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The antioxidants α-lipoic acid and *N*-acetylcysteine reverse memory impairment and brain oxidative stress in aged SAMP8 mice

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Abstract

Oxidative stress may play a crucial role in age-related neurodegenerative disorders. Here, we examined the ability of two antioxidants, α-lipoic acid (LA) and *N*-acetylcysteine (NAC), to reverse the cognitive deficits found in the SAMP8 mouse. By 12 months of age, this strain develops elevated levels of Aβ and severe deficits in learning and memory. We found that 12-month-old SAMP8 mice, in comparison with 4-month-old mice, had increased levels of protein carbonyls (an index of protein oxidation), increased TBARS (an index of lipid peroxidation) and a decrease in the weakly immobilized/strongly immobilized (W/S) ratio of the protein-specific spin label MAL-6 (an index of oxidation-induced conformational changes in synaptosomal membrane proteins). Chronic administration of either LA or NAC improved cognition of 12-month-old SAMP8 mice in both the T-maze footshock avoidance paradigm and the lever press appetitive task without inducing non-specific effects on motor activity, motivation to avoid shock, or body weight. These effects probably occurred directly within the brain, as NAC crossed the blood–brain barrier and accumulated in the brain. Furthermore, treatment of 12-month-old SAMP8 mice with LA reversed all three indexes of oxidative stress. These results support the hypothesis that oxidative stress can lead to cognitive dysfunction and provide evidence for a therapeutic role for antioxidants.

Keywords: *N*-acetylcysteine, blood–brain barrier, learning, α-lipoic acid, oxidative stress, SAMP8.

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Free radical damage from oxidative stress has long been thought to play an important role in age-related neurodegenerative disorders (Harman 1995). It has been suggested that free radical damage compromises composition integrity of cell membranes, which decreases membrane fluidity (Zs-Nagy 1990). Although the specific mechanism for free radical generation and consequent oxidative stress differ between normal aging and neurodegenerative diseases, a consensus is emerging that free radical processes do play an important role in the etiology of many disorders (Hensley *et al*. 1995a; Butterfield and Stadtman 1997; Butterfield *et al*. 2001a; Butterfield and Lauderback 2002). Free radical-mediated damage to neuronal membrane components has been implicated in the etiology of diseases of aging such as Alzheimer's disease (AD). For example, the molecular basis of AD is unclear, but numerous lines of genetic and biochemical evidence suggest that a 39–43 amino acid peptide, amyloid β peptide (Aβ) that is the principal component of senile plaques in the AD brain, is central to the pathogenesis of AD (Butterfield *et al*. 2001a; Butterfield *et al*. 2001b; Butterfield and Lauderback 2002). Aβ is

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Abbreviations used: Aβ, amyloid β peptide; AD, Alzheimer's disease; BBB, blood–brain barrier; DNP, 2,4-dinitrophenyl hydrazone; FFA, free fatty acids; LA, α-lipoic acid; NAC, *N*-acetylcysteine; SDS, sodium dodecyl sulfate; W/S, weakly immobilized/strongly immobilized.

involved in free-radical formation that induces damage to neurons *in vitro* (Varadarajan *et al*. 2001; Yatin *et al*. 1999; Varadarajan *et al*. 2000; Kanski *et al*. 2002).

Oxidative stress can probably result in cognitive impairments. Antioxidants have been found to both prevent and reverse learning and memory deficits induced by free radicals (Bickford *et al*. 2000; Kastin *et al*. 1979; Introini *et al*. 1985; Shih *et al*. 1986; Jankovic *et al*. 1990; Guerrero *et al*. 1999; Abe and Saito 2000; Emilien *et al*. 2000; Rivas-Arancibia *et al*. 2000). α-Lipoic acid (LA) and *N*-acetylcysteine (NAC) are two antioxidants used to combat oxidative stress-induced damage (Nagamatsu *et al*. 1995; Yao *et al*. 1989; Maziere *et al*. 1999). Studies indicate that both LA and NAC protect against oxidative stress in peripheral tissues and in the central nervous system (Maziere *et al*. 1999; Yehuda and Youdim 1981; Drust and Crawford 1983; Greeley *et al*. 1989; Stoll *et al*. 1993; Packer *et al*. 1997; Martinez *et al*. 2000; Pocernich *et al*. 2002). In addition, both compounds have been found to reverse age-related impairments in memory (Martinez *et al*. 2000; Yehuda and Youdim 1981; Stoll *et al*. 1993) and LA prevents the increase in lipid peroxidase levels that occurs with age (Arivazhagan *et al*. 2000).

The SAMP8 strain of mice develops deficits in learning and memory by 12 months of age (Woods and Porte. 1983; Yagi *et al*. 1988; Flood and Morley 1998). Such deficits are not seen in other strains at this age. SAMP8 mice have elevated levels of Aβ caused by the overexpression of amyloid precursor protein (APP) (Morley *et al*. 2000; Cserr 1984; Kumar *et al*. 2000; Morley *et al*. 2002). Decreasing Aβ with antibody or antisense in 12-month-old SAMP8 mice improves learning and memory (Van Bree *et al*. 1990; Kumar *et al*. 2000; Morley *et al*. 2000). In addition, SAMP8 mice have been found to have increased free radical production in the central nervous system (Sato *et al*. 1996; Butterfield *et al*. 1997) associated with mitochondrial dysfunction (Fujibayashi *et al*. 1998). In the current study, we examined the effects of two potent antioxidants, LA and NAC, on acquisition in the T-maze footshock avoidance task and lever press appetitive task, the ability of NAC to cross the blood–brain barrier (BBB), and the ability of LA to reverse markers of oxidative stress.

Materials and methods

Subjects

Experimentally naive, 4- and 12-month-old male SAMP8 mice were obtained from our breeding colony. The colony is derived from siblings generously provided by Dr Takeda of (Kyoto University, Japan), and has been maintained as an inbred strain for 12 years under clean-room procedures (i.e. use of sterile gloves in handling mice, sterilized cages and bedding, restricted access to breeding area), and housed in microisolator HEPA filter units (Allentown Caging, PA, USA). The colony routinely undergoes serological testing for viral and bacterial contamination and has remained free of pathogens for over 5 years. Mice are housed in rooms with a 12 : 12 light/dark cycle (lights on at 06.00 h) at 20–22°C with water and food (Richmond Laboratory Rodent Diet 5001) available *ad libitum*. All experiments were conducted after institutional approval of the animal use subcommittee, which subscribes to the NIH Guide for Care and Use of Laboratory Animals.

Drug administration

LA and NAC were a gift from Jarrow Formulas, Inc. (Los Angeles, CA, USA). All drugs were dissolved in saline at pH 7.0. LA (100 mg/kg), NAC (100 mg/kg), or saline were administered subcutaneously once daily for 4 weeks at 1 PM. At the end of week one, animals were tested in the T-maze footshock avoidance paradigm. The following day, activity was tested in an open field and a 1-week habituation to milk was begun. At the end of the 1-week habituation, mice were trained in the lever press. Behavioral measures and training were conducted between 07.30 and 11.30 h.

T-maze training

Training procedures for the T-maze footshock avoidance apparatus have been described (Farr *et al*. 1999, 2000). The maze consisted of a black plastic start alley with a start box at one end and two goal boxes at the other. A stainless steel rod floor ran throughout the maze. The start box was separated from the start alley by a plastic guillotine door that prevented the mouse from moving down the alley until the training started.

A training trial began when a mouse was placed into the start box. The guillotine door was raised and the buzzer was sounded simultaneously; 5 s later, footshock was applied. The goal box the mouse first entered on the first trial was designated as 'incorrect'. Footshock was continued until the mouse entered the other goal box, which on all subsequent trials was designated 'correct' for the particular mouse. At the end of each trial, the mouse was removed from the goal box and returned to its home cage. A new trial began by placing the mouse back in the start box, sounding the buzzer, and raising the guillotine door. Footshock was applied 5 s later if the mouse did not leave the start box or failed to enter the correct goal box.

Training used an intertrial interval of 45 s and a door-bell type buzzer at 65 dB as the conditioned stimulus warning of onset of foot shock at 0.40 mA (Coulbourn Instruments scrambled grid floor shocker model E13-08). Mice were trained until they made one avoidance.

Open field

In order to eliminate the possibility that differences in acquisition were produced by changes in activity in treated mice, we examined activity in an open field. Mice were given one trial each in which they were allowed to explore freely in an open field for 15 min to determine their activity level. The open field apparatus consisted of a circular field 45 cm in diameter with sides that were 30 cm high. A testing session started with the entrance of the mouse into the side of the field facing the wall. The distances each mouse traveled during the single 15 min session were recorded in centimeters using a Polytrak recording system (San Diego Instruments).

Habituation to milk solution

Appetitive tasks used a solution of one part evaporated milk and two parts water. Mice were initially habituated to this novel food by allowing them access to it in their home cages overnight. During

three nights of habituation, food and water were removed to encourage drinking of the milk solution. After the third session, mice not consuming at least 20 mL of milk solution were excluded (less than 10% of the population). In appetitive training tasks, mice were run after overnight food and water deprivation.

Acquisition of lever press for milk reinforcement

Mice were placed into a fully automated lever press chamber. Pressing a lever on one wall of the compartment caused a light and liquid dipper with 100 μL of milk to appear on the opposite wall. On days 1 and 2, mice had 11 s to obtain the reward; on days 3 and 4, mice had 7 s to obtain the reward; on all subsequent days, mice had 4 s to obtain a reward. Mice were given a 30-min training session each day until all groups in the particular study received an average of 100 rewards in a 30-min session. The measure of acquisition was the number of reinforced lever presses.

Blood–brain barrier studies

[^{14}C]*N*-Acetylcysteine (C-NAC) with a specific activity of 55 mCi/mmol was purchased from ICN Biomedicals, Inc. (Irvine, CA, USA). Bovine serum albumin was labeled with ^{99m}Tc (T-Alb) by adding 0.12 mg of stannous tartrate and 1 mg of bovine serum albumin to 1-mL distilled water. The pH was adjusted to 2.5–3.3 with 0.2 M HCl. The mixture was incubated at room temperature (22°C) for 20 min before use.

Measurement of influx into brain

The unidirectional influx rate (K_i) from blood to brain was determined in mice by multiple-time regression analysis (Blasberg *et al*. 1983; Patlak *et al*. 1983; Kastin *et al*. 2001). Male ICR mice weighing 20–25 g were anesthetized with urethane and the left jugular vein and right carotid artery exposed. Mice received an injection into the jugular vein of 0.2 mL of lactated Ringer's solution with 1% bovine serum albumin and 2 (10^6) cpm of (C-NAC). Arterial blood was collected at 2, 3, 5, 7.5, 10, 15 or 20 min after intravenous injection through a cut in the carotid artery. Mice were immediately decapitated after collection of arterial blood and the whole brain removed. Whole blood was centrifuged at 5000 *g* at 4°C for 10 min and 50 μL removed. The levels of radioactivity in serum and brain were determined in a beta counter. The K_i, expressed in μL/g/min, and the apparent volume of distribution (V_i, in μL/g) was determined from the equation

$$Am/Cpt = K_i\left[\int_0^t Cp(T)\mathrm{d}T\right]/Cpt + V_i$$

where *Am* is cpm/g of brain, *Cpt* is cpm/μL of arterial serum at time *t*, and T is the dummy variable for time. *Am*/*Cpt* is the brain/blood ratio in μL/g and exposure time is measured by the term [ι_o^t *Cp*(T) dT]/*Cpt*. Only the linear portion of the relation between *Am*/*Cpt* versus exposure time is used to compute K_i and V_i. The percentage of the intravenously injected dose taken up per gram of brain was determined from the equation:

$$\%\,Inj/g = 10^{-3}(Am/Cpt)(\%\,Inj/\mathrm{mL}).$$

Capillary depletion

Mice received intravenous injections of 6.6 (10^6) cpm of (C-NAC) and 10^6 cpm of T-Alb. Ten minutes after intravenous injection, the vascular space of the brain was washed free of blood. This washout was preceded by opening the abdomen and taking an arterial blood sample from a cut in the abdominal aorta. The jugular veins were than severed, the thorax opened, and 20 mL of lactated Ringer's solution perfused through the left ventricle of the heart while the descending thoracic aorta was occluded. Washout took less than 1 min. After washout, the mouse was immediately decapitated and the cerebral cortex removed, weighed, and homogenized with a glass homogenizer (10 strokes) in 0.8 mL of physiological buffer (10 mM Hepes, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4 and 10 mM D-glucose adjusted to pH 7.4). Dextran solution (1.6 mL of a 26% solution) was added to the homogenate, which was vortexed and homogenized again (three strokes). Homogenization was performed on ice before centrifuging at 5400 *g* for 15 min at 4°C in a Beckman Allegra 21R centrifuge with a swinging bucket rotor (Fullenton, CA, USA). The pellet containing brain capillaries and the supernatant representing the brain parenchymal/brain interstitial fluid space were carefully separated and the levels of radioactivity determined in gamma and beta counters for ^{99m}Tc and ^{14}C, respectively. Levels of ^{14}C and ^{99m}Tc were also measured in serum. Levels of ^{14}C were determined after degradation of ^{99m}Tc to non-detectable levels. The fractions were expressed as volumes of distribution (μL/g).

Oxidative stress measures

SAMP8 brain samples were flash frozen in liquid nitrogen in St. Louis and sent to Lexington on wet ice overnight. The samples were homogenized in 0.32 M sucrose isolation buffer (2 mM EDTA, 2 mM EGTA, 20 mM Hepes, 20 μg/mL trypsin inhibitor, 4 μg/mL leupeptin, 4 μg/mL pepstatin, 5 μg/mL aprotinin) by sonication and the protein concentration was determined by the BCA method. Three indices of oxidative stress were used: (i) protein carbonyl levels, which index protein oxidation (Butterfield and Stadtman 1997); (ii) the weakly immobilized/strongly immobilized (W/S) ratio of the protein-specific spin label MAL-6, which when bound to synaptosomal membrane proteins yields a lower value of the W/S ratio, indexing oxidative stress-induced alterations in membrane protein conformation (Hensley *et al*. 1994; Butterfield and Stadtman 1997); and (iii) thiobarbituric acid reactive substance (TBARS), an index of lipid peroxidation. Protein carbonyl levels have been shown to be increased in aging (Butterfield *et al*. 1997; Hensley *et al*. 1994; Butterfield and Stadtman 1997). The W/S ratio is an EPR parameter reflective of protein–protein interactions, which decreases in oxidative stress (Hensley *et al*. 1994). The W/S ratio is the ratio of intensity of the $M_I = +1$ low-field weakly immobilized line and $M_I = +1$ low-field strongly immobilized resonance line of a protein-specific spin label, MAL-6 (Butterfield 1982). TBARS provide a measure of lipid peroxidation damage (Ohkawa *et al*. 1979) that was shown to be involved in aging (Tappel 1968; Harman 1969) because of the high reactivity of thiobarbituric acid with the lipid peroxidation end product, malondialdehyde (Ohkawa *et al*. 1979).

Carbonyl level

Protein carbonyl levels of proteins were determined immunochemically as adducts of 2,4-dinitrophenylhydrazine (Oliver *et al*. 1987). Five microliters of the samples were treated with an equal volume of 12% sodium dodecyl sulfate (SDS). Samples were then derivatized

with 10 μL of 20 mM 2,4-DNPH for 20 min. The reaction was stopped by addition of neutralizing reagent (7.5 μL of 2 M Tris/30% glycerol buffer, pH = 8.0). Levels of protein carbonyls were measured by using the slot-blot technique with 250 ng of protein loaded per slot. The 2,4-dinitrophenyl hydrazone (DNP) adduct of the carbonyls is detected on the nitrocellulose paper using a primary rabbit antibody (Intergen) specific for DNP-protein adducts (1 : 100) and then a secondary goat anti-rabbit IgG (Sigma) antibody. The resulting stain was developed by SCION-IMAGE software package.

TBARS

The concentration of TBARS in brain tissue was determined according to the method of Ohkawa *et al*. (1979). Fifty milliliters of 10% w/v of ice cold trichloroacetic acid was added into 0.25 mL of 4 mg/mL tissue homogenate. The samples were spun in an Eppendorf centrifuge tube for 5 min at 3000 *g*. The supernatant was collected (0.5 mL) and treated with TBA reagent (20 mM TBA in 50% v/v glacial acetic acid). The samples were then heated at 100°C for 1 h. After the cooling period, butanol was added, and the organic layer was removed and redistributed to a black microtiter plate (Corning Inc, Acton, MA, USA). End point fluorescence was measured at $\lambda_{ex} = 515$ nm and $\lambda_{em} = 585$ nm.

W/S ratio

Synaptosomes were purified as described (Butterfield *et al*. 1994; Hensley *et al*. 1994; Hensley *et al*. 1995b). The crude homogenate was removed and respun at 20 000 *g* at 4°C for 10 min. The resulting pellet was resuspended in the 0.32 M sucrose isolation buffer and layered on a discontinuous sucrose gradient (10 mL of 1.18 M sucrose, pH 8.5/10 mL of 1.0 M sucrose, pH 7.4/10 mL of 0.85 M sucrose, pH 7.4, each containing 2 mM EDTA/2 mM EGTA/10 mM Hepes). The samples were then spun at 82 500 *g* at 4°C for 60 min in a Beckman swinging-bucket rotor. Synaptosomes were removed from the 1.18/1.0 M sucrose interface and resuspended in 20 mL of lysing buffer (10 mM Hepes/2 mM EDTA/2 mM EGTA, pH 7.4). The samples were then centrifuged at 32 000 *g* at 4°C for 10 min. The pellet was removed and resuspended in PBS buffer and spun down twice more. After the third wash, the protein concentration was determined by the BCA method.

Spin labeling of synaptosomal membrane proteins was performed as described (Umhauer *et al*. 1992; Hensley *et al*. 1994). Isolated synaptosomes were suspended in lysing buffer for 30 min. Lysed synaptosomal membranes were labeled with the protein-specific spin label MAL-6. After incubation for 18 h at 4°C with 20 μg MAL-6/mg protein, samples were washed six times in lysing buffer to remove excess spin label. The pellet was then resuspended in approximately 400 μL lysing buffer and allowed to come to room temperature. EPR spectra were acquired on a Bruker model EMX EPR spectrometer (Bruker, Billerica, MA, USA) operating at an incident microwave power of 18 mW, a modulation amplitude of 0.4 G, a time constant of 1.28 ms, and a conversion time of 10 ms.

Statistics

For behavioral studies, results are expressed as means with their standard errors. All groups had 10 mice. The acquisition test scores for T-maze (mean trials to make first avoidance) and lever press (number of reinforced lever presses) for each group were analyzed with one-way analysis of variance (ANOVA) and a two-way analysis of variance, respectively. Latencies to escape shock, activity, food intake, and weight change were analyzed by a one-way ANOVA. The control and treatment groups were compared with Tukey's *t*-test or Tukey's HSD (Keppel and Zedeck 1989).

For BBB pharmacokinetic analysis, regression lines were calculated by the least squares method and compared statistically with the PRIZM 3.0 program (GraphPad Software, Inc, San Diego, CA, USA). Regression lines are reported with their slope, standard error of the mean, correlation coefficient (r), the number of mice per line (n), and the level of the statistical significance (p). Means are reported with their standard error and n.

Oxidative stress measures were analyzed by Student's *t*-tests. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of LA on cognition

Administration of LA improved acquisition as tested in the T-maze footshock avoidance paradigm (Fig. 1a). The ANOVA for the trials to first avoidance measure indicated a significant effect ($F_{2,27} = 34.31$, $p < 0.001$). Tukey's *t*-test *post-hoc* analysis revealed 12-month-old SAMP8 mice that had received LA took significantly fewer trials to reach criterion than the 12-month-old SAMP8 mice which received saline. The group of mice administered LA did not significantly differ from the 4-month-old SAMP8 mice administered saline. The ANOVA for latencies to escape shock on the first trial did not indicate a significant difference.

The ANOVA for activity in an open field indicated a statistically significant effect ($F_{2,27} = 11.48$, $p < 0.001$). Tukey's HSD *post-hoc* analysis indicated that the 4-month-old SAMP8 mice (1897 ± 65 cm, $n = 10$) were significantly more active in an open field than the 12-month-old SAMP8 mice which received either saline (1583 ± 42 cm, $n = 10$) or LA (1656 ± 33 cm, $n = 11$). The groups of 12-month-old SAMP8 mice receiving either saline or LA did not differ from one another.

The two-way ANOVA for the number of rewarded lever presses showed a significant effect for group ($F_{2,260} = 50.86$, $p < 0.0001$), day ($F_{9,260} = 42.80$, $p < 0.0001$), and the interaction group × day ($F_{18,260} = 4.18\, p < 0.0001$). Tukey's *post-hoc* analysis indicated that 12-month-old SAMP8 mice administered LA achieved significantly more rewards on days 7–9 compared with the mice administered saline (Fig. 1b). Four-month-old SAMP8 mice achieved significantly more rewards than either of the 12 month groups on days 7–10.

The ANOVA analyzing food intake in the mice administered LA indicated a statistically significant effect ($F_{2,27} = 3.62$, $p < 0.05$). Tukey's HSD *post-hoc* analysis indicated that the 12-month-old SAMP8 mice administered LA ate significantly more than the 4-month-old SAMP8 mice.

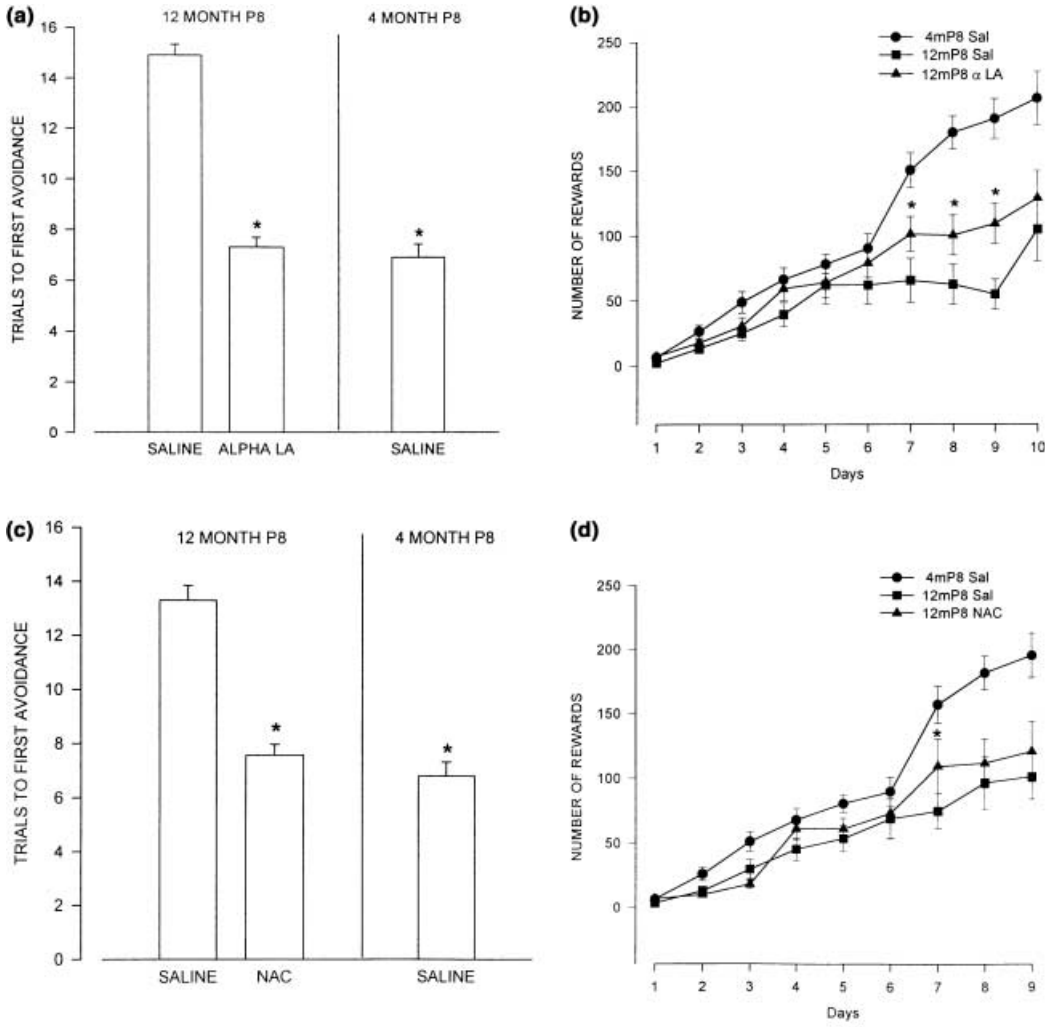

Fig. 1 Effects of LA and NAC on acquisition of T-maze footshock avoidance and lever press. (a) LA improved acquisition of T-maze footshock avoidance in 12-month-old SAMP8 mice. *Indicates that the value differs from the saline treated 12-month-old SAMP8 mice at $p < 0.01$. (b) LA improved acquisition of lever press in 12-month-old SAMP8 mice. On days 7–9 of training, 12-month-old SAMP8 mice receiving LA achieved more rewards than the day-matched 12-month-old SAMP8 mice which received saline $p < 0.01$. Four-month-old SAMP8 mice achieved more rewards than either 12-month-old SAMP8 groups on days 7–10 $p < 0.01$. *$p < 0.01$ in comparison to day-matched 12-month-old SAMP8 mice receiving saline. (c) NAC improved acquisition of T-maze footshock avoidance in 12-month-old SAMP8 mice. *Indicates that the value differs from the saline treated 12-month-old SAMP8 mice at $p < 0.01$. (d) NAC improved acquisition of lever press in 12 month SAMP8 mice. On day 7, 12-month-old SAMP8 mice receiving NAC achieved more rewards that the 12-month-old SAMP8 mice receiving saline *$p < 0.01$. Four-month-old SAMP8 mice achieved more rewards than both 12-month-old SAMP8 groups on days 7–9, $p < 0.01$.

Twelve-month-old SAMP8 mice administered saline were not significantly different from the 12-month-old SAMP8 mice administered LA nor the 4-month-old SAMP8 mice. The ANOVA for body weight change during treatment did not show a significant effect (see Table 1).

Effects of NAC on cognition

The ANOVA analyzing T-maze acquisition showed a significant effect ($F_{2,25} = 61.29$, $p < 0.001$). Tukey's *post-hoc* analysis showed that the 12-month-old SAMP8 mice which received NAC performed significantly better than the 12-month-old SAMP8 mice which received saline and were not different from the 4 month SAMP8 mice (Fig. 1c). The ANOVA for latencies to escape shock on first trial did not show a significant effect.

The ANOVA for activity showed a significant effect $F(2,26 = 4.07, p < 0.05)$. Tukey's *post-hoc* analysis showed the only difference to be that the 4-month-old SAMP8 mice administered saline (1897 ± 65, $n = 10$) were significantly more active than the 12-month-old SAMP8 mice

Table 1 Effects of antioxidants on food intake and body weight

	Average daily food intake (g)	Weight change (g)
Study 1		
12 M P8 saline	5.11 ± 0.83[a,b]	+ 0.07 ± 0.90[a]
12 M P8 α-lipoic	5.78 ± 0.76[a]	− 0.36 ± 1.22[a]
4 M P8 saline	4.93 ± 0.63[b]	− 0.54 ± 0.62[a]
Study 2		
12 M P8 saline	5.79 ± 0.84[a,b]	+ 0.40 ± 0.47[a,b]
12 M P8 *N*-acetylcysteine	6.07 ± 0.71[a]	+ 0.88 ± 0.72[a]
4 M P8 saline	4.99 ± 0.55[b]	− 0.46 ± 0.84[b]

M refers to age in months; P8 refers to SAMP8 mice. [a,b]Groups with different letters are significantly differ $p < 0.05$ for that study.

administered NAC (1668 ± 66 cm, $n = 10$). Twelve-month-old-mice given saline (1720 ± 52 cm, $n = 9$) were not different from 12-month-old mice given NAC.

The two-way ANOVA for rewarded presses in the lever press study showed a significant effect for group ($F_{2,234} = 28.76$, $p < 0.0001$), day ($F_{8,234} = 47.00$, $p < 0.0001$), and the interaction group × day ($F_{2,8} = 2.42$ $p < 0.002$). Tukey's *post-hoc* analysis indicated that 12-month-old SAMP8 mice administered NAC achieved significantly more rewards on day 7 compared with the 12-month-old SAMP8 mice administered saline (Fig. 1d). Four-month-old SAMP8 mice achieved significantly more rewards than both 12-month-old groups on the other days.

The ANOVA for food intake did not show a significant effect. The ANOVA for body weight change showed a statistically significant difference ($F_{2,27} = 9.57$, $p < 0.001$). Tukey's HSD *post-hoc* analysis indicated that the 12-month-old SAMP8 mice which received saline and NAC gained weight, whereas the 4-month-old SAMP8 mice lost weight (see Table 1).

Blood–brain barrier permeability to NAC

Figure 2(a) shows the relation between the log of levels of radioactivity in arterial serum expressed as the percentage/milliliter of injected dose (%*Inj*/mL) versus time after the intravenousinjection of C-NAC. This relation had a slope of −0.0222 and an intercept 1.051 ($n = 13$, $r = 0.908$, $p < 0.0001$). This gave a half-time disappearance from blood of 13.5 min and a volume of distribution of 8.89 mL.

Figure 2(b) shows the relation between the brain/blood ratios (*Am*/*Cpt*) and exposure time for mice which received C-NAC. This relation was statistically significant ($r = 0.955$, $n = 13$, $p < 0.0001$) with $K_i = 2.41 \pm 0.226$ μL/g-min and $V_i = 39.3 \pm 2.79$ μL/g. The percentage of the injected dose

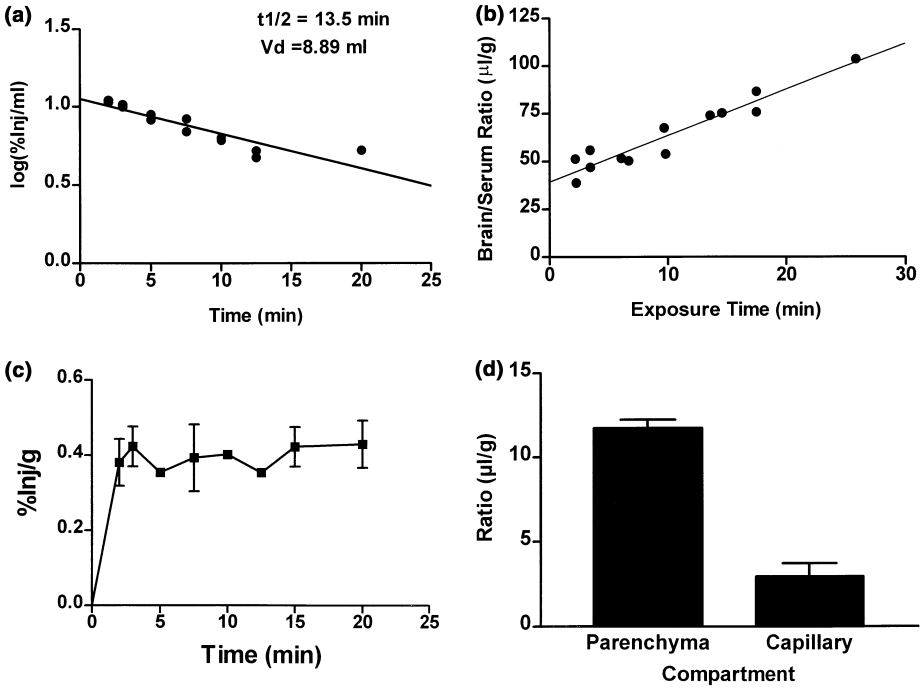

Fig. 2 (a) Clearance of NAC from blood after intravenous injection. Half-time disappearance from blood was 13.5 min and the volume of distribution was 8.89 mL. (b) Multiple-time regression analysis of NAC transport across the BBB. The unidirectional influx rate was measured to be 2.41 ± 0.226 μL/g-min. (c) The percentage of an intravenously injected dose taken up per gram of brain (%*Inj*/g) for NAC. Values were about 0.4 %*Inj*/g. (d) Capillary depletion. Results show that most of the NAC taken up by brain completely crossed the BBB to enter the paranchymal space of the brain.

taken up per gram of brain is shown in Fig. 2(c) as determined from the equation:

$$\%Inj/\mathrm{g} = 10^{-3}\,(Am/Cpt)(\%Inj/\mathrm{mL}).$$

These results show that about 0.4 %*Inj*/g was taken up by brain.

C-NAC crossed the endothelial barrier of the cerebral cortex as shown by recovery of radioactivity from the parenchymal space of the brain (Fig. 2d). About 2.5 μL/g of C-NAC was present in capillaries and may reflect uptake by or binding to endothelium. For parenchyma, about 12.5 μL of C-NAC was present, indicating that the majority of C-NAC crossed the BBB.

Measures of oxidative stress

Protein carbonyl levels

To determine whether the protein carbonyl levels of cortical synaptosomal membranes were increased in the 12-month-old SAMP8 mice relative to those from 4-month-old SAMP8 mice, the 2,4-dinitrophenylhydrazone adducts were measured immunochemically. The results showed that the protein carbonyl levels of brain proteins in 12-month-old SAMP8 were significantly greater than those of 4-month-old SAMP8 by 29% (Fig. 3aA; $p < 0.01$). However, treatment with LA reduced this difference to a statistically non-significant 17% increase (Fig. 3aB).

W/S ratio

Consistent with the result for protein carbonyl levels, the W/S ratio was decreased significantly in the synaptosomal membrane proteins from 12-month-old SAMP8 mice when compared with 4-month-old SAMP8 mice (Fig. 3bA; $p < 0.001$). As noted above, the W/S ratio is lowered by oxidative stress (Hensley *et al*. 1994; Hall *et al*. 1995a; Hall *et al*. 1995b; Hall *et al*. 1995c; Butterfield *et al*. 1997). Treatment with LA abolished the difference between aged and young SAMP8 mice (Fig. 3bB).

TBARS

Increased TBARS levels were observed in the brains of 12-month-old SAMP8 mice when compared with 4-month-old SAMP8 mice (Fig. 3cA; $p < 0.05$). This result suggested an increased lipid peroxidation in 12-month-old SAMP8 mice. Treatment with LA abolished the difference between young and old mice (Fig. 3cB).

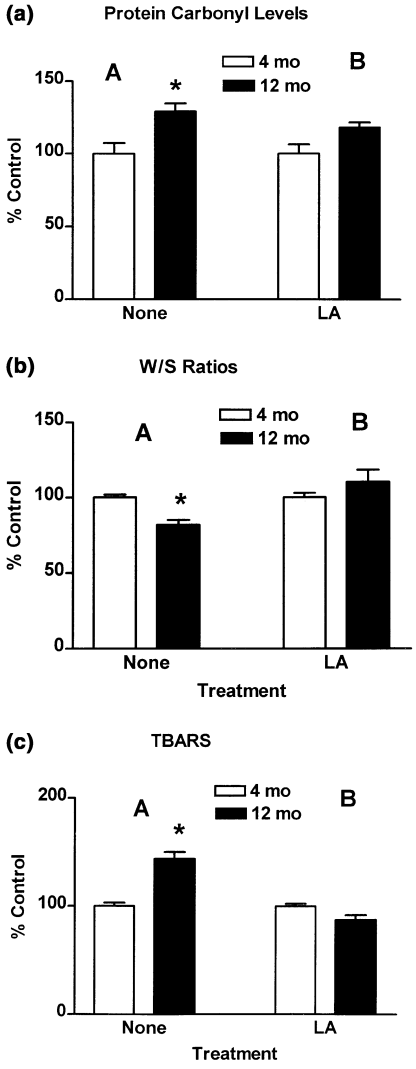

Fig. 3 (a) Protein carbonyl levels for 4 and 12-month-old SAMP8 mice. A, Untreated mice. Error bars indicate SEM for eight animals in each group. B, LA treated mice. Error bars indicate SEM for seven animals in each group. Measured value is normalized with the 4-month-old values. *$p < 0.01$. (b) W/S ratios for 4 and 12-month-old SAMP8 mice. A, Untreated mice. Error bars indicate SEM for six and seven animals, respectively. B, LA-treated mice. Error bar indicates SEM for six animals in each group. Measured value is normalized to the 4-month-old values. *$p < 0.001$. (c) TBARS of 4 and 12-month-old SAMP8 mice. (i) Untreated mice. Error bars indicate SEM for seven and six animals, respectively. (ii) LA treated mice. Error bar indicates SEM for seven and six animals in each group. Measured value is normalized with the 4-month-old values. *$p < 0.01$.

Discussion

Oxidative stress and damage induced by free radicals has been proposed as an important mechanism for both normal aging and the cognitive decline of neurodegenerative diseases (Zs-Nagy 1978; Zs-Nagy 1990). Here, we investigated whether antioxidant treatment could reverse the cognitive decline and oxidative damage seen in the aged SAMP8 mouse. This strain of mouse starts at about 6–8 months of age to overexpress Aβ, the peptide postulated to be the cause of AD (Kumar *et al*. 2000; Morley *et al*. 2000). With Aβ overexpression, these mice develop cognitive impairments which precedes by several months physical decline (Takeda

et al. 1991; Flood and Morley 1993; Nomura *et al*. 1996; Tanaka *et al*. 1998; Butterfield *et al*. 2001b; Morley *et al*. 2002). By 12 months of age, SAMP8 mice are healthy but have developed severe impairments in learning and memory, which are reversed by antibody or antisense directed at Aβ (Kumar *et al*. 2000; Morley *et al*. 2000; Banks *et al*. 2001). Other strains of mice at this age show little or no cognitive decline.

Here, we found that both LA and NAC could reverse impaired learning in 12-month-old SAMP8 mice in two separate behavioral paradigms. In the footshock avoidance T-maze, LA and NAC each returned learning to a level that was not different from unimpaired 4-month-old SAMP8 mice. LA and NAC were less effective in the lever press test. Although either antioxidant improved performance in this food-reward paradigm to a statistically significant degree, 12-month-old SAMP8 mice still underperformed when compared with 4-month-old mice.

The reason why antioxidants were not as potent in a food-reward-based learning paradigm is not clear, but one possibility is that anorexia of aging, which is thought to be unrelated to free radical production (Morley 2001; Morley 1997). Antioxidant treatment did not affect motor activity, response to shock, or body weight. Effects on these parameters could have acted as confounders and an absence of effect further supports a direct action of antioxidants on cognition.

These results are similar to those that found diets rich in antioxidants improved learning in aged rats (Bickford *et al*. 2000). In addition, two of three diets tested significantly increased brain concentrations of glutathione, the most potent known intracellular antioxidant. LA and NAC are precursors to glutathione (Overton and Fisher 1991; Wernerman and Hammarqvis 1996; Exner *et al*. 2000). The cognitive impairments of the SAMP8 mouse, however, are much greater than those occurring with normal aging.

The ability of antioxidants to reverse cognitive impairments depends upon their ability to reach the brain. This, in turn, depends on an ability to cross the BBB. The accumulation of free fatty acids (FFA) in brain results from a complex interplay between free and serum protein bound FFA, BBB influx transporters, BBB efflux transporters, and brain utilization (Banks *et al*. 1997b; Rapoport and Robinson 1995). Amino acid uptake by the brain depends on BBB transporters such as the one for large neutral amino acids (Davson and Segal 1996). Here, we showed that NAC entered the brain at a rate of about 2.41 μL/g-min, a modest rate in comparison to essential amino acids and about the same rate as many centrally active peptides (Begley 1994; Banks *et al*. 1995a; Banks *et al*. 1995b; Banks *et al*. 1997a; Banks 1999). Results of the capillary depletion experiment showed NAC could completely cross the capillary wall to enter the brain tissue and extracellular space. To determine the percentage of an intravenous dose of NAC taken up by a g of brain (%*Inj*/g), we first had to calculate circulating pharmacokinetic parameters (shown in Fig. 5). These values and the influx characteristics were then used to calculate %*Inj*/g. The results showed that about 0.4% of an injected dose was taken up by brain. This is about 4, 5, 20, and 200 times greater than the values for acetaminophen, interleukin-1 alpha, morphine, and domoic acid, respectively (Banks *et al*. 1991; Preston and Hynie 1991; Banks and Kastin 1994; Courade *et al*. 2001), all CNS-active agents. Therefore, the amount of NAC transported across the BBB is well within the therapeutic range of compounds known to exert effects on the brain.

We assessed the effect of antioxidant treatment on measures of oxidative stress in the aged SAMP8 mouse. We found that 12-month-old SAMP8 mice had more oxidative stress than 4-month-old SAMP8 mice. This confirms previous findings from another colony of SAMP8 mice and is consistent with studies showing that the SAMP8 mouse accumulates more oxidative damage than animals which age normally (Butterfield *et al*. 1997; Stadtman 1992; Hensley *et al*. 1994; Fujibayashi *et al*. 1998). Additionally, the brains of aged SAMP8 mice have a 44–50% decrease in delta-9 desaturase activity and a corresponding decrease in unsaturated free fatty acids. (Butterfield *et al*. 2001a).

The results suggest that oxidative stress is widespread, affecting measures of protein oxidation, lipid peroxidation, and oxidation-dependent changes in membrane protein conformation. These parameters were all reversed by treatment with LA. Ames and coworkers have shown that LA is able to partially reverse memory loss in normal aging rats by delaying mitochondrial dysfunction and RNA/DNA oxidation (Liu *et al*. 2002). Mitochondrial dysfunction is accompanied by a leakage into cytoplasm of O_2 and H_2O_2. That LA is readily taken up into mitochondria where it acts as a cofactor in oxidative decarboxylation of α-keto acids has led to the view that LA maybe a useful therapeutic agent in diseases characterized by mitochondria dysfunction or oxidative stress (Packer *et al*. 1997; Lynch 2001).

LA derives its antioxidant capability from its ability to (i) act as a scavenger of reactive oxygen species (ROS); (ii) chelate metals; and (iii) recycle endogenous antioxidants (Lynch 2001). LA can scavenge singlet oxygen, H_2O_2, $OH^{\cdot}$, NO and $ONOO^{-}$. The reduced form of LA, dihyrolipoic acid, can further scavenge $O_2^{\cdot}$ and peroxyl radicals (Kagan *et al*. 1992). LA can also chelate several divalent cations, e.g. Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}. Therefore, LA can inhibit ascorbate-induced production of H_2O_2 by Cu^{+} (Ou *et al*. 1995). LA can recycle endogenous antioxidants, such as GSH (Ou *et al*. 1995) and vitamin C (Drake *et al*. 2002), which regenerate vitamin E. GSH, vitamin C, and vitamin E all protect the brain from oxidative stress (Drake *et al*. 2002).

In conclusion, we found treatment with the antioxidants LA and NAC reversed the age-related cognitive impairment in SAMP8 mice. These substances probably act directly on

the brain, as NAC crossed the BBB and accumulated in brain to a significant degree. Treatment with LA reversed the oxidative stress seen in 12-month-old SAMP8 mice to levels that were not different from those seen in 4-month-old SAMP8 mice. These results support the hypothesis that oxidative stress can lead to cognitive dysfunction and provide evidence for a therapeutic role for antioxidants.

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