RNA was extracted using Tri-Reagent (Molecular Research Center). RNA was reverse transcribed with the Superscript III First Strand Synthesis kit (Invitrogen) to generate cDNA as per the manufacturer's instructions. Relative gene expression was determined using TaqMan Gene Expression Master Mix (Invitrogen) and commercially available TaqMan primers (Invitrogen) for nuclear factor (erythroid-derived 2)-like 2 (NFE2L2; NRF2), NAD(P)H dehydrogenase-quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase, catalytic subunit (GCLC), heme oxygenase 1 (HMOX1), mitochondrially encoded NADH dehydrogenase 1 (Mt-ND1), mitochondrially encoded ATP synthase 6 (Mt-ATP6), mitochondrially encoded cytochrome c oxidase 1 (Mt-CO1), mitochondrially encoded cytochrome B (Mt-CYB), synaptophysin, post-synaptic density protein 95 (PSD95) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative PCR (qPCR) was performed on a StepOne Plus Machine (Applied Biosystems) and analyzed using the delta-delta Ct method.

Statistics

Statistical significance was determined using one- and two-way analysis of variance. Bonferroni post-hoc tests were also conducted. Student t-tests were also conducted for mitochondrial and antioxidant gene expression when comparisons were made separately for each gender and age. Significance was defined as $p \leq 0.05$. Analyses were performed using Excel or GraphPad Prism 6.

Results

CAW improves MWM performance in aged mice but only modestly affects performance in young mice

Aged C57BL/6 mice (20 months) were treated with CAW in their drinking water (2g/L) for two weeks prior to behavioral testing, and exposure to CAW continued throughout testing (Figure 1). No difference was observed between any of the groups of mice in the visual platform phase of the MWM (data not shown). In the hidden platform phase, although no differences were observed in aged male mice on days 1-4, CAW-treated males found the hidden platform significantly faster on day 5 than did the control male mice (Figure 2A). Similarly aged female mice treated with CAW also showed significantly improved performance relative to female controls in time to find the hidden platform but again this improvement was not evident until the fifth day of testing (Figure 2B). Young male animals treated with CAW also improved on the fifth day of hidden platform testing (Figure 2C) but CAW treatment had no effect on young female mice (Figure 2D).

A similar pattern was observed for the distance the male mice swam before finding the platform, where a treatment effect of CAW was not apparent until day 5 of hidden platform testing in aged mice (Figure 2E and 2F) and again this difference was also observable in young male but not young female mice (Figure 2G and 2H).

There were no differences on days 1 and 2 of the probe trials between CAW-treated animals and controls (data not shown). Beginning on day 3 of the probe phase of the MWM, aged male mice treated with CAW spent significantly more time in the quadrant that previously contained the platform than did control males (Figure 3A). This increase persisted on day 4 and 5 and was still evident 24h and 72h after the final hidden platform trial. In the aged females, CAW treatment also increased the amount of time spent in the target quadrant but this difference didn't occur until day 4 and while it continued on day 5 and 24h after hidden platform testing, by 72h post-platform testing there was no difference between controls and CAW treated aged female mice.

CAW did not have as robust an effect on the memory retention of the young animals. In the probe phase of the MWM young male CAW-treated mice did spent significantly more time in the target quadrant than controls on days 4 and 5 (Figure 3B), and while there was a non-significant trend toward increased time in the target quadrant 24h after hidden platform testing ($p=0.09$), by 72h post-hidden platform testing there was no discernible difference between control and CAW-treated young male mice. In young CAW-treated female mice a significant increase in the percent time in the target quadrant was seen only seen 72h post-hidden platform testing, although a non-significant trend toward greater time in the target quadrant was observable at day 5 ($p=0.09$) of hidden platform testing and 24h post-hidden platform testing ($p=0.07$).

CAW increases expression of mitochondrial and antioxidant genes in the brain and liver of aged mice

CAW treatment significantly increased the expression of mitochondrial genes encoding enzymes in the electron transport chain (ETC) in the hippocampus of both male and female aged mice (Figure 4A). This up-regulation was also observed in the frontal cortex, cerebellum and liver of CAW-treated male and female animals (Figure 4B, 4C and 4D). CAW also increased the expression of the antioxidant response gene NRF2 as well as several of its downstream target genes. This increase was observed in the hippocampus, cortex, cerebellum and liver of both male and female CAW-treated aged animals (Figure 4E, 4F, 4G and 4H).

CAW increases expression of mitochondrial and antioxidant genes in the brain and liver of young mice

CAW treatment coordinately induced the expression of several ETC genes in the hippocampus of male and female young mice (Figure 5A). CAW similarly increased the expression of these genes in the frontal cortex, cerebellum and liver of these animals (Figure 5B, 5C and 5D). An increase in the expression of NRF2 and its target genes was also seen in the hippocampus, frontal cortex, cerebellum and liver of CAW-treated male and female young mice (Figure 5E, 5F, 5G and 5H).

CAW increases the expression of synaptic genes in the hippocampus and frontal cortex, but not cerebellum of both aged and young mice

The expression of the pre- and post-synaptic markers, synaptophysin and post-synaptic protein 95 (PSD95), was assessed in both young and old CAW-treated animals. Significant

increases in the expression of both genes were observed in the hippocampus of both young and old male CAW-treated mice (Figure 6A). These increases were also seen in the frontal cortex of young and old male CAW-treated mice (Figure 6B) but notably not in the cerebellum of these animals (Figure 6C). Similar inductions in the expression of these synaptic genes were detected in the hippocampus and frontal cortex of both young and old CAW-treated female mice (Figure 6D and 6E). Again however, there were no differences in the expression of either PSD95 or synatpophysin in the cerebellum in either young or old female animals (Figure 6F). Interestingly, basal expression of these genes did not differ between old and young mice of either gender in any brain region (Figure 6A-F). Likewise in the hippocampus and cortex the magnitude of induction of these synaptic genes was similar in the young and old mice of both genders (Figure 6A, 6B, 6D, 6E).

Discussion

We have previously shown that CAW can attenuate behavioral deficits in a mouse model of Aβ accumulation (Soumyanath, 2012) and can modulate antioxidant and mitochondrial pathways in *in vitro* models of Aβ toxicity (Gray, 2015). Here we explore the cognitive enhancing effects of CAW in young and old healthy animals and the effect of the extract on mitochondrial and antioxidant pathways *in vivo*. Although the cognitive-enhancing effects of *Centella asiatica* and its modulation of these pathways have been confirmed in other animal models of dementia and neurotoxicity (Gupta, 2003; Kumar, 2011; Prakash, 2013; Shinomol, 2008; Subathra, 2005; Veerendra Kumar, 2003), to our knowledge this is the first study to demonstrate these effects in both aged and young healthy mice.

In this study we showed that CAW can improve performance in the MWM test of learning and memory in aged mice. There were only modest effects of CAW on learning and memory acquisition that were not observable until the final day of the hidden platform phase. In the final three days of hidden platform training the performance of control mice appears to plateau while the CAW mice continue to improve resulting in the slight, but statistically significant, difference in performance seen on the fifth day. The probe phase revealed a more significant effect of CAW on memory retention in the older animals beginning on days 3 or 4 and persisting 24h and 72h post-hidden platform. The effect of CAW in the younger animals was noticeably less pronounced than in the aged mice. The very subtle effect on learning and memory acquisition observable on the final day of the hidden platform phase in young male CAW-treated animals was not apparent at all in young female CAW-treated mice. Similarly in the probe phase while young male mice displayed a blunted effect of CAW on memory retention relative to older mice, female mice hardly responded at all. The differential behavioral response to CAW between the young and old mice may be due to the fact that young mice are cognitively intact and therefore do not have as much room to improve as do the older mice with their age-related behavioral deficits. It is more difficult to speculate as to the cause of the gender discrepancy in response to CAW. This is not the first report of differences between the genders in response to *Centella asiatica.* A small clinical intervention with *Centella asiatica* has also documented a differential cognitive response between male and female participants. However in that study female subjects responded more robustly than did males (Dev, 2009). More extensive future investigations will need to be designed to better understand these gender differences.

We also observed that CAW treatment can substantially increase the expression of antioxidant and mitochondrial genes in these animals. This is consistent with our previous in vitro data in rat primary neurons and human neuroblastoma cells (Gray, 2015). These gene expression changes were detected in the brain as well as the liver of both young and old CAW-treated animals of both genders suggesting that the effects of CAW are systemic and not restricted to the central nervous system. If indeed the mitochondrial gene expression is increased in all tissues, including muscle, we might have expected to see differences in body weight in the CAW-treated animals compared to controls. This did not occur (data not shown), however, we did not closely monitor food intake or activity which can also contribute to body weight. *Centella asiatica* has been reported to have sedative properties (Veerendra Kumar, 2002) and to play a potential role in the modulation of hunger (Ramaswamy, 1970) so it is possible these variables compensated for the increased metabolic rate in the CAW-treated animals. Future studies utilizing metabolic cages are necessary to more fully understand the effects of CAW treatment on whole body metabolism.

CAW-treatment also increased the expression of synaptic genes. Like the antioxidant and mitochondrial genes, these expression changes were observed in both young and old animals of both genders. Synaptophysin and PSD95 are widely-used markers of spine density, which is thought to be the structural basis for changes in cognition (Harris, 1999). In fact alterations in spine density have been associated with cognitive ability in various models of cognitive impairment (Ittner, 2010; Kolb, 1997; Roberson, 2010; Rogers, 2011). It is notable that while we did see changes in synaptic gene expression in young animals there was not as robust an improvement in the behavioral task in these animals. Again this may be due to the fact that the young animals are cognitively intact and therefore have very little room for improvement on the MWM. It's possible that by employing other, more sensitive, behavioral assessments of cognitive function, an improvement could be detected in these young animals. The MWM assesses hippocampal learning and memory. Given the similar gene expression changes in the frontal cortex, changes in more cortically-dependent tasks, like executive function, may also be improved with CAW treatment. It will be interesting to see, in future studies, if age and gender differences persist across different behavioral paradigms.

It is also interesting that we only observed an effect of CAW on the expression of synaptic markers in the hippocampus and frontal cortex but not the cerebellum possibly indicating a regionally selective effect of CAW. The hippocampus and cortex are highly plastic regions (Burke, 2006; Jellinger, 2013; Laroche, 2000; Li, 2009) therefore it is possible that the alterations caused by CAW are sufficient to induce this synaptic remodeling in those areas but not others. Future studies remain necessary to confirm that these expression changes are also observable at the protein level but additional work is already underway to determine if changes in spine density or neuronal morphology are observable in these same brain regions in animals treated with CAW.

We do not yet know whether the gene expression changes in the brains of CAW treated animals are a direct effect of compounds from CAW crossing the blood brain barrier or the downstream result of peripheral effects of the extract. CAW is a complex mixture, the

composition of which we are still in the process of defining. We have identified several caffeoylquinic acids (CQAs) in CAW which appear to contribute to its neuroprotective, antioxidant and mitochondrial effects *in vitro* (Gray, 2014, 2015). Studies have shown that CQAs can improve cognitive function in senescence-accelerated prone mice (Han J, 2010; Sasaki K, 2013) but again whether this is because of direct action on the brain or a secondary effect of the CQAs is unknown. Studies are underway in our lab to try to detect CQAs in the brains of animals treated with CAW.

It is notable that the CAW-induced gene expression changes were similar between ages and genders even though behavioral effects differed in these groups. This discrepancy may be due to the fact that in addition to being cognitively intact, young mice also do not have the mitochondrial dysfunction and oxidative stress that accompany aging (Haider, 2014; Liu, 2002). Again the current study is limited by the sole use of gene expression data. As with the synaptic gene expression, additional studies are necessary to confirm that these expression changes actually reflect increased mitochondrial and antioxidant protein levels. More relevant still will be future investigations into whether expression changes result in alterations in mitochondrial function and morphology which are essential for validating and interpreting these preliminary findings.

If mitochondrial and antioxidant changes do in fact contribute to cognitive decline then perhaps increasing mitochondrial and antioxidant gene expression may not affect cognitive performance until mitochondrial dysfunction and oxidative damage are apparent. Future experiments to determine if chronic exposure to CAW, initiated in young animals, can delay or prevent age-related mitochondrial dysfunction, oxidative damage and cognitive decline would be informative.

Finally, although the gene expression changes we observed in this study are provocative, they do not definitively identify a mechanism of action of CAW. Metabolomic analyses are ongoing using tissue from this study to identify additional mechanistic pathways that may contribute to the beneficial effects of CAW.

Conclusion

Our findings demonstrate that CAW improves cognitive performance and affects mitochondrial and antioxidant response pathways *in vivo*. The contribution that the mitochondrial and antioxidant changes have to the overall cognitive-enhancing effects of CAW remains to be seen, but as mitochondrial dysfunction and oxidative stress are common to many conditions associated with cognitive impairment (Emerit, 2004; Lin, 2006) the potential utility of CAW may be quite broad.

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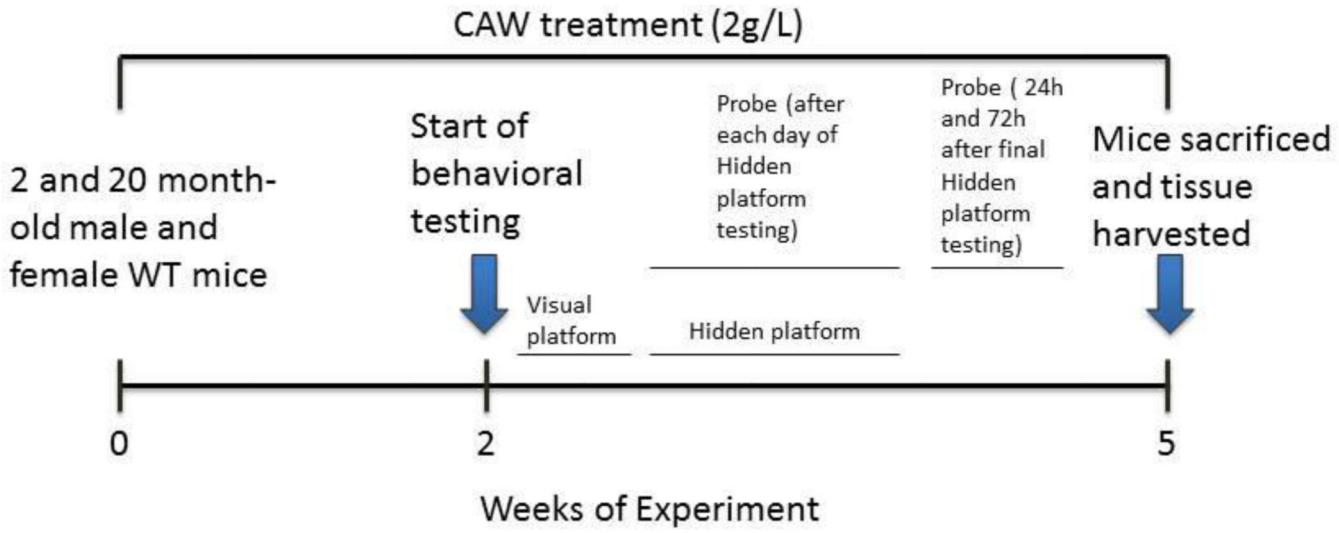

Figure 1. Timeline of CAW treatment and behavioral assessment

Mice were treated with CAW two weeks prior to the beginning of behavioral testing and continued throughout the experiment. After testing, animals were sacrificed and tissue was harvested. CAW treatment lasted a total of 5 weeks.

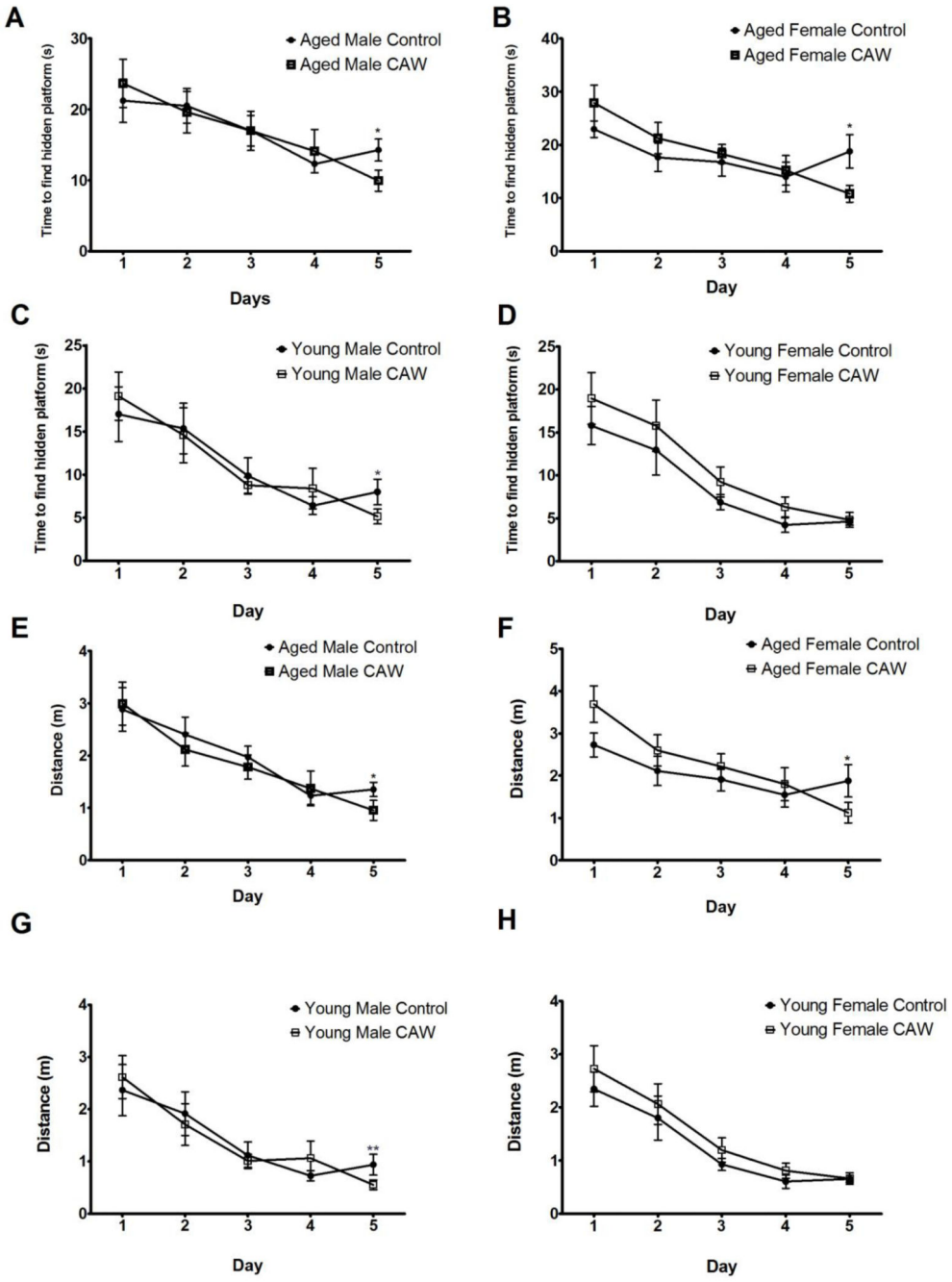

Figure 2. CAW-induced improvements in memory acquisition are more pronounced in aged animals than young animals

A) Time to find the hidden platform was significantly decreased in aged CAW-treated male mice (20 months) on day 5 of testing. B) CAW treatment decreased the time to find the platform in aged female mice on day 5 of testing. C) Time to find the hidden platform was slightly decreased in young CAW-treated male mice (2 months) on day 5 of testing, but was not improved in young CAW-treated female mice (D). Distance traveled to find the hidden platform was also significantly decreased on day 5 for aged male (E) and female (F) mice. In

young mice distance to find the hidden platform decreased in CAW-treated male (G) but not (H) female animals. (n=9-13; *p<0.05)

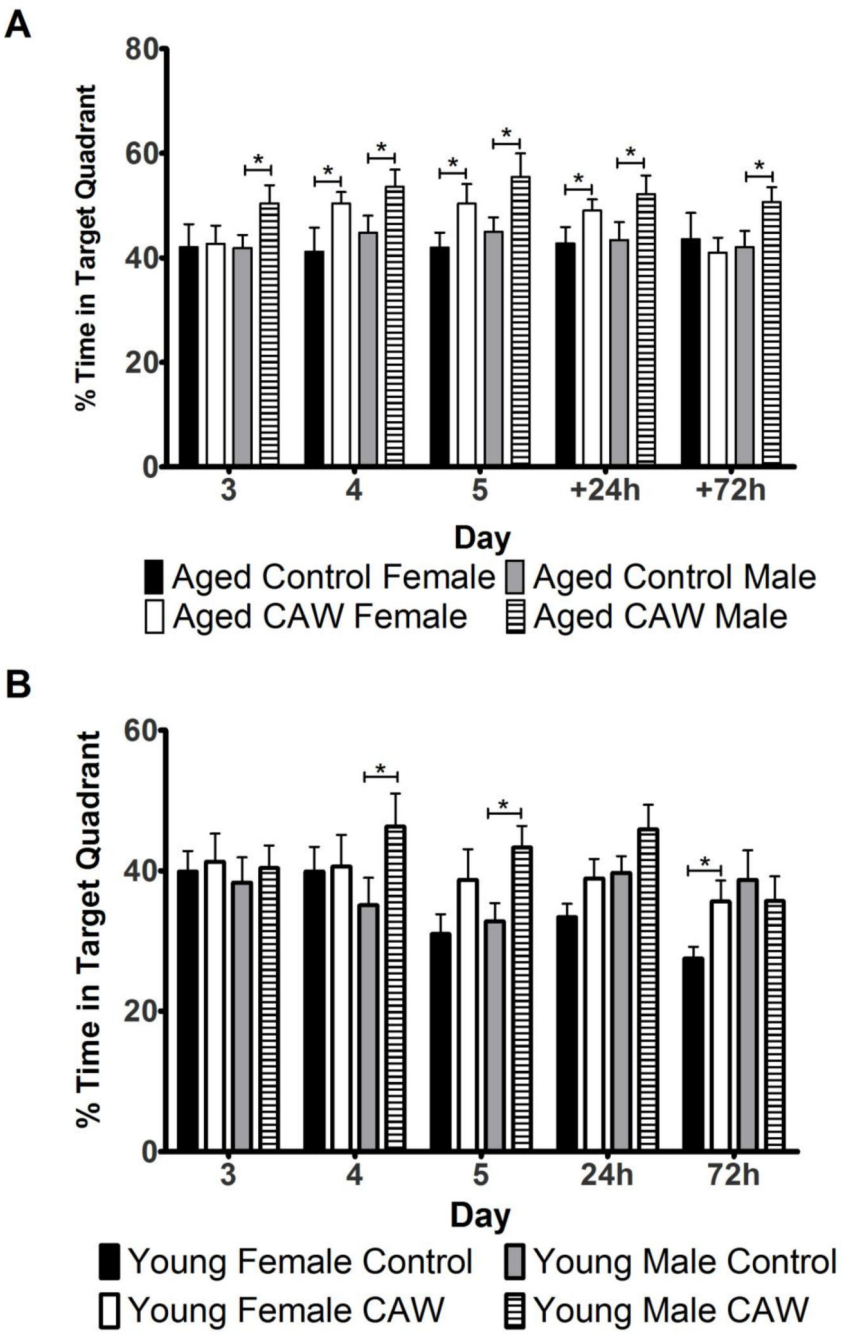

Figure 3. CAW improvements in memory retention are also more pronounced in aged animals

A) In the probe phase of the MWM test, aged CAW treated male mice spent significantly longer in the target quadrant than controls beginning on day 3 and continuing through 72h post-hidden platform testing. Aged CAW-treated females also spent more time in the target quadrant but not until day 4 and only lasting 24h post-hidden platform testing. n=12-13 of each gender per treatment condition. B) In the probe phase of the MWM, young CAW treated male mice spent significantly longer in the target quadrant than controls on days 4 and 5 but not 24h or 72h post-hidden platform testing. CAW treatment did not increase the

amount of time young female mice spent in the target quadrant until 72h post-hidden platform testing. (n=9; *p<0.05)

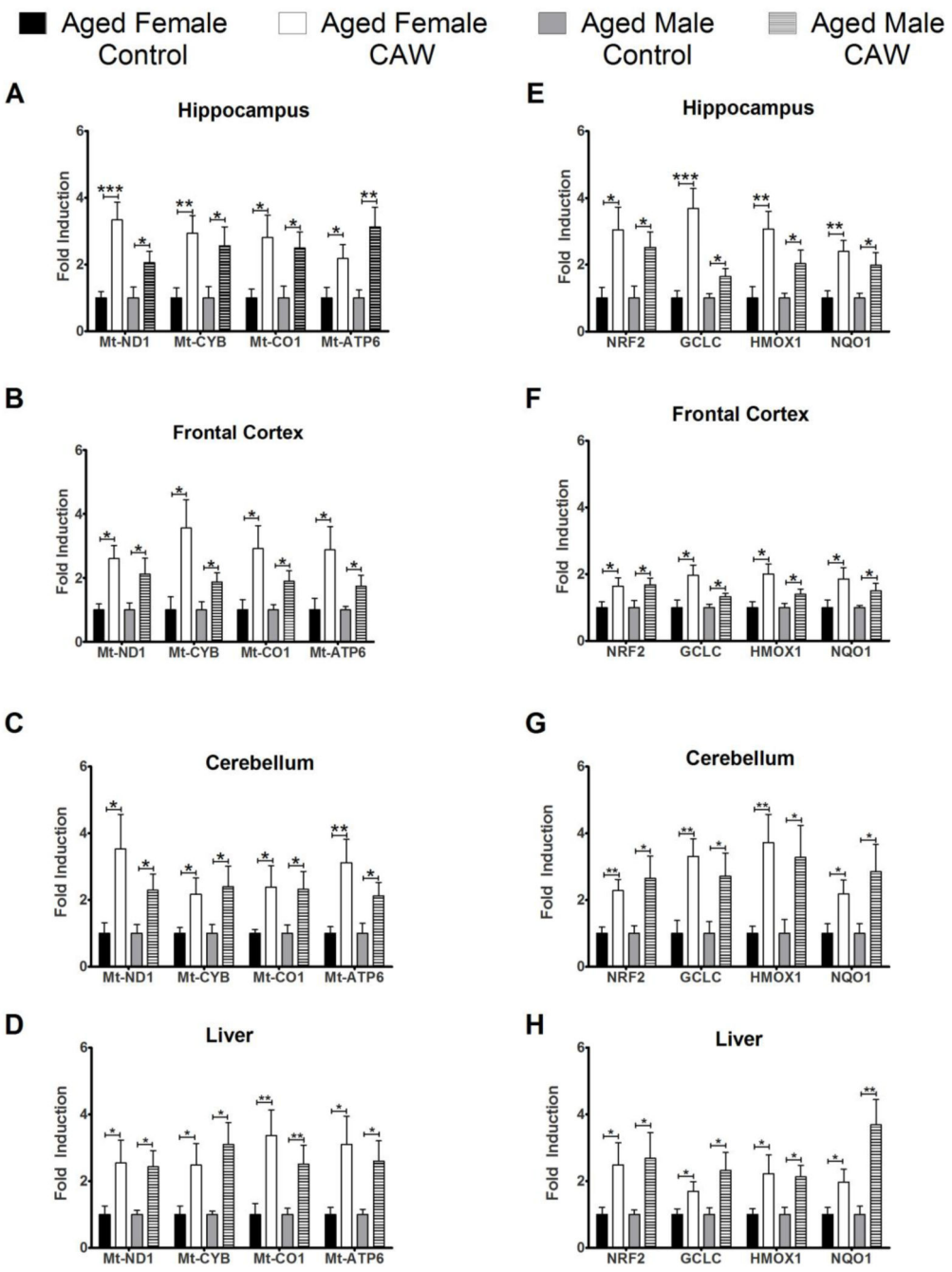

Figure 4. CAW increases mitochondrial and antioxidant gene expression in aged animals

A) CAW increased expression of ETC genes in the hippocampus of aged CAW-treated male and female mice. CAW also increased expression of ETC genes in the B) frontal cortex and C) cerebellum of CAW-treated animals. D) CAW treated animals had increased ETC expression in the liver as well. CAW increased expression of antioxidant response genes in the E) hippocampus, F) frontal cortex, G) cerebellum and H) liver of aged male and female mice. n=12-13 of each gender per treatment condition. *p<0.05; **p<0.01

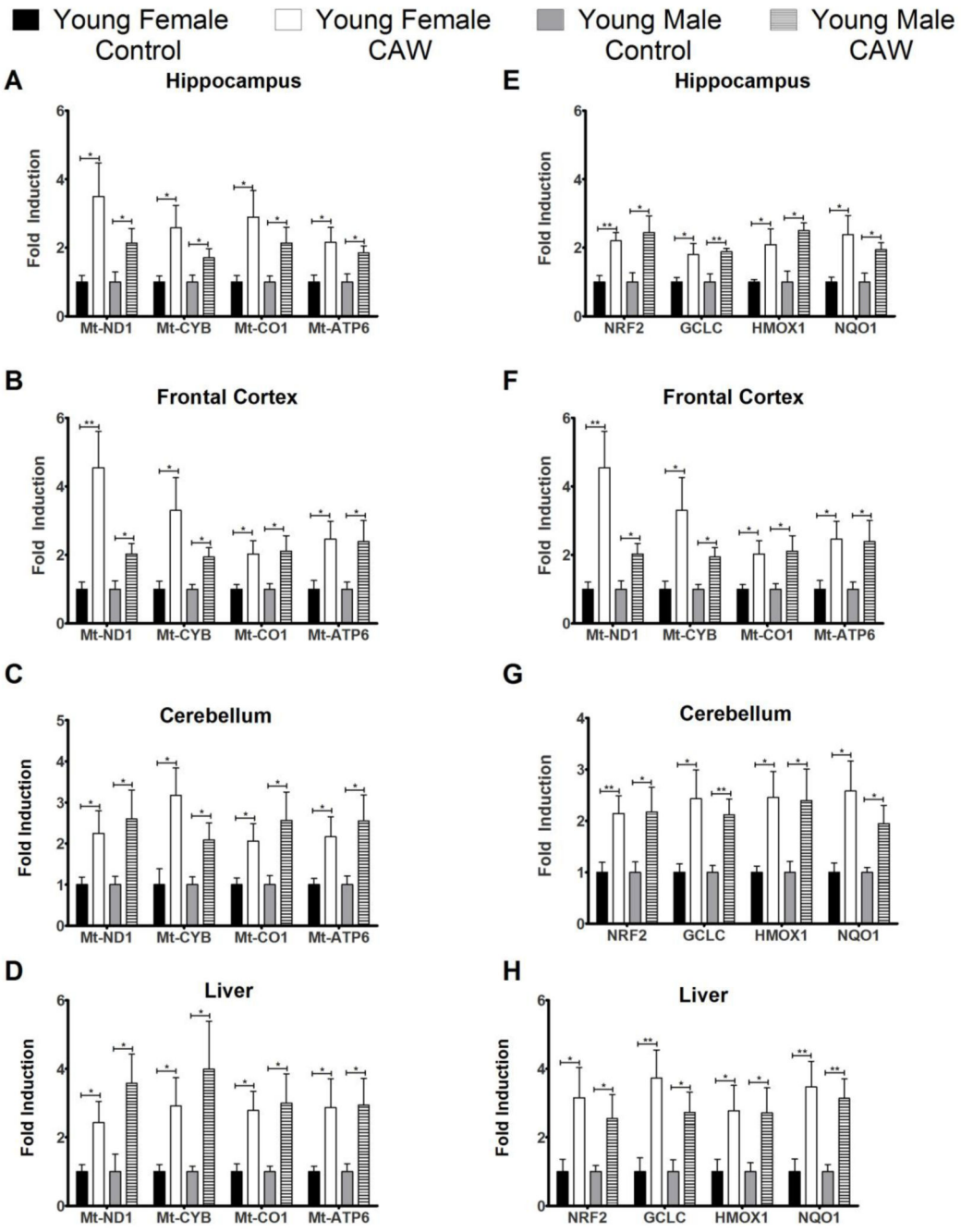

Figure 5. CAW increases mitochondrial and antioxidant gene expression in young animals

CAW increased expression of ETC genes in the A) hippocampus, B) frontal cortex and C) liver of young CAW-treated male and female mice. CAW also increased expression of antioxidant response genes in the D) hippocampus, E) frontal cortex, and F) liver of young male and female mice. n=9 of each gender per treatment condition. *p<0.05; **p<0.01

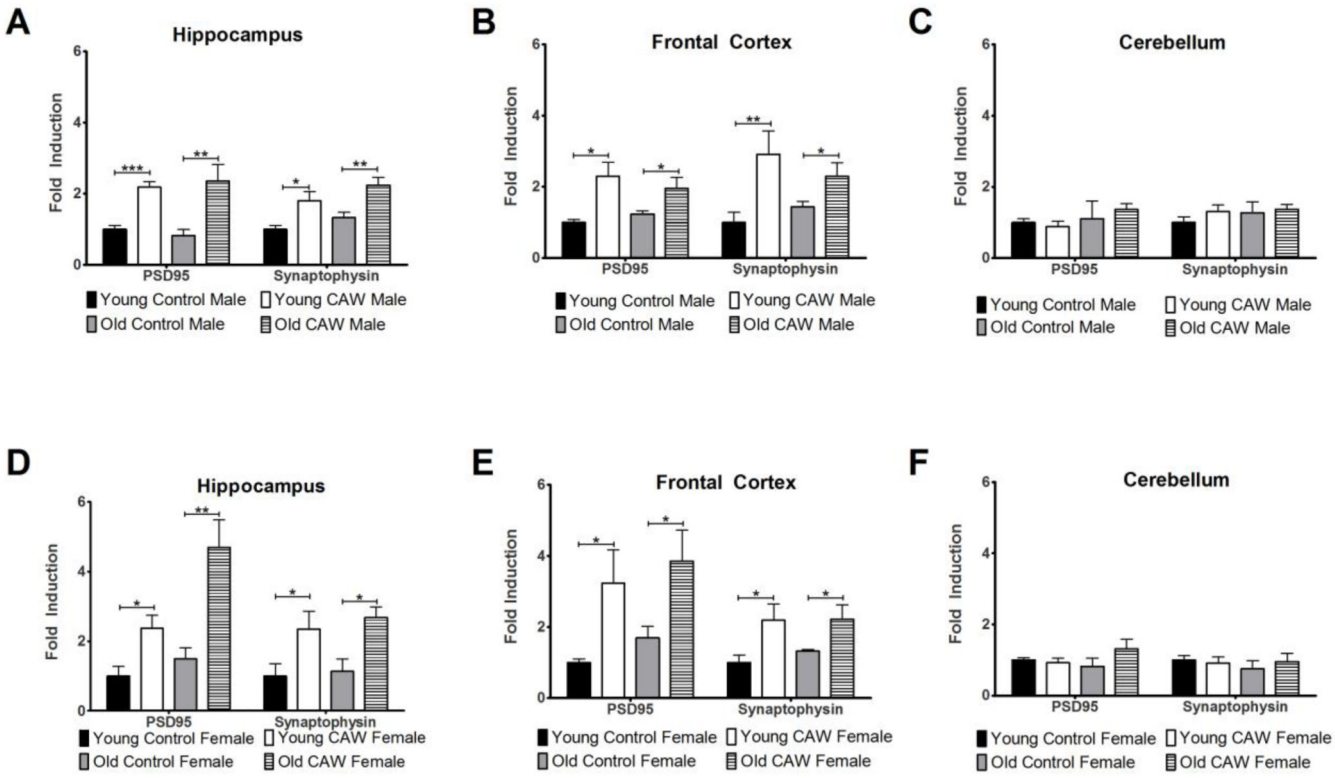

Figure 6. CAW increases expression of synaptic genes in hippocampus and cortex but not cerebellum of aged and young animals

CAW increased expression of PSD95 and synaptophysin in the A) hippocampus and B) frontal cortex but had no effect in the C) cerebellum of aged and young male mice (values normalized to young control treated animals of each gender). CAW similarly induced the expression of PSD95 and synaptophysin in the D) hippocampus and E) frontal cortex but not F) cerebellum of aged and young female mice. n=9-13 of each gender per treatment condition. *p<0.05; **p<0.01