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Research Article

Mountain-cultivated ginseng protects against cognitive impairments in aged GPx-1 knockout mice via activation of Nrf2/ChAT/ERK signaling pathway

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ABSTRACT

Background: Escalating evidence shows that ginseng possesses an antiaging potential with cognitive enhancing activity. As mountain cultivated ginseng (MCG) is cultivated without agricultural chemicals, MCG has emerged as a popular herb medicine. However, little is known about the MCG-mediated pharmacological mechanism on brain aging.
Methods: As we demonstrated that glutathione peroxidase (GPx) is important for enhancing memory function in the animal model of aging, we investigated the role of MCG as a GPx inducer using GPx-1 (a major type of GPx) knockout (KO) mice. We assessed whether MCG modulates redox and cholinergic parameters, and memory function in aged GPx-1 knockout KOmice.
Results: Redox burden of aged GPx-1 KO mice was more evident than that of aged wild-type (WT) mice. Alteration of Nrf2 DNA binding activity appeared to be more evident than that of NFκB DNA binding activity in aged GPx-1 KO mice. Alteration in choline acetyltransferase (ChAT) activity was more evident than that in acetylcholine esterase activity. MCG significantly attenuated reductions in Nrf2 system and ChAT level. MCG significantly enhanced the co-localization of Nrf2-immunoreactivity and ChAT-immunoreactivity in the same cell population. Nrf2 inhibitor brusatol significantly counteracted MCG-mediated up-regulation in ChAT level and ChAT inhibition (by k252a) significantly reduced ERK phosphorylation by MCG, suggesting that MCG might require signal cascade of Nrf2/ChAT/ERK to enhance cognition.
Conclusion: GPx-1 depletion might be a prerequisite for cognitive impairment in aged animals. MCG-mediated cognition enhancement might be associated with the activations of Nrf2, ChAT, and ERK signaling cascade.

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1. Introduction

Escalating evidence indicated that Korean ginseng (Panax ginseng Meyer) plays a role as antioxidant [1–4] and anti-inflammatory agents [4,5]. In general, ginseng supply is mainly available via the culture in the field [6]. Mountain cultivated ginseng (MCG), however, is grown in mountains before application. This wild-simulated method is conducted without additional agricultural chemicals [7,8]. Therefore, MCG is highly required for medical use.

Previously we demonstrated that MCG mitigated cognitive decline [2,3]. More information on MCG application is shown in Supplementary information (I). In particular, MCG requires up-regulation of glutathione to protect against cognitive impairment induced by phencyclidine in mice [3]. It is recognized that GSH is an essential substrate of glutathione peroxidase (GPx). Importantly, compelling evidence indicated that glutathione peroxidase-1 (GPx-1) gene, the major subtype of GPx in most tissues, ameliorated diverse aging conditions [9–11].

It is recognized that cognitive impairment is considered one of the most predominant outcomes of aging [12]. Thus, it is meaningful to prevent cognitive impairments for healthy aging [13]. Much of the study on aging and age-related diseases mainly focused on the role of the cerebral redox system. We [14–22] and others [23,24] proposed that the GPx-1 gene significantly ameliorated diverse conditions of cognitive impairments. We also suggested that ginsenosides might be a GPx-1 inducer against neuropsychotoxic conditions [1,25]. However, until now, it is unclear whether MCG itself modulates cognitive impairments in aging organisms. Therefore, we investigated here whether MCG up-regulates GPx-1-related redox mechanism to modulate memory dysfunction in aged mice. For the better understanding on the Nrf2/ChAT/ERK pathway, please refer to Supplementary information (II).

2. Materials and methods

2.1. Animals

All mice used, treated per the National Institutes of Health (NIH) Public Health Service Policy on Humane Care and Use of Laboratory Animals (2015 Edition; grants.nih.gov/grants/olaw/references/PHSPolicyLabAnimals.pdf) and according to the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (KW-210803-1). C57BL/6J (wild-type, WT) mice (Bio Genomics, Inc., Charles River Technology, Gapyung-Gun, Gyeonggi-Do, Republic of Korea), were bred in a temperature-controlled facility (24 ± 2 °C) under a 12-h light/dark cycle and *fed ad libitum*. Mice were allowed under these conditions for 2 weeks prior to the experiment. 12 months old (12 M) male mice were used as aged mice. Glutathione peroxidase-1 knockout (GPx-1 KO) mice were generated by HO et al [26] (Supplementary Materials and Methods 1.1).

2.2. Drug treatment

MCG was provided by Prof. Sung Kwon Ko (Semyung University, Jecheon, Republic of Korea) (Supplementary Fig. S2, and Supplementary Table S1) and was stored at −20°C.

Since changes in redox and cholinergic parameters were most significant in aged mice (please refer to Figs. 1 and 3), we focused on aged mice for further study. Nrf2 inhibitor brusatol (Sigma-Aldrich, USA), Trk inhibitor k252a (Enzo Life Sciences, Inc., NY, U.S.A.), and ERK inhibitor U0126 (Tocris Bioscience; Avonmouth, Bristol, UK) were dissolved in dimethyl sulfoxide (DMSO). The last concentration of DMSO was 5% (v/v) [27]. Aged GPx-1 and WT mice were treated with MCG (20 mg/kg, i.p. /day) for 30 successive days. The dosing term and administration route of MCG are comparable to those of ginsenoside Re [22].

Brusatol (1mg/kg, i.p.), k252a (0.3 mg/kg, i.p.) or U0126 (20μg/2μl, i.c.v/brain) was treated 90min after MCG and 90 min before every memory tests (novel object recognition and passive avoidance tests). The neurochemical assessments were conducted 2 h after the conclusion of the passive avoidance test (Supplementary Fig. S1). Simultaneously, double-labeling immunostaining was conducted using aged GPx-1 KO mice.

2.3. Reactive oxygen species (ROS)

ROS was examined by assessing the conversion of dichlorofluorescin (DCF) from 2′,7′-dichlorofluorescin diacetate (DCFH-DA) [22,28]. Please refer to Supplementary Materials and Methods 1.3.

2.4. 4-Hydroxynonenal (HNE)

For the extent of lipid peroxidation, HNE was measured using an OxiSelect™ HNE adduct ELISA kit (Cell Biolabs, Inc., San Diego, CA, U.S.A.) [22]. Supplementary Materials and Methods 1.4.

2.5. Protein carbonyl

Protein carbonyl level was examined for the understanding of protein oxidation was examined as demonstrated by Oliver et al [29]. Please refer to Supplementary Materials and Methods 1.5.

2.6. GSH and GSSG

Hippocampal tissues were dissected immediately after decapitation. Then, GSH and GSSG were examined upon tissue dissection as previously described [20,22]. As previously described, HPLC-UV/Vis detection system (Model LC-20AT and SPD-20A, Shimadzu) was used to separate and analyze the residual aqueous phase containing derived glutathione [30]. Please refer to Supplementary Materials and Methods 1.6.

2.7. Nuclear fraction

The extraction of nuclear fraction from hippocampal tissue was performed in accordance to the manufacturer's instructions of the Nuclear Extraction Kit (#40410; Active Motif, Carlsbad, CA, U.S.A.) [31]. Please refer to Supplementary Materials and Methods 1.7.

2.8. NF-κB DNA-binding activity

Following the manufacturer's instructions, the NF-κB p65 DNA-binding activity was determined by using the TransAM® NF-κB transcription factor ELISA kit (Active Motif) [16]. Please refer to Supplementary Materials and Methods 1.8.

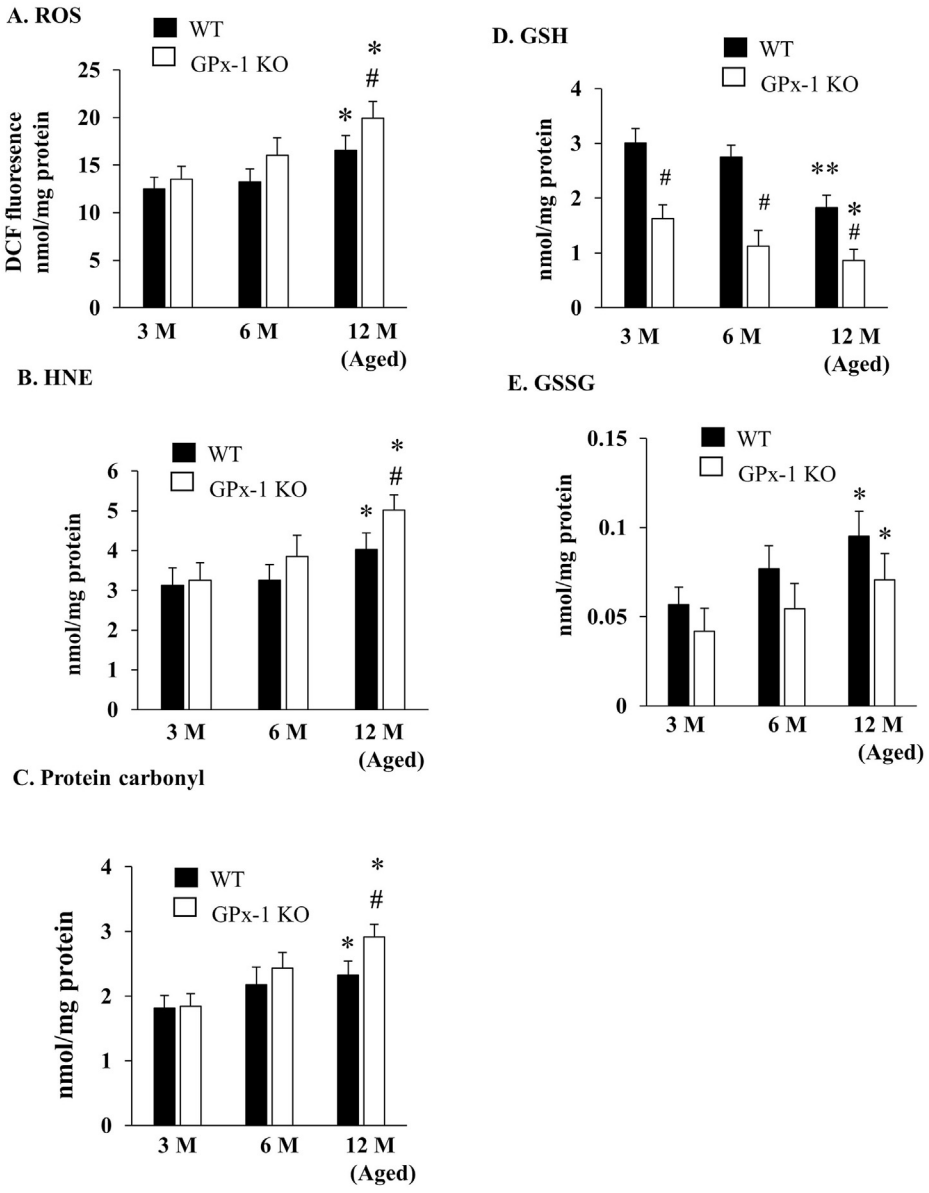

Fig. 1. Aging facilitates oxidative burden in the hippocampus of WT and GPx-1 KO mice; effects of MCG. Changes in reactive oxygen species (ROS) (A), 4-hydroxynonenal (HNE) (B), protein carbonyl (C) over time. Time-course of changes in GSH (D) and GSSG (E). The effects of MCG against alterations in ROS (F), HNE (G), protein carbonyl (H), GSH (I), and GSSG (J). M = months old. Each value represents the mean ± S.E.M. of 8 animals. *P < 0.05 vs. corresponding 3 M. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 vs. corresponding WT. †P < 0.05, ††P < 0.01 vs. Saline / WT. §P < 0.05, §§P < 0.01 vs. Saline / GPx-1 KO. Two-way ANOVA followed by Fisher's LSD pairwise comparisons were used to analyze the data.

2.9. Nuclear factor erythroid-2-related factor 2 (Nrf2) DNA-binding activity

According to manufacturer's instructions, Nrf2 DNA-binding activity was measured using a TransAM® Nrf2 Transcription Factor ELISA Kit (Active Motif) [32]. Please refer to Supplementary Materials and Methods 1.9.

2.10. Acetylcholine (ACh) level

The hippocampal tissues were homogenated as described previously [19]. ACh levels in the supernatant were assessed by using an Amplex® Red Acetylcholine/Acetylcholinesterase Assay Kit (A-12217; Invitrogen, USA) [19]. Please refer to Supplementary Materials and Methods 1.10.

2.11. Acetylcholine esterase (AChE) and choline acetyltransferase (ChAT) activities

A spectrophotometer was used to measure the absorbance at 324 nm using an Amplex® Red Acetylcholine/Acetylcholinesterase Assay Kit (A-12217; Invitrogen) [19]. Please refer to Supplementary Materials and Methods 1.11.

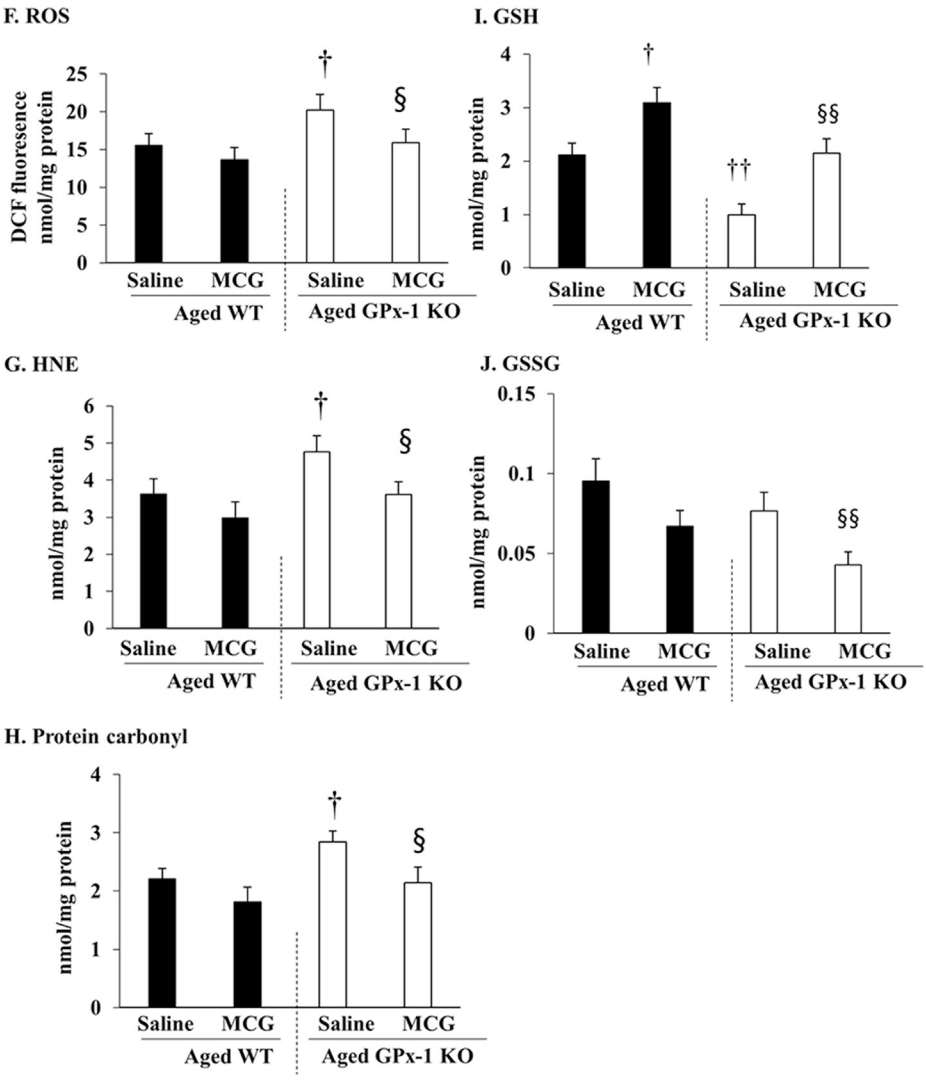

Fig. 1. (*continued*).

2.12. Double-labeled immunocytochemistry

Five μm thickness of brain sections from GPx-1 KO mice were positioned on the same slide and processed for immunostaining. Adhered tissues on poly-L-lysine-precoated coverslips, were fixed in PBS-4% para-formaldehyde (PFA), and were permeabilized with 0.1% Triton X-100 in PBS for 15 min. After saturation with PBS-1% BSA, tissues were incubated for 40 min with the primary antibody and were incubated for 40 min with the secondary antibody as follows: mouse anti-Nrf2 (1:100) (Santa Cruz, TX, USA), goat anti-ChAT (1:100) (Sigma-Aldrich, St. Louis, MO, USA). Secondary antibodies were anti-mouse IgG H&L (Alexa Fluor® 488) (1:200) (Abcam, Cambridge, MA, USA), anti-goat IgG H&L (Alexa Fluor® 546) (1:200) (Invitrogen, Carlsbad, CA, USA) [33]. Please refer to Supplementary Materials and Methods 1.12.

2.13. Western blot analysis

Whole proteins extracted from hippocampal tissues were quantified and electrophoresed as described previously [4]. After that, the membranes were preincubated with 3% non-fat milk for 30 min and incubated overnight at 4 °C with primary antibody against ChAT (1:1000, Sigma-Aldrich), ERK (1:10000, Cell Signaling), p-ERK (1:1000, Cell Signaling), Keap-1 (1:1000; Abcam), HO-1 (1:2000; Abcam), NQO-1 (1:1000; Abcam) or β-actin (1:30000, Sigma-Aldrich) for 1 night. Membranes were incubated with HRP-conjugated secondary anti-rabbit IgG (1:5000, GE healthcare, Piscataway, NJ, USA), anti-goat IgG (1:5000, Sigma-Aldrich) or anti-mouse IgG (1:5000, Sigma-Aldrich) for 2 h. Please refer to Supplementary Materials and Methods 1.13.

2.14. Novel object recognition test (NORT)

The NORT was conducted as shown previously [34]. The apparatus consisted of a Plexiglas open-field box (40 × 40 × 40 cm). Please refer to Supplementary Materials and Methods 1.14.

2.15. Passive avoidance test

Using a Gemini Avoidance System (San Diego Instrument, San Diego, CA) the passive avoidance test was assessed according to the protocol described previously [17,35],. The apparatus was divided

into a two-compartment shuttle chamber with a constant current shock generator. For both acquisition and retention trials (cut-of time, 300 s), the latencies in seconds were measured as the time between placement into the lighted chamber and entry into the dark chamber [17,35]. Please refer to Supplementary Materials and Methods 1.15.

2.16. Data analysis

IBM SPSS ver.24.0 (IBM, Chicago, IL, U.S.A) software was used to analyze data using a one-way ANOVA or two-way ANOVA for repeated measures followed by Fisher's LSD pairwise comparisons. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Aging facilitates oxidative burden, GSH, and GSSG in the hippocampus of GPx-1 KO mice; effects of MCG

As shown in Supplementary Fig. S1A of experimental design, redox parameters were examined (Fig. 1). Oxidative markers were significantly increased in aged WT mice (ROS, HNE, and protein carbonyl; $P < 0.05$ vs. those of 3 M WT mice) and aged GPx-1 KO mice (ROS, HNE, and protein carbonyl; $P < 0.05$ vs. those of 3 M WT mice) than 3 M WT and GPx-1 KO mice, respectively. Oxidative parameters of aged GPx-1 KO mice were consistently higher (ROS, HNE, and protein carbonyl; $P < 0.05$ vs. those of aged WT mice) than those of aged WT mice (Fig. 1A–C). On the other hand, aged WT mice significantly decreased ($P < 0.01$ vs. 3 M mice; $P < 0.05$ vs. 6 M mice) GSH levels. GPx-1 KO significantly lowered GSH levels (3 M WT vs. GPx-1 KO; $P < 0.01$.6 M WT vs. GPx-1 KO; $P < 0.01$. Aged WT vs. GPx-1 KO; $P < 0.01$) in aged mice. On the other hand, GSSG level was significantly increased aged mice (WT or GPx-1 KO; $P < 0.05$ vs. 3 M mice) (Fig. 1D and E).

Thus, we focused on aged animals for further study. Although MCG did not significantly attenuate oxidative parameters of aged WT mice, MCG significantly attenuated (ROS, HNE, and protein carbonyl; $P < 0.05$ vs. those of aged GPx-1 KO mice, respectively) oxidative parameters of aged GPx-1 KO mice (Fig. 1F–H). MCG significantly attenuated reduced GSH levels in aged mice. This attenuation seemed to be more underlined in aged GPx-1 KO mice ($P < 0.01$ vs. saline/GPx-1 KO) than aged WT mice ($P < 0.05$ vs. saline/WT). Although MCG did not significantly affect GSSG level in aged WT mice, MCG significantly attenuated GSSG level in aged GPx-1 KO mice ($P < 0.01$ vs. saline/aged GPx-1 KO mice) (Fig. 1I and J).

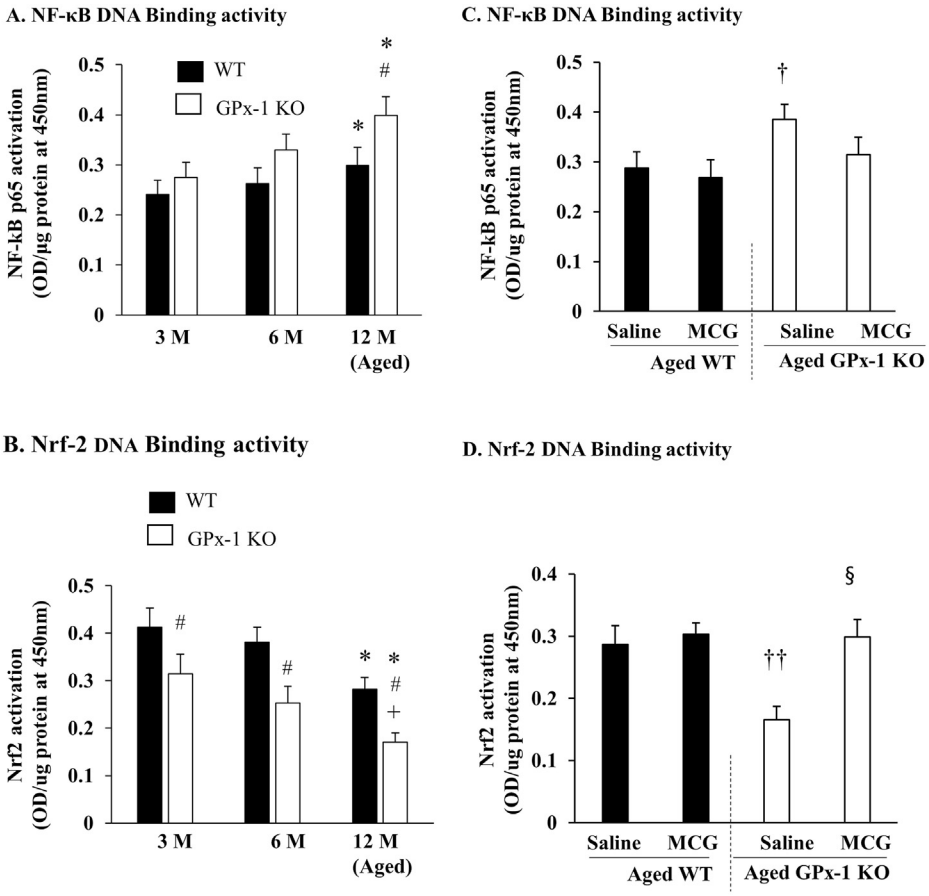

Fig. 2. Aging alters NF-κB DNA binding and Nrf2 DNA binding activities in the hippocampus of WT and GPx-1 KO mice; effects of MCG. Changes in NF-κB DNA binding activity (A) and Nrf2 DNA binding activity (B) over time. Effects of MCG against aging-induced changes in NF-κB DNA binding activity (C) and in Nrf2 DNA binding activity (D). M = months old. Each value represents the mean ± S.E.M. of 8 animals. *$P < 0.05$ vs. corresponding 3 M. #$P < 0.01$ vs. corresponding WT. +$P<0.05$ vs. 6 M. †$P < 0.05$, ††$P < 0.01$ vs. Saline / WT. §$P < 0.01$ vs. Saline / GPx-1 KO. Two-way ANOVA followed by Fisher's LSD pairwise comparisons were used to analyze the data.

3.2. Aging alters DNA binding activities of NF-κB and Nrf2 in the hippocampus of WT and GPx-1 KO mice; effects of MCG

As shown in Fig. 2A and B, NF-κB DNA binding activity of aged WT ($P < 0.05$) and GPx-1 KO mice ($P < 0.05$) were significantly higher than that of 3 M WT mice, respectively. NF-κB DNA binding activity of aged GPx-1 KO mice is significantly higher ($P < 0.05$) than that of aged WT mice (Fig. 2A). On the other hand, Nrf2 DNA binding activity appeared to be decreased over time (Fig. 2B). Nrf2 DNA binding activity of aged WT and GPx-1 KO mice was significantly lower ($P < 0.05$) than that of 3 M WT and GPx-1 KO mice, respectively. Six M ($P < 0.05$ vs. corresponding WT) and aged GPx-1 KO mice ($P < 0.01$ vs. corresponding WT) were lower than corresponding WT mice. Thus, it is plausible that alteration of Nrf2 DNA binding activity is more pronounced than that of NF-κB DNA binding activity during aging. Since changes in aged animals are most significant, we focused on aged animals to assess MCG-mediated activity. As shown in Fig. 2C, NF-κB DNA binding activity of aged GPx-1 KO mice is higher ($P < 0.05$) than that of aged WT mice. As manifested in Fig. 2D, Nrf2 DNA binding activity of aged GPx-1 KO mice was lower ($P < 0.01$) than that of aged WT mice. MCG appeared to be increased Nrf2 DNA binding activity in aged WT mice. In contrast, MCG significantly increased ($P < 0.01$) Nrf2 DNA binding activity in aged GPx-1 KO mice. Consistently, MCG attenuated the alteration in Keap-1, HO-1, and NQO-1 expressions in aged-WT and aged GPx-1 KO mice (Supplementary Fig. S4).

3.3. Aging alters acetylcholine (ACh) level, choline acetyltransferase (ChAT), and acetylcholine esterase (AChE) activities in the hippocampus of WT and GPx-1 KO mice

As shown in Fig. 3A, ACh level of aged WT ($P < 0.05$ vs. 3 M WT mice) and GPx-1 KO mice ($P < 0.05$ vs. 3 M GPx-1 KO mice. $P < 0.05$ vs. 6 M GPx-1 KO mice) were significantly lower than that of corresponding mice, respectively. ACh levels of aged GPx-1 KO mice were significantly lower ($P < 0.01$) than that of aged WT mice. Time course of change of ACh level might be in line with that of ChAT activity.

As shown in Fig. 3B, ChAT activity of aged WT ($P < 0.01$ vs. 3 M old WT mice) and GPx-1 KO mice ($P < 0.01$ vs. 3 M GPx-1 KO mice) were significantly lower than that of corresponding mice, respectively. ChAT activity of 6 M old GPx-1 KO mice was lower ($P < 0.01$) than that of 6 M WT mice. Consistently, ChAT activity of aged GPx-1 KO mice was significantly lower ($P < 0.01$) than that of aged WT mice. In contrast, AChE activity of aged GPx-1 KO was significantly higher ($P < 0.05$) than 3 M GPx-1 KO or aged WT mice (Fig. 3C). Thus, ChAT activity seems to be more sensitive than AChE activity during the aging process.

3.4. MCG enhances Nrf2- and ChAT-immunoreactivities (IRs) in the same cellular population of aged GPx-1 KO mice

Because we found that MCG significantly increased Nrf2 DNA binding activity and ChAT activities mainly in aged GPx-1 KO mice, we conducted double-labelling immunocytochemistry to understand the immunodistribution of Nrf2 and ChAT-IRs.

As shown in representative photomicrograph on the double-labelling immunocytochemistry of Nrf2 and ChAT (Fig. 4A), MCG significantly increased Nrf2-IR in the dentate gyrus (DG) ($P < 0.01$) and in the CA1 ($P < 0.01$) and CA3 ($P < 0.01$) regions in the aged GPx-1 KO mice (Fig. 4B). Consistently, ChAT-IR in the DG ($P < 0.01$) and in the CA1 ($P < 0.01$) and CA3 ($P < 0.01$) in the aged GPx-1 KO mice (Fig. 4C). Most Nrf2-IR was significantly co-localized in the ChAT-immunoreactive cells (Fig. 4D).

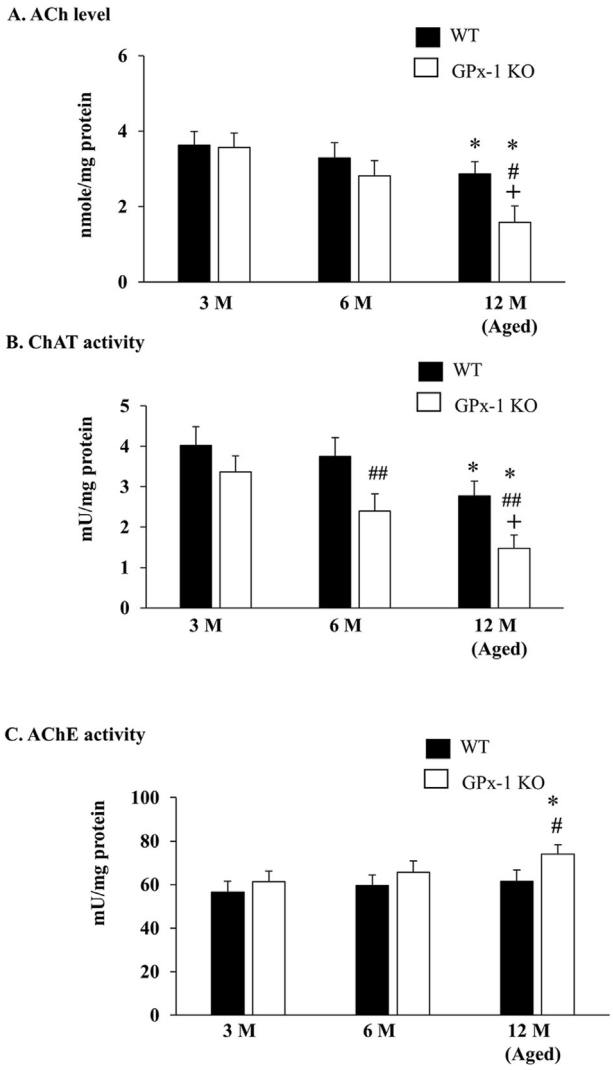

Fig. 3. Aging alters acetylcholine (ACh) level, choline acetyltransferase (ChAT), and acetylcholine esterase (AChE) activities in the hippocampus of WT and GPx-1 KO mice; effects of MCG. Changes in ACh level (A), ChAT activity (B), and AChE activity (C) over time. M = months old. Each value represents the mean ± S.E.M. of 8 animals. *$P < 0.05$ vs. corresponding 3 M. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. corresponding WT. $^{+}P<0.05$ vs. corresponding 6 M. Two-way ANOVA followed by Fisher's LSD pairwise comparisons were used to analyze the data.

3.5. MCG requires hippocampal activation of Nrf2, ChAT, and ERK signalings in aged GPx-1 KO mice

Because we [27] and others [36–38] suggested that BDNF receptor tyrosine kinase B (TrkB) inhibitor k252a also inhibits ChAT, we used k252a to inhibit ChAT level.

As shown in Fig. 5A and B, we asked whether MCG-related pharmacological activity is associated with Nrf2 signaling for inducing ChAT. Although aged GPx-1 KO showed significantly reduced ChAT activity in mice, MCG significantly attenuated this reduction. This attenuation was significantly counteracted by brusatol or k252a suggesting that Nrf2 mediates up-regulation of ChAT activity in the presence of MCG (Fig. 5A). On the other hand, MCG-mediated attenuation was not altered by U0126. This profile of ChAT activity is comparable to that of ChAT expression in the current experimental condition (Fig. 5B). ERK inhibitor U0126 did not affect MCG-mediated ChAT induction (Fig. 5A and B). As shown in Fig. 5C, Gpx-1 KO itself significantly decreased ERK

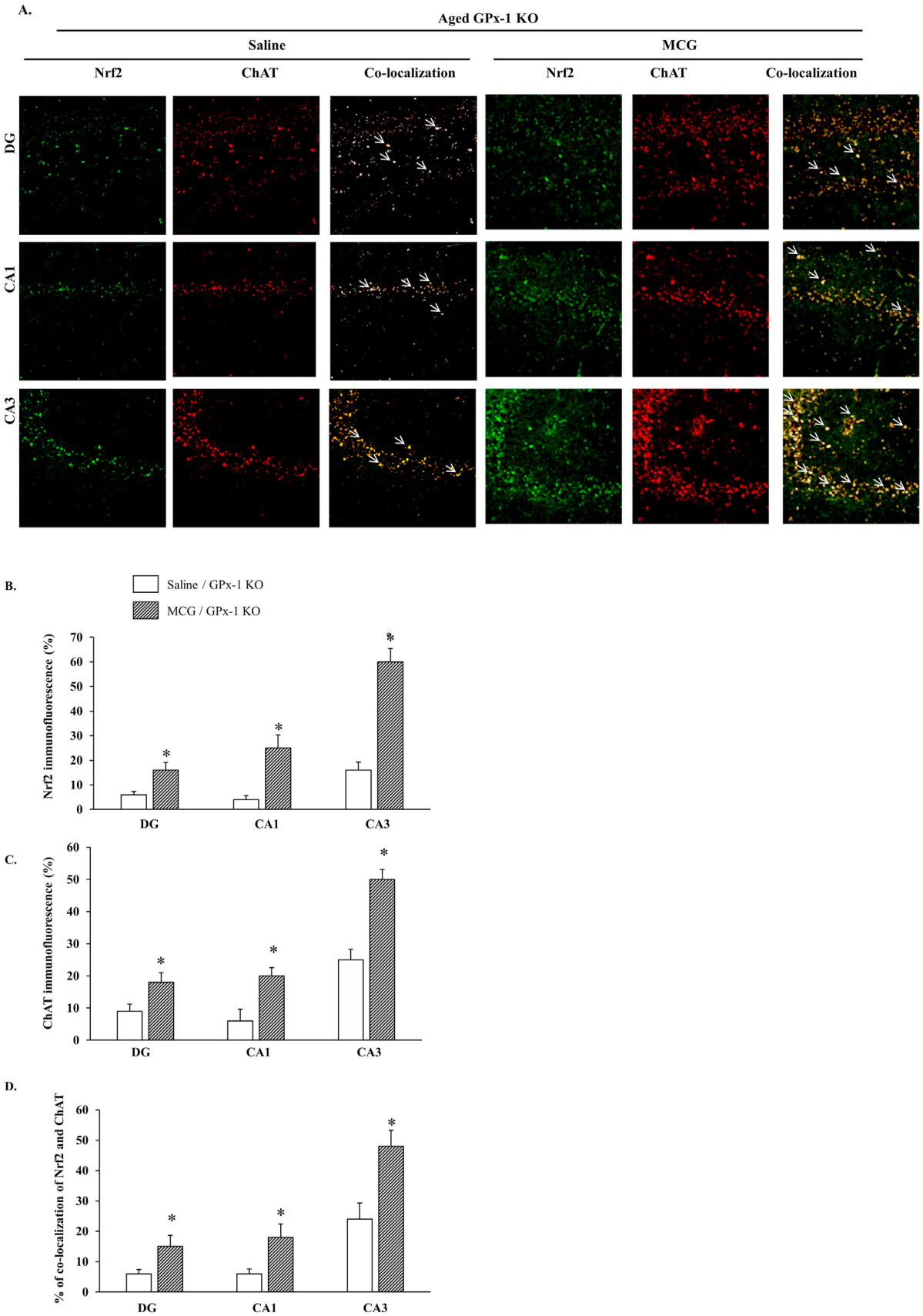

Fig. 4. MCG enhances co-localized immunoreactivities of Nrf2- and ChAT in the same hippocampal cells of GPx-1 KO mice. The representative photomicrograph of the double-labeling immunocytochemistry of Nrf2 and ChAT (A). Effects of MCG on aging-induced changes in Nrf2-IR (B), ChAT-IR (C), and co-localization of Nrf2 and ChAT (D). Each value represents the mean ± S.E.M. of 4 animals. *P < 0.01 vs. corresponding Saline. One-way ANOVA followed by Fisher's LSD pairwise comparisons were used to analyze the data.

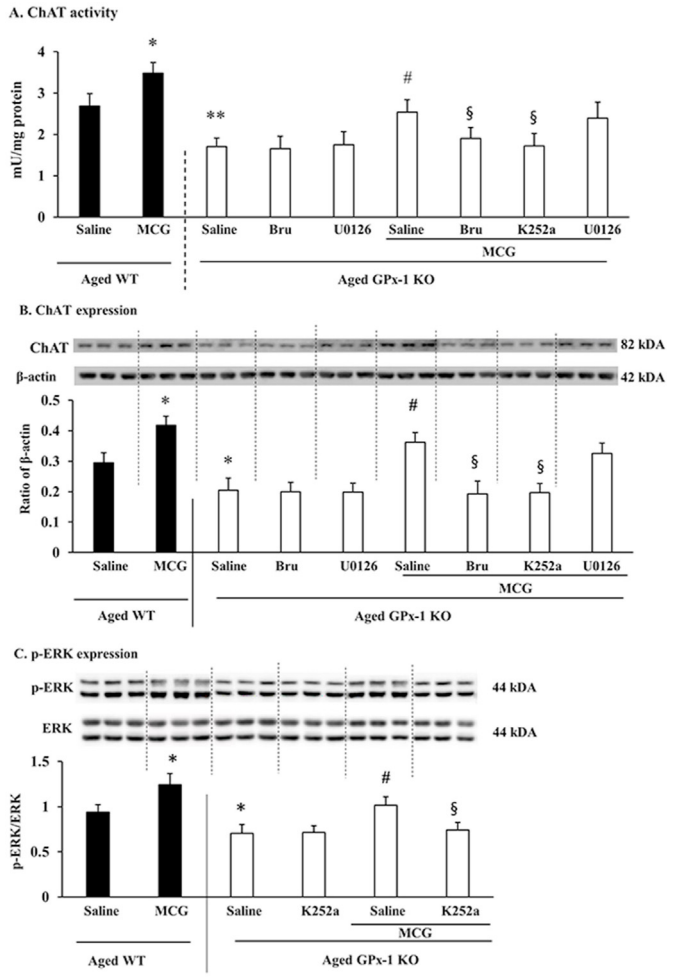

Fig. 5. Effects of brusatol, k252a, and U0126 on MCG-mediated pharmacological activity against ChAT activity (A) and ChAT expression (B) and effect of k252a on MCG-mediated pharmacological activity against ERK phsphorylation in the hippocampus of aged GPx-1 KO mice. Each value represents the mean ± S.E.M. of 5 animals (ChAT activity) and 3 animals (ChAT and ERK expressions). *P < 0.05, **P < 0.01 vs. Saline / WT. #P < 0.05 vs. Saline / GPx-1 KO. §P < 0.05 vs. MCG / GPx-1 KO. One-way ANOVA followed by Fisher's LSD pairwise comparisons was used to analyze the data.

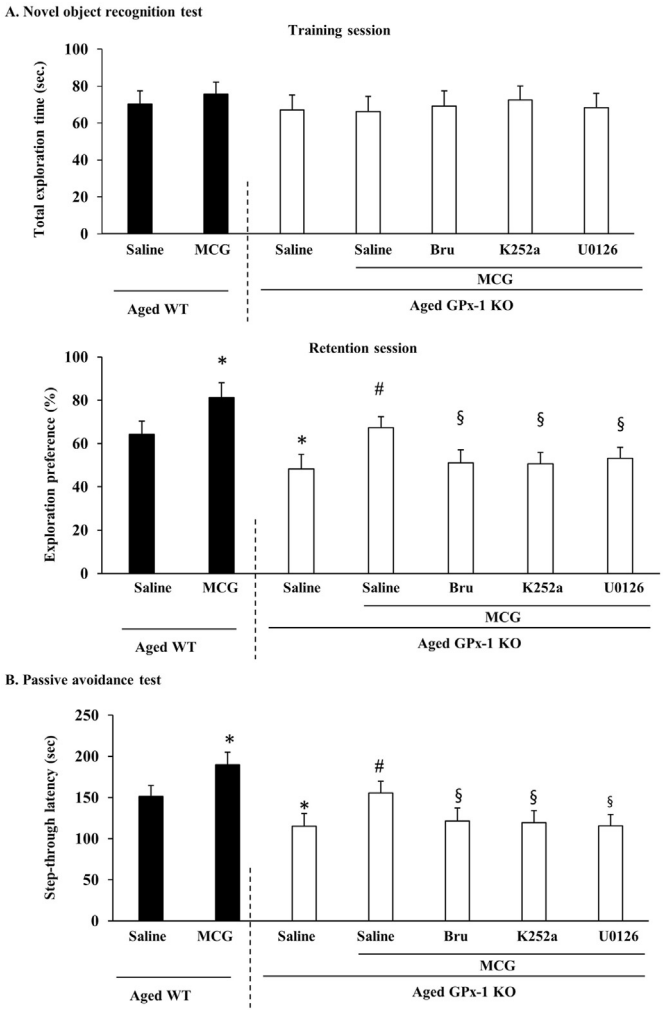

Fig. 6. Effects of brusatol, k252a, and U0126 on MCG-mediated pharmacological activity against novel object recognition (A) and passive avoidance tests (B) in aged GPx-1 KO mice. Each value represents the mean ± S.E.M. of 8 animals. *P < 0.05 vs. Saline / WT. #P < 0.05 vs. Saline / GPx-1 KO. §P < 0.05 vs. MCG / GPx-1 KO. Two-way ANOVA followed by Fisher's LSD pairwise comparisons were used to analyze the data.

phosphorylation (P < 0.05 vs. aged WT mice) in aged mice. MCG significantly mitigated (P < 0.05) this decrease. This mitigation was significantly counteracted by k252a (Fig. 5C), suggesting that ChAT can be an upstream molecule for ERK signaling.

3.6. MCG-mediated memory enhancement is associated with activations of Nrf, TrkB/ChAT, and ERK in aged GPx-1 KO mice

As shown in Fig. 6. we asked whether MCG modulates signaling cascades of Nrf2, ChAT, and ERK for attenuating cognitive dysfunction in aged GPx-1 KO mice. As shown in Fig. 6A, GPx-1 KO showed impaired (P < 0.05 vs. aged WT mice) performance in novel object recognition test (NORT) in aged mice. MCG significantly ameliorated (P < 0.05 vs. Saline/aged GPx-1 KO mice) memory impairment in NORT in aged GPx-1 KO mice. The memory function with or without MCG as evaluated by passive avoidance tests (Fig. 6B) is comparable to that by NORT. MCG-mediated memory-enhancing effects were significantly inhibited by brusatol, k252a, or U0126, indicating that MCG requires activations of Nrf2, ChAT, and ERK for cognitive enhancements in aged GPx-1 KO mice. In addition, please refer to Supplementary Fig. S3 for behavioral data in the absence of MCG.

4. Discussion

Unexpectedly, we observed that GPx-1 KO did not show any compensative induction in Nrf2 DNA binding activity in the aged mice, but GPx-1 KO showed significant inhibition of Nrf2 DNA binding activity [For the better understanding, please refer to Supplementary information (III)]. The degree of inhibition in Nrf2 system induced by GPx-1 KO in aged mice appeared to be more pronounced than that of activation in NFκB system. In addition, altered activity in ChAT was more pronounced than that in AChE activity in the aged GPx-1 KO mice. MCG significantly attenuated reductions in Nrf2 system and ChAT level in aged GPx-1 KO mice. Co-localization of Nrf2-IR and ChAT-IR was noted in the same cell of the hippocampus. Therefore, we suggest that GPx-1 KO is a pre-requisite for cognitive impairment in the aging organism and that MCG-mediated cognition enhancement is associated with the activation of the Nrf2, ChAT, and ERK signaling cascade.

Since cerebral catalase activity is low [39], GPx is deemed as one of the most important peroxide (i.e., H_2O_2) scavengers in the brain

[16]. Particularly, the Se-dependent GPx-1 isoform is the major one in the brain [16,40,41]. Previously, we [16] and others [42] demonstrated that GPx-1 depletion increased systemic oxidative stress in mice. Furthermore, increased senescence was demonstrated for fibroblasts from GPx-1 KO mice [42]. Similarly, we showed mechanistic links between hippocampal alteration of redox and cholinergic systems in aged GPx-1 KO animals. Importantly, we demonstrated that GPx-1 gene-encoded adenoviral vector significantly blocked β-amyloid (1-42)-induced cholinergic decline in GPx-1 KO mice [17,19], suggesting that the GPx-1 gene is an endogenous factor for enhancing cognitive/cholinergic functions [16,17,19].

Consistently, compelling evidence suggested that the regulations of GPx levels by ginseng treatment can be responsible for protecting ROS-associated disorders [43], suggesting that ginsenosides attenuate oxidative burden, via GPx induction [44]. Similarly, we reported that MCG significantly attenuates recognition memory impairments induced by psychotoxic insult (i.e. phencyclidine treatment) via a GSH synthetic system including GPx in the prefrontal cortex of mice [3]. More importantly, we demonstrated that the phencyclidine-induced recognition memory deficit is associated with the inhibition of the GPx/GPx-1-mediated Nrf2/GSH synthetic pathway [20] and that ginsenoside Re plays a major role in MCG-mediated efficacy [1,3].

We showed the major component of ginsenosides in MCG expressed an anti-inflammatory potential by antioxidant activity [4,45]. By the way, it was considered that Nrf2 induced anti-oxidant and anti-inflammatory genes. Consistently, here we showed that Nrf2 inhibitor brusatol counteracted MCG-mediated signaling cascade, suggesting that MCG facilitates Nrf2-mediated memory-enhancing signaling in aged GPx-1 deficient conditions. Indeed, the Nrf2 system has been declared not only as an essential modulator of aging and species longevity but also as a critical molecular target against senescence [46]. Furthermore, it was also suggested that the Nrf2 was necessary for the antiaging gene *klotho* itself to protect against senescence [47,48]. Therefore, we propose that both GPx-1 and Nrf2 can be potential protective targets of MCG for up-regulating GSH-related antioxidant capacity and attenuating cognitive dysfunction of the aging process.

ChAT is significantly reduced with increasing age in the hippocampus [49] and cerebral cortex [50]. Importantly, the loss of ChAT, but not alteration of AChE, in aging may be critical for abnormalities of cholinergic nerve terminals [51]. Here we also observed that alteration of ChAT is more sensitive than that of AChE in response to the aging process, although it remains to be further clarified.

Indeed, ChAT activity was reduced after exposure to H_2O_2 in vitro [52]. Therefore, it is plausible that H_2O_2 formation by GPx-1 depletion might mediate free radical processes and significantly inhibit ChAT activity probably via ROS acting on ChAT level in the brain [19]. We [53] and others [54] showed that exposure to ginseng up-regulated cholinergic parameters including ChAT level in the mice model. Here, we for the first time demonstrate that MCG also consistently up-regulates ChAT/ACh levels in aged GPx-1 KO mice, suggesting that MCG requires ChAT/ACh and GPx-1 inductions for anti-aging and cognitive enhancing potential.

Consistently, it has been demonstrated that the genetic overexpressing ChAT into the neural stem cells significantly enhanced the cognitive function of aged mice [27]. This finding is, at least in part, in line with the current result using MCG.

Indeed, we also found that aging-mediated oxidative stress mainly affects the impairment of Nrf2 transcription factor, followed by down-regulation of ChAT, suggesting that GPx-1 deficiency triggers oxidative stress, followed by inhibitions of Nrf2 and ChAT during the aging process. We observed that Nrf2- and ChAT-IRs were co-localized in the same neuronal populations as [55] reported in a different neurotoxic animal model, and MCG significantly enhanced these co-localizations [55]. Thus, it is considered that Nrf2-immunoreactive cells significantly release the ChAT gene in the presence of MCG for enhancing cognitive function, although the cellular scenario between Nrf2 and ChAT remains to be fully explored.

Importantly, we showed that MCG-induced Nrf2-IR, ChAT-IR, and co-localization of Nrf2 and ChAT-IRs were most conspicuous in the CA3 region out of CA1, DG, and CA3 regions in aged GPx-1 KO mice. Similarly, Bae et al [56] demonstrated that ginsenosides modulate CA3 neurons to modulate the physiological function of the hippocampus. Indeed, the CA3 neurons are important for modulating BDNF [57] and ERK [57] signaling, and long-term potentiation [58]. We observed here that k252a, a BDNF receptor tyrosine kinase B (TrkB) inhibitor, counteracted MCG-mediated ChAT/ERK levels and memory dysfunction in aged GPx-1 KO mice. As reflected by the previous reports [57,58], we cannot rule out the possibility that MCG might up-regulate and BDNF-, and ERK-signaling in the CA3 to protect against cognitive impairments, and that MCG improves memory in aged mice via enlargement of long-term potentiation in the CA3 region [58], However, it remains to be elucidated.

In conclusion, we propose that genetic depletion of GPx-1 might be an optimal model for studying geriatric memory dysfunction in aged mice via consistent oxidative burden in the hippocampus and that MCG ameliorates cognitive impairments mainly via up-regulation of Nrf2, ChAT, and ERK signaling pathway (Supplementary Fig. S5) However, the precise mechanism mediated by the critical component in the MCG remains to be further explored.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Declaration of competing interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2023.01.005.

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