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Sulforaphane alleviates scopolamine-induced memory impairment in mice

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ABSTRACT

Sulforaphane, an organosulfur compound present in cruciferous vegetables, has been shown to exert neuroprotective effects in experimental *in vitro* and *in vivo* models of neurodegeneration. To determine whether sulforaphane can preserve cognitive function, we examined its effects on scopolamine-induced memory impairment in mice using the Morris water maze test. Sulforaphane (10 or 50 mg/kg) was administered to C57BL/6 mice by oral gavage for 14 days (days 1–14), and memory impairment was induced by intraperitoneal injection of scopolamine (1 mg/kg) for 7 days (days 8–14). Mice that received scopolamine alone showed impaired learning and memory retention and considerably decreased cholinergic system reactivity in the hippocampus and frontal cortex, as indicated by a decreased acetylcholine (ACh) level and an increased acetylcholinesterase (AChE) activity. Sulforaphane significantly attenuated the scopolamine-induced memory impairment and improved cholinergic system reactivity, as indicated by an increased ACh level, decreased AChE activity, and increased choline acetyltransferase (ChAT) expression in the hippocampus and frontal cortex. These effects of sulforaphane on cholinergic system reactivity were confirmed *in vitro*. Sulforaphane (10 or 20 μM) increased the ACh level, decreased the AChE activity, and increased ChAT expression in scopolamine-treated primary cortical neurons. These observations suggest that sulforaphane might exert a significant neuroprotective effect on cholinergic deficit and cognitive impairment.

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1. Introduction

Sulforaphane (1-isothiocyanato-4-methylsulfinylbutane; Fig. 1A) is an organosulfur compound found in cruciferous vegetables *e.g.*, broccoli, cabbage, watercress, and Brussels sprouts [1]. Sulforaphane first attracted interest because of its potential anti-cancer activity [2]. However, because of its diverse pharmacological properties including cardiovascular protection, protection against diabetic nephropathy, neuropathy, and *Helicobacter pylori* infection, and restoration of skin integrity [2], this compound is currently being tested in several clinical trials (www.clinicaltrials.gov). Recent *in vitro* and *in vivo* studies have examined the potential neuroprotective actions of sulforaphane. For example, sulforaphane has been shown to protect cortical neurons against 5-*S*-cysteinyl-dopamine-induced toxicity [3] and Neuro2A and N1E 115 cells against amyloid β-induced toxicity [4]. In rodents, sulforaphane reduces infarct volume following focal cerebral ischemia [5] and attenuates brain edema following traumatic brain injury [6]. However, the potential effects of sulforaphane on cholinergic system and cognitive function have not yet been elucidated.

The central cholinergic system is important in the regulation of cognitive function. The neurotransmitter acetylcholine (ACh),

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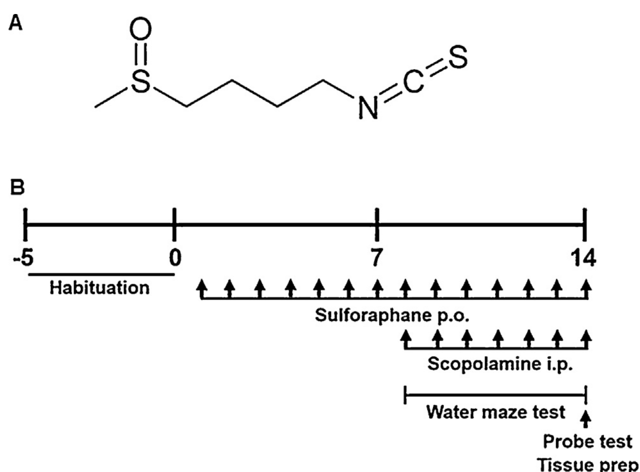

Fig. 1. Chemical structure of sulforaphane and study design. (A) Structure of sulforaphane. (B) After a 5-day adaption period, the mice were given sulforaphane (10 or 50 mg/kg, p.o.) for a total of 14 days. Beginning on day 8, scopolamine (1 mg/kg, i.p.) was also administered for 7 days, and the Morris water maze test was used to assess behavior during this period (days 8–14). On day 14, each mouse was sacrificed, and the hippocampus and frontal cortex were removed for the analysis of acetylcholine level, acetylcholinesterase activity, and choline acetyltransferase expression.

which is synthesized by choline acetyltransferase (ChAT) in cholinergic neurons [7], is required for central and peripheral control of multiple cognitive processes including timing, attention, learning, and memory [8]. Accordingly, lesions of the cholinergic neurons that decrease ACh release into the synaptic cleft result in learning and memory dysfunction [9]. The duration of ACh action is dependent upon the activity of acetylcholinesterase (AChE), which hydrolyzes ACh after its release [10,11]. Inhibition of AChE is the therapeutic approach used for Alzheimer's disease, other types of dementia, traumatic brain injury, and delirium, and it may potentially be useful in the treatment of schizophrenia [12]. ChAT is the most specific marker of cholinergic neuron function in the central and peripheral nervous systems [13]. The activity of ChAT is strongly reduced in aged brains, and the degree of reduction in activity is significantly correlated with the severity of cognitive impairment [14].

Scopolamine is a nonselective muscarinic ACh receptor (mAChR) antagonist that mainly targets M1AChR and M2AChR, thereby impairing learning acquisition and short-term memory in rodents and humans [15–17]. Scopolamine-induced amnesia has been used to generate experimental animal models for the screening of antiamnesic drugs [10]. To tease out the effects that result in cognitive benefits, it is essential to test drugs using a memory impairment model that is nondegenerative and shows cholinergic dysregulation. The hippocampus and frontal cortex are particularly vulnerable to scopolamine-induced neuronal injury [18].

To elucidate the potential effects of sulforaphane on cognitive function and the cholinergic system, we evaluated learning and memory retention in C57BL/6 mice using the Morris water maze test and assessed cholinergic markers (ACh, AChE, and ChAT) in C57BL/6 and primary cortical neurons exposed to scopolamine.

2. Materials and methods

2.1. Materials

Sulforaphane was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Scopolamine hydrochloride, fetal bovine serum (FBS), and antibodies against β-actin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium, Neurobasal medium, Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS), L-glutamine, B-27 supplement, and penicillin/streptomycin were purchased from Gibco BRL (Carlsbad, CA, USA). The Amplex Red ACh/AChE assay kit was purchased from Invitrogen (Grand Island, NY, USA). Antibodies against ChAT were purchased from Abcam (Cambridge, United Kingdom).

2.2. Animals

Female C57BL/6 mice (age, 8 weeks; weight, 25–27 g) were purchased from Orient Bio (Seoul, South Korea) and housed in a regulated environment (21 ± 2 °C, 12-h light/dark cycle, light period starting at 8 AM) with free access to food and water. All experiments were conducted in compliance with Konkuk University's Council Directive for the care and use of laboratory animals (KU11025). To test the effect of sulforaphane on cognitive function, the mice were randomly assigned to four treatment groups ($n = 10$ per group): (1) vehicle (phosphate buffered saline [PBS]), (2) scopolamine (1 mg/kg/day, 100 μl/day), (3) sulforaphane (10 mg/kg/day, 100 μl/day) + scopolamine (1 mg/kg/day, 100 μl/day), and (4) sulforaphane (50 mg/kg/day, 100 μl/day) + scopolamine (1 mg/kg/day, 100 μl/day). Sulforaphane was dissolved in PBS and administered by oral gavage (p.o.) on days 1–7, prior to training for the Morris water maze test, and continued on days 8–14, during which the mice underwent the Morris water maze test. Scopolamine was dissolved in PBS and administered by intraperitoneal (i.p.) injection on days 8–14. On days 8–14, sulforaphane was administered 60 min before the trial, and scopolamine was injected 30 min before the trial. On day 14, the mice were decapitated immediately following the Morris water maze test. The hippocampus and frontal cortex were removed, dissected on ice, and frozen at −80 °C until analysis. The experimental design is summarized in Fig. 1B.

2.3. Cell culture

Primary cortical neurons were prepared from ICR mice at embryonic day 15. Cerebral cortices from the embryos were dissected and placed in ice-cold Ca^{2+}/Mg^{2+}-free HBSS (4 ml/embryo). The tissue was centrifuged (300 × g, 2 min), dissociated with 0.05% trypsin in HBSS for 10 min at 37 °C, resuspended in minimal essential medium containing 10% FBS, 10% heat-inactivated horse serum, 2 mM L-glutamine, and 1% penicillin/streptomycin and then filtered twice through a 70-μm cell strainer. Cells were seeded on 0.2-mg/ml poly-D-lysine-coated plates and incubated in a 37 °C humidified atmosphere. The cells were allowed to adhere to the plates for 45 min before the culture media was changed to Neurobasal medium supplemented with B27, 1% L-glutamine, and 1% penicillin/streptomycin. Neurites were observed sprouting from neuronal cell bodies on day 3 after the initial plating. Neurobasal medium was changed every other day. The cortical neurons were treated with sulforaphane (10 or 20 μM) for 1 h, followed by 20 μM scopolamine for 6 or 12 h; PBS was used as the vehicle.

2.4. Morris water maze test

The water maze test was conducted in a circular tank (diameter 183 cm, height 58 cm) filled with water maintained at 25 ± 2 °C. The tank was divided into four quadrants with a hidden escape platform (diameter, 20 cm; height, 48 cm) submerged 1.5 cm below the water surface in the center of one quadrant. The mice were trained to find the hidden platform by learning and memorizing several visual cues placed outside the maze. The position of the cues remained unchanged throughout the experiments. Four trials were conducted on each day of the training period (days 8–13). Each mouse was given 60 s to find the hidden platform and allowed to remain on it for another 30 s. The mean time used to find the

platform (mean escape latency), mean distance traveled to locate the platform (mean path length), and mean distance from the platform (mean search error) during training trials were determined. Mice that failed to escape from the water within 60 s were guided to the platform and allowed to remain there for 30 s. On day 14, the platform was removed from the tank, and the mice underwent a spatial probe trial in which they were given 30 s to search for the removed platform. The number of times the mice crossed the previous platform location (number of crossings) and the time spent in a 2× (40-cm diameter) concentric area surrounding the platform (time in platform annulus) were recorded. A camera located above the center of the maze relayed images to a videocassette recorder. Data from the water maze trials were analyzed using human visual system Image Software (HVS Image, Hampton, UK).

2.5. ACh and AChE activity analysis

The Amplex Red ACh/AChE assay kit was used to determine ACh level and AChE activity. Working solutions of 400 μM Amplex Red reagent containing 2 U/ml horseradish peroxidase and 0.2 U/ml choline oxidase was prepared from stock solutions. To measure the effect of sulforaphane on ACh level and AChE activity in tissues and cells, 100 U/ml AChE and 100 μM ACh were added to measure ACh level and AChE activity, respectively. To measure the cell-free effect of sulforaphane on AChE activity, sulforaphane was prepared with 10 mU/ml AChE and 100 μM ACh. The reaction began when 100 μl of the working solution was added to microplate wells containing samples. Fluorescence emitted by the individual samples was detected using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 560 nm and emission wavelength of 590 nm. Background fluorescence was eliminated by subtracting values derived from the negative control.

2.6. Western blot analysis

Tissues and cells were homogenized in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Beverly, MA, USA), and the protein concentration was determined using Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). The protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 5% fat-free dry milk or 5% FBS and then incubated with primary antibodies against ChAT and β-actin. After incubation with horseradish-peroxidase-conjugated secondary antibodies, protein bands were detected using an enhanced chemiluminescence detection kit (GE Healthcare, St. Giles, UK).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from tissues and cells using RNAiso reagent (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA (1 μg/μl) served as a template for the synthesis of cDNA using a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio). ChAT and β-actin cDNA were amplified using TaKaRa Ex Taq™ DNA polymerase and the following PCR primers (Bioneer, Daejeon, Korea): ChAT, 5′-CTTGGATGGTCCAGGCAC-3′ and 5′-GTCATACCAACGATTCGCTCC-3′; β-actin, 5′-TGGTGGGTATGGGTCAGAAG-3′ and 5′-CATGGCTGGGGTTGAAGG-3′. PCR amplification of ChAT consisted of 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, yielding a 106 base-pair product. PCR amplification of β-actin consisted of 25 cycles of 30 s at 94 °C, 30 s at 63 °C, and 1 min at 72 °C, yielding a 266 base-pair product. The PCR products were separated on 1.5% agarose gels, which were stained with ethidium bromide.

2.8. Statistical analysis

Statistical analyses were performed using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA). Results were expressed as mean ± standard error of the mean (SEM). Behavioral data were analyzed by one-way repeated analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. The remaining data were analyzed by one-way ANOVA followed by Fisher's LSD test.

3. Results

3.1. Sulforaphane alleviates scopolamine-induced memory impairment

The Morris water maze test was used to test the ability of sulforaphane to preserve cognitive function in mice treated with scopolamine. Analysis of mean escape latency (*i.e.*, time required to locate the escape platform) revealed significant between-group effects (vehicle control, scopolamine, sulforaphane + scopolamine) ($F(3,36) = 31.789$, $p < 0.001$) and training session effects ($F(3,139) = 10.699$, $p < 0.001$). However, a significant interaction between treatment and training was not detected ($F(11,139) = 0.572$, $p = 0.857$). As shown in Fig. 2A, the control mice learned to locate the submerged platform more quickly during the training sessions compared with scopolamine-treated mice, which showed little improvement over the course of training. *Post hoc* analysis confirmed that the mean escape latency of control mice was significantly shorter than that of the scopolamine-treated mice ($p < 0.001$). However, scopolamine-treated mice that also received sulforaphane (10 or 50 mg/kg/day) performed significantly better than those that received scopolamine alone ($p = 0.031$ and $p = 0.029$, respectively) (Fig. 2A).

Analysis of mean path length (*i.e.*, distance traveled to locate the escape platform) revealed significant between-group effects (vehicle control, scopolamine, sulforaphane + scopolamine) ($F(3,36) = 18.111$, $p < 0.001$) and training session effects ($F(5,180) = 5.037$, $p < 0.001$). However, a significant interaction between treatment and training session was not detected ($F(15,180) = 0.670$, $p = 0.811$). As shown in Fig. 2B, the control mice learned to take a shorter path to the submerged platform during the training sessions. In contrast, the scopolamine-treated mice showed little improvement over the course of training. *Post hoc* analysis confirmed that the mean path length of control mice was shorter than that of scopolamine-treated mice ($p < 0.001$). However, scopolamine-treated mice that also received 50 mg/kg/day sulforaphane performed significantly better than those that received scopolamine alone ($p = 0.020$) (Fig. 2B).

Analysis of mean search error (*i.e.*, mean distance from the escape platform during the search) revealed significant between-group effects (vehicle control, scopolamine, sulforaphane + scopolamine) ($F(3,36) = 29.748$, $p < 0.001$) and training effects ($F(5,180) = 11.942$, $p < 0.001$). However, a significant interaction between treatment and training session was not detected ($F(15,180) = 0.615$, $p = 0.860$). As shown in Fig. 2C, the control mice showed improved search error during the training sessions; however, scopolamine-treated mice showed little improvement over the course of training. *Post hoc* analysis confirmed that the mean search error of the control mice was significantly lower than that of scopolamine-treated mice ($p < 0.001$). However, scopolamine-treated mice that also received sulforaphane (10 or 50 mg/kg/day) performed significantly better than those that received scopolamine alone ($p = 0.030$ and $p = 0.048$, respectively) (Fig. 2C).

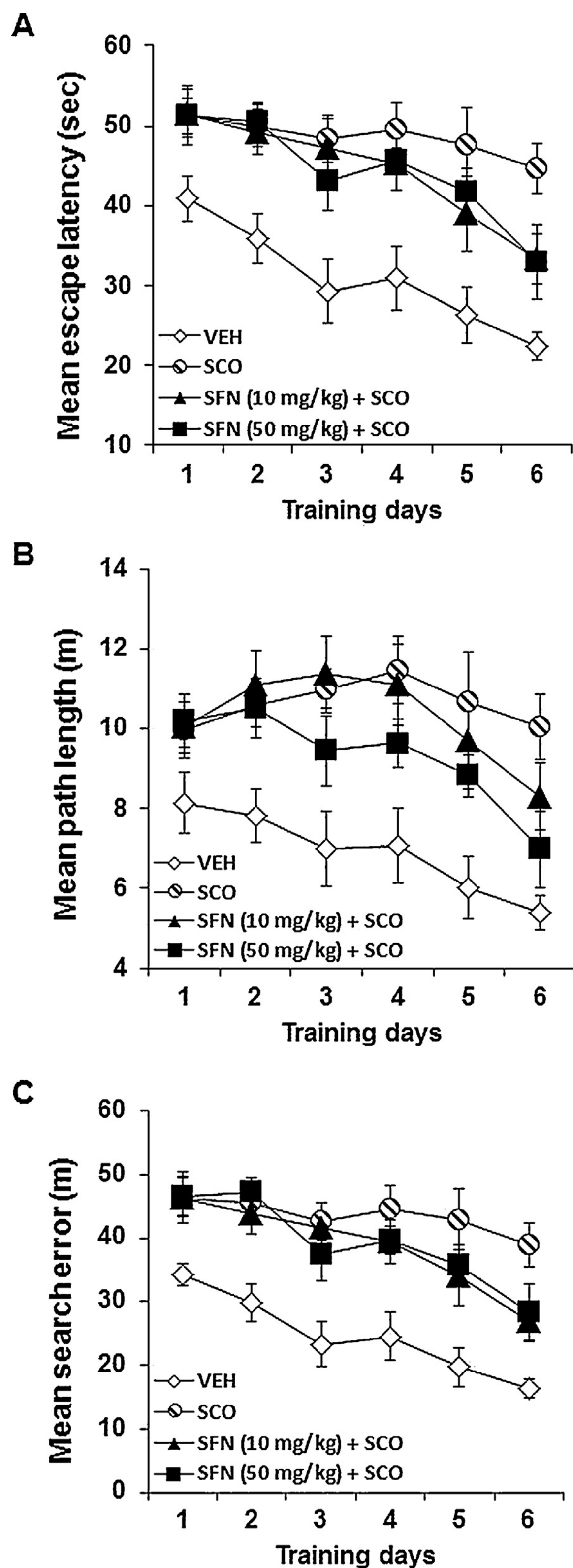

Fig. 2. Effect of sulforaphane on scopolamine-induced memory impairment during the training period for the Morris water maze test. Sulforaphane (SFN, 10 or 50 mg/kg, p.o.) was administered prior to the training trials for the Morris water maze test (days 1–7) and continued on days 8–14, during which the Morris water maze test was carried out. Memory impairment was induced by scopolamine (SCO, 1 mg/kg, administered by i.p. injection days 8–14). During the training period (days 8–13), we evaluated (A) mean escape latency, (B) mean path length, and (C) mean search error. (♦) Vehicle (VEH) control; (▨) SCO; (▲) SFN (10 mg/kg) + SCO; (■) SFN/(50 mg/kg) + SCO.

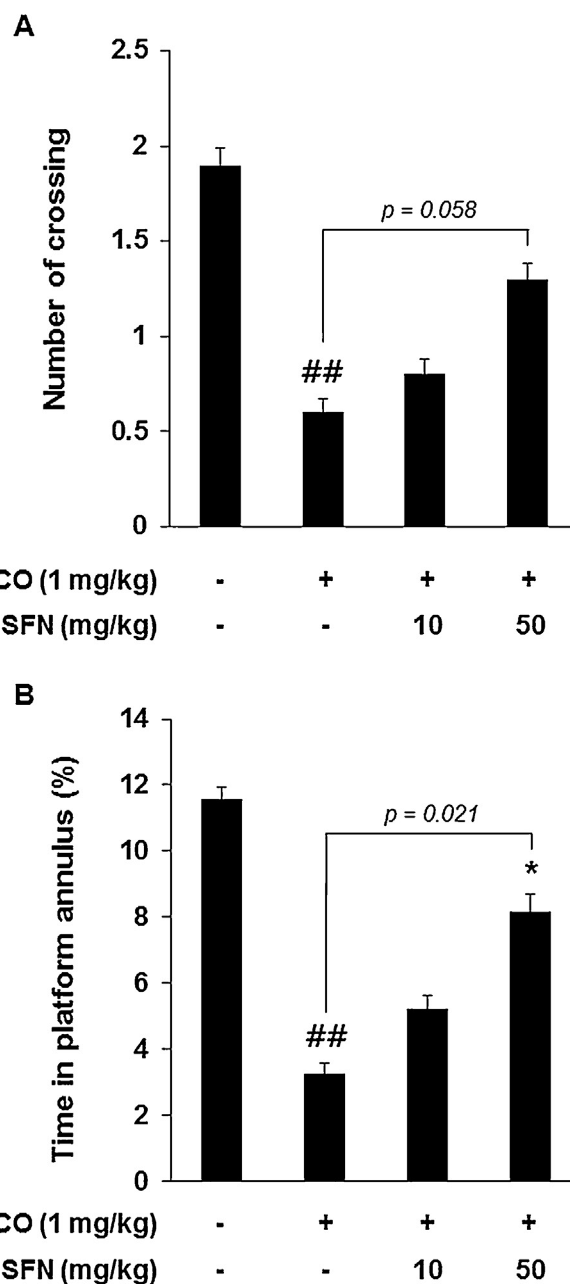

Fig. 3. Effect of sulforaphane on scopolamine-induced memory impairment, as assessed by the Morris water maze spatial probe test. On day 14, the platform was removed from its previous location, and the mice underwent the spatial probe trial, which evaluated the (A) number of crossings and (B) time in platform annulus. Data are presented as mean ± SEM ($n = 10$). $^{\#\#}p < 0.01$ compared with vehicle control; $^{*}p < 0.05$ compared with scopolamine alone.

On day 14 (24 h after the final training session), the mice underwent the spatial probe test to determine whether they remembered the platform location. The mean number of crossings (*i.e.*, number of times the previous platform location was crossed during the search) was significantly lower for the scopolamine-treated mice than for the control mice (Fig. 3A). However, the scopolamine-treated mice that also received sulforaphane (50 mg/kg/day) crossed the platform location more often than those that received scopolamine alone ($p = 0.058$) (Fig. 3A). Similarly, mean time in platform annulus (*i.e.*, percentage of time spent in a 2× concentric area surrounding the platform location) was significantly lower for the scopolamine-treated mice than the vehicle control mice (Fig. 3B). However, the scopolamine-treated mice that also received sulforaphane

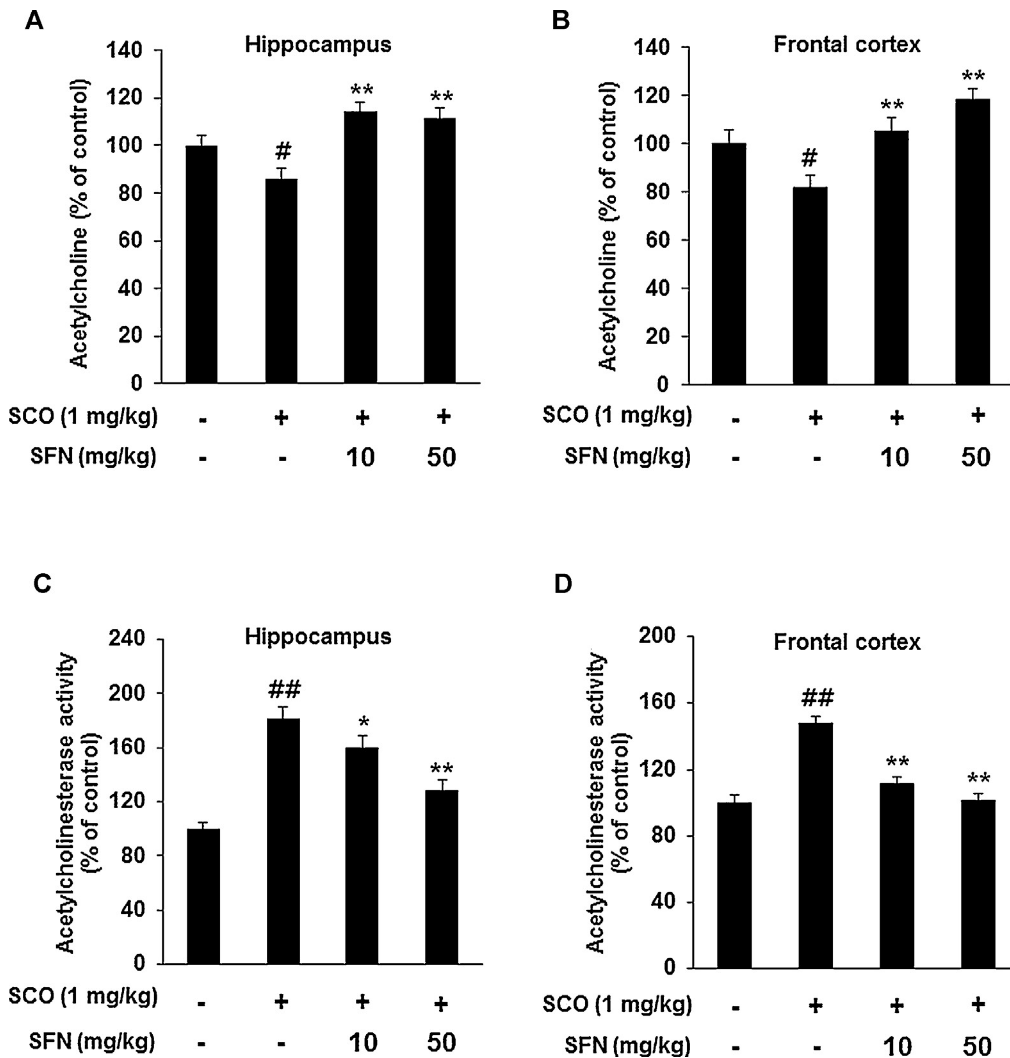

Fig. 4. Effect of sulforaphane on scopolamine-induced reduction in acetylcholine (ACh) level and increase in acetylcholinesterase (AChE) activity in the hippocampus and frontal cortex. Mice were decapitated immediately after the Morris water maze test, and the hippocampus and frontal cortex were removed to assay ACh level and AChE activity using Amplex red Ach/AChE assay kit as described in the methods section. (A) ACh level in the hippocampus. (B) ACh level in the frontal cortex. (C) AChE activity in the hippocampus. (D) AChE activity in the frontal cortex. Data are presented as mean ± SEM ($n = 10$). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with vehicle control; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with scopolamine alone.

(50 mg/kg/day) spent significantly more time in the platform annulus than those that received scopolamine alone (Fig. 3B).

3.2. Sulforaphane attenuates the scopolamine-induced decrease in ACh level and increase in AChE activity in the hippocampus and frontal cortex

Analysis of the hippocampus and frontal cortex showed that ACh levels were significantly lower in the scopolamine-treated mice than in the control mice (Fig. 4A and B). However, scopolamine-treated mice that also received sulforaphane (10 or 50 mg/kg/day) had significantly higher ACh levels than those that received scopolamine alone (Fig. 4A and B). Similarly, AChE activity was significantly higher in scopolamine-treated mice than in control mice (Fig. 4C and D), but scopolamine-treated mice that also received sulforaphane (10 or 50 mg/kg/day) had significantly lower AChE activity than those that received scopolamine alone (Fig. 4C and D).

3.3. Sulforaphane increases ChAT expression in the hippocampus and frontal cortex

The effect of sulforaphane on ChAT expression was investigated in the hippocampus and frontal cortex. Although ChAT protein and mRNA levels in the hippocampus did not differ significantly between scopolamine-treated mice and vehicle control mice (Fig. 5A–D), hippocampal ChAT protein and mRNA levels of scopolamine-treated mice that also received sulforaphane were significantly higher than ChAT levels of mice that received scopolamine alone (Fig. 5A–D). In the frontal cortex, ChAT protein and mRNA levels of scopolamine-treated mice were significantly lower than that of vehicle control mice (Fig. 5E–H). Furthermore, scopolamine-treated mice that also received sulforaphane had significantly higher ChAT protein and mRNA levels in the frontal cortex compared with mice that received scopolamine alone (Fig. 5E–H).

3.4. Sulforaphane attenuates the decrease in ACh level and increase in AChE activity and ChAT expression induced by scopolamine in primary cortical neurons

The effects of sulforaphane on ACh level, AChE activity, and ChAT expression were then determined in primary cortical neurons, which fully develop morphologically and are completely functional in culture [19]. Our results showed that sulforaphane (10 or 20 μM) significantly attenuated the decrease in ACh level (Fig. 6A) and increase in AChE activity induced by 20 μM scopolamine in

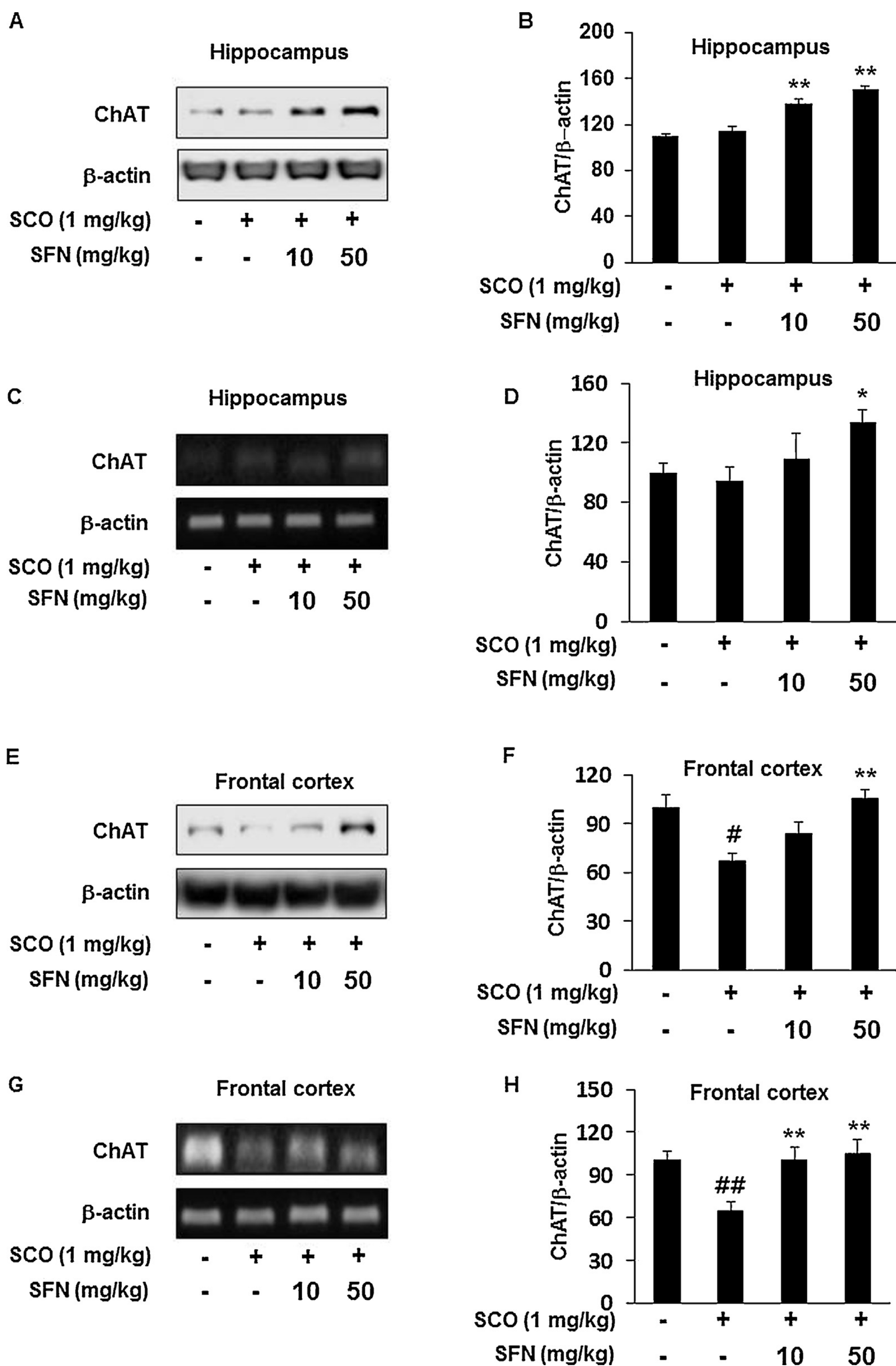

Fig. 5. Effect of sulforaphane on choline acetyltransferase (ChAT) expression in the hippocampus and frontal cortex. Mice were decapitated immediately after the Morris water maze test, and the hippocampus and frontal cortex were removed to determine ChAT expression. (A) Representative Western blot gel and (B) densitometry analysis of ChAT protein level in the hippocampus. (C) Representative RT-PCR gel and (D) densitometry analysis of ChAT mRNA level in the hippocampus. (E) Representative Western blot representative gel and (F) densitometry analysis of ChAT protein level in the frontal cortex. (G) Representative RT-PCR gel and (H) densitometry analysis of ChAT mRNA level in the frontal cortex. β-actin was used as a loading control. Data are presented as mean ± SEM ($n = 10$). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with vehicle control; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with scopolamine alone.

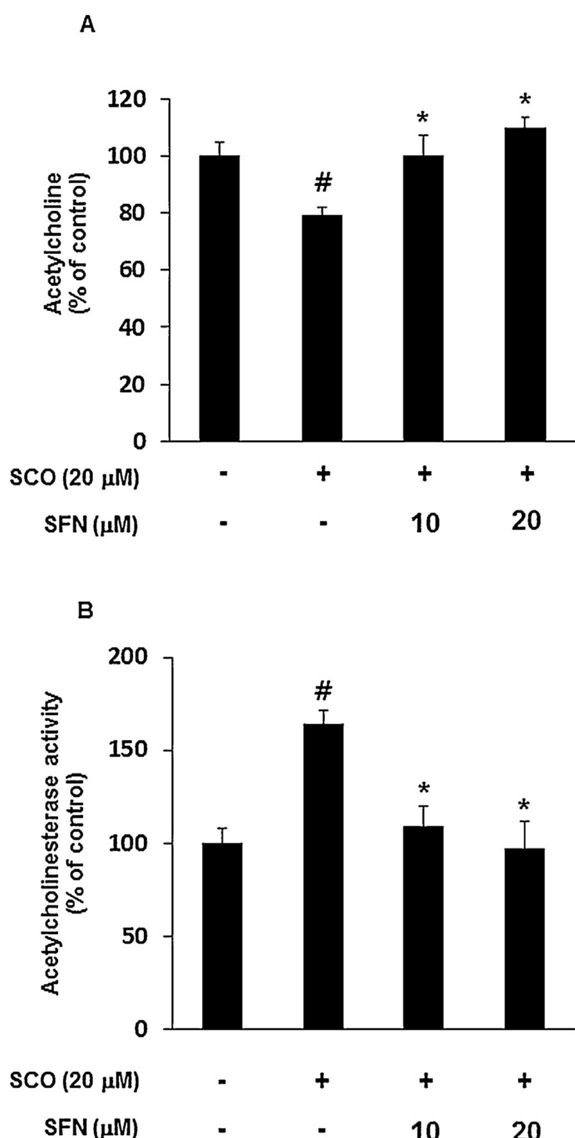

Fig. 6. Effect of sulforaphane on scopolamine-induced reduction in acetylcholine (ACh) level and increase in acetylcholinesterase (AChE) activity in primary cortical neurons. The neurons were treated with or without sulforaphane (SFN, 10 or 20 μM) for 1 h, followed by scopolamine treatment (SCO, 20 μM) for 24 h. (A) ACh level in cortical neurons. (B) AChE activity in cortical neurons. Data are presented as mean ± SEM ($n = 3$). #$p < 0.05$ compared with vehicle control; *$p < 0.05$ compared with scopolamine alone.

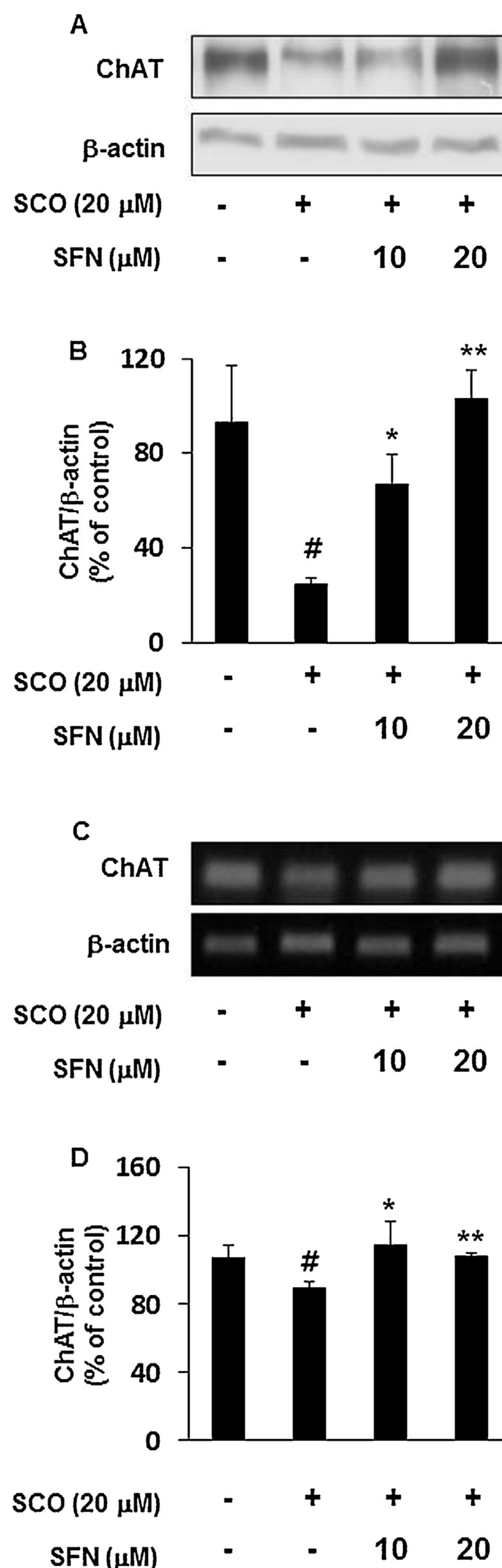

Fig. 7. Effect of sulforaphane on choline acetyltransferase (ChAT) expression in primary cortical neurons. (A) Representative Western blot gel and (B) densitometry analysis of ChAT protein level after treating neurons with or without sulforaphane (SFN, 10 or 20 μM) for 1 h, followed by scopolamine (SCO, 20 μM) for 12 h. (C) Representative RT-PCR representative gel and (D) densitometry analysis of ChAT mRNA level after treating neurons with or without sulforaphane (SFN, 10 or 20 μM) for 1 h, followed by scopolamine (SCO, 20 μM) for 6 h. β-actin was used as a loading control. Data are presented as mean ± SEM ($n = 3$). #$p < 0.05$ compared with vehicle control; *$p < 0.05$ and **$p < 0.01$ compared with scopolamine alone.

cortical neurons (Fig. 6B). Similarly, sulforaphane (10 or 20 μM) significantly attenuated the scopolamine-induced decrease in ChAT protein level (Fig. 7A and B) and ChAT mRNA level (Fig. 7C and D).

3.5. Sulforaphane inhibits AChE activity in cell-free condition

Cell-free effect of sulforaphane on AChE activity was measured. In cell-free condition, sulforaphane (0–1280 μM) inhibited AChE activity in a dose-dependent manner (Fig. 8). Sulforaphane inhibited AChE activity with an IC_{50} 36.86 μM. This observation suggests that sulforaphane directly interacts with AChE and inhibits its activity.

4. Discussion

We used scopolamine to investigate the effects of sulforaphane in the cholinergic system because it has been shown to induce

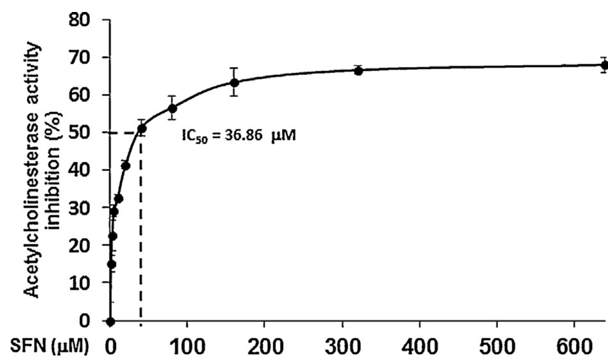

Fig. 8. Cell-free effect of sulforaphane on acetylcholinesterase (AChE) activity. Percentage of AChE activity inhibition by sulforaphane (SFN, 0, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320, or 640 μM). Data are presented as mean ± SEM ($n = 6$). The IC_{50} value was calculated by means of regression analysis.

ACh depletion in the mouse hippocampus and frontal cortex [18]. Reduced cholinergic transmission may be responsible, at least in part, for the cognitive deficits in models of scopolamine-induced amnesia [20,21]. The present study demonstrated that sulforaphane is able to protect mice from scopolamine-induced learning and memory impairment as assessed by the Morris water maze test. In addition, sulforaphane prevented the decrease in ACh level caused by scopolamine-induced upregulation of AChE activity and downregulation of ChAT expression in the hippocampus and frontal cortex. In scopolamine-treated primary cortical neurons, sulforaphane also increased ChAT and ACh levels and reduced AChE activity (Fig. 9).

Because ACh is an essential neurotransmitter in learning and memory processes, strategies to enhance ACh level can improve these abilities [22]. ACh is unique among the classical neurotransmitters because its synaptic action is terminated by enzymatic hydrolysis of AChE [23]; thus, the turnover rate of ACh is much higher than that of other neurotransmitters [23], and ACh needs to be continuously resynthesized by ChAT. A previous study reported that transgenic mice expressing the human *ChAT* gene exhibited

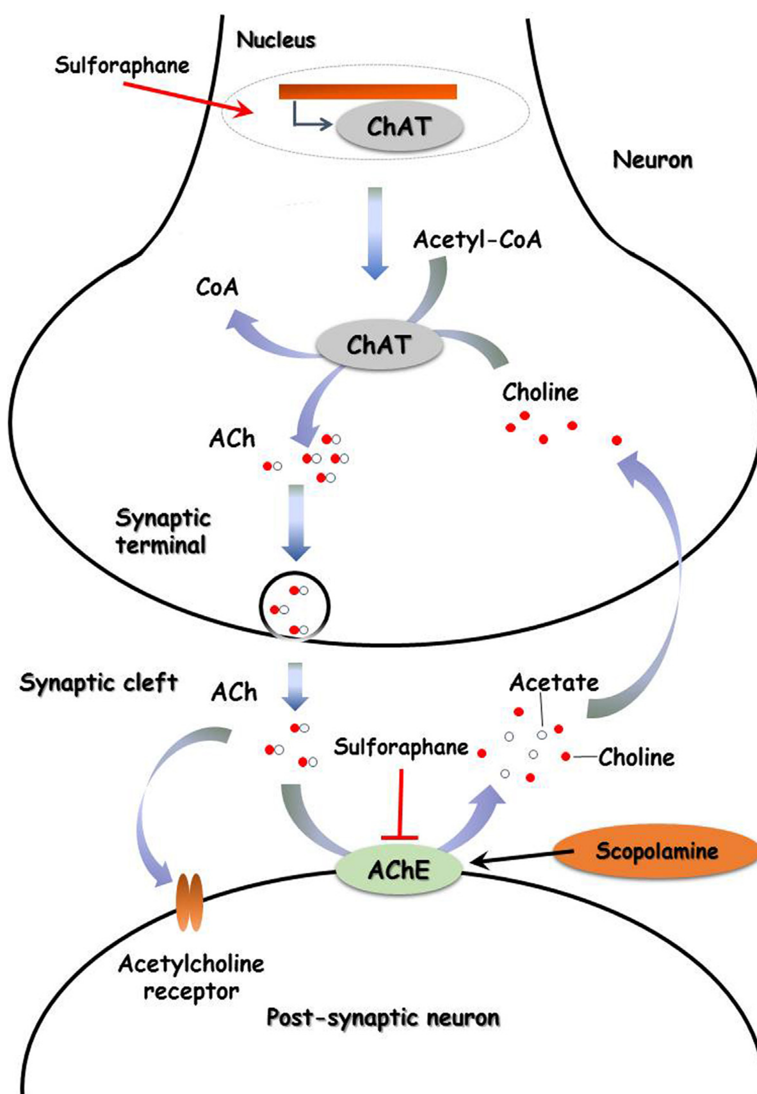

Fig. 9. Proposed model for sulforaphane-mediated alleviation of memory impairment induced by scopolamine. In cholinergic neurons, sulforaphane increases acetylcholine (ACh) levels by stimulating choline acetyltransferase (ChAT) expression. ACh is synthesized by ChAT from acetyl-CoA and choline, and released by the cholinergic nerve terminal into the secretory synaptic cleft. Receptors in postsynaptic neurons are activated by ACh, thereby promoting learning and memory retention. Sulforaphane also directly interferes and inhibits the activity of acetylcholinesterase (AChE), which hydrolyzes ACh to acetate and choline. The increased level of ACh may be responsible, at least in part, for ability of sulforaphane to alleviate scopolamine-induced memory impairment.

decreased learning and memory deterioration as they aged [24]. Exogenous ChAT can effectively increase ACh levels, thereby improving cognitive function [25,26]. These findings indicate that, when taken together, the improvement in learning and memory in mice treated with sulforaphane was likely due to the prevention of scopolamine-induced dysregulation of ACh level, AChE activity, and ChAT expression.

Many natural products including alkaloids, coumarins, flavonoids, and stilbenes, have been reported as AChE inhibitors and suggested as sources of new lead compounds for the treatment of the Alzheimer's disease symptoms [27]. We found that cell-free sulforaphane (IC_{50} = 36.86 μM) exhibits the inhibitory activity on AChE, suggesting that sulforaphane directly interacts with and inhibits AChE activity in cells and tissues. Previous studies have shown that ChAT activity and/or expression can be up-regulated by a variety of extracellular signals, including leukemia inhibitory factor [28], ciliary neurotrophic factor [29,30], and brain-derived neurotrophic factor [31,32]. Our group recently revealed that sulforaphane increases the expression of BDNF *in vitro* and *in vivo* (data no shown), sulforaphane-mediated BDNF expression might contribute to increase ChAT expression. The results we observed on AChE inhibition and ChAT expression suggest that sulforaphane might be a promising natural product to improve the cholinergic system and cognitive function.

Recent advances have shown the neuroprotective activity of sulforaphane in various experimental models of brain injury and neurodegeneration. Sulforaphane protected memory and cognitive function in a traumatic brain injury animal model as assessed by the Morris water maze test [33]. When administered following traumatic brain injury, sulforaphane attenuated blood–brain barrier permeability and reduced cerebral edema [33]. Sulforaphane also protected cognitive function in amyloid β-induced Alzheimer's disease acute mouse models, as assessed by the Y-maze and passive avoidance behavior tests [34]. In mice treated with sulforaphane after the unilateral intrastriatal injection of 6-hydroxydopamine, the increase in 6-hydroxydopamine-induced rotations and deficits in motor coordination were ameliorated significantly by sulforaphane treatment, suggesting that sulforaphane has neuroprotective activity in mouse model of Parkinson's disease [35]. In most cases, the neuroprotective effects of sulforaphane were accompanied by activation of the transcription factor NF-E2-related factor 2 (Nrf2) and up-regulation of its detoxifying and antioxidant target genes [2]. However, our observation using Nrf2 knockout primary cortical neurons found that sulforaphane significantly increases ChAT expression both in Nrf2 knockout and wild-type neurons (data not shown), suggesting that the neuroprotective effects of sulforaphane on the cholinergic system were not accompanied by activation of Nrf2.

Although the distribution of sulforaphane throughout the body has been investigated *in vitro* and in animal and human studies, the brain bioavailability of sulforaphane is unclear. Pharmacokinetic studies in both rats and humans have reported only that sulforaphane can reach micromolar concentrations in the blood [2,36,37]. After administration of sulforaphane (50 μmol) by oral gavage, plasma levels of sulforaphane were detectable after 1 h and peaked at ~20 μmol/l at 4 h in rats, with a half-life of ~2.2 h [37]. It has been reported that the important parameters determining free diffusion across the blood–brain barrier are molecular weight of the drug and H-bonding based on the drug chemical structure [38]. Since the molecular weight of sulforaphane is <400 Da and forms less than eight H-bonds, sulforaphane is probably a brain-penetrating molecule, which is an important area for future research.

In summary, our study showed that sulforaphane alleviates scopolamine-induced memory impairment. Given that pretreatment with sulforaphane counteracted the scopolamine-induced decrease in cholinergic activity, modulation of ACh, AChE, and ChAT may be the mode of action underlying the improved memory and learning ability. Together with previously reported neuropharmacological activity of sulforaphane, this study has provided the first evidence that sulforaphane enhances the cholinergic system, thereby improving learning and memory. Our observations suggest sulforaphane as a promising natural product for the future prevention of Alzheimer's disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2014.05.003.

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