

REVIEW ARTICLE

**The S-Adenosyl Homocysteine Hydrolase Inhibitor  
3-Deaza-Adenosine Prevents Oxidative Damage  
and Cognitive Impairment Following Folate and Vitamin E  
Deprivation in a Murine Model of Age-Related,  
Oxidative Stress-Induced Neurodegeneration**

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**Abstract**

Deficiencies in folate promote neurodegeneration and potentiate the influence of other risk factors for neurodegeneration. This is accomplished at least in part by increasing levels of the neurotoxin homocysteine (HC). The S-adenosyl homocysteine (SAH) hydrolase inhibitor 3-deaza-adenosine (DZA) prevents HC accumulation following folate deprivation. We tested the ability of dietary supplementation with DZA to counteract the deleterious influence of folate deprivation. Folate deficiency has previously been shown to potentiate the impact of apolipoprotein E (ApoE); ApoE<sup>-/-</sup> mice deprived of folate demonstrated increased oxidative damage in brain tissue and impaired cognitive performance as compared to normal mice or to ApoE<sup>-/-</sup> mice receiving folate. Herein, we demonstrate that dietary supplementation with DZA prevented both the increase in oxidative damage and impaired cognition characteristic of ApoE<sup>-/-</sup> mice following folate deprivation. These findings suggest that manipulation of the methionine cycle by DZA can counteract folate deficiency. Because folate deprivation, increased HC, and apolipoprotein E deficiency are all risk factors for Alzheimer's disease, these findings also underscore that DZA might be useful in a therapeutic approach to delay neurodegeneration in Alzheimer's disease.

**Index Entries:** Homocysteine; folate; 3-deaza adenosine; neurodegeneration; oxidative stress; apolipoprotein E.

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## Introduction

One factor contributing to the age-related decline in cognitive performance is increased oxidative stress, which can arise from dietary, environmental, and/or genetic sources. (Berr, 2002; Butterfield et al., 2002; Floyd and Hensley, 2002; Perry et al., 2002a,b). Folate deficiency contributes to age-related neurological and psychological disorders including dementia, impaired cognition, depression, psychosis, Alzheimer's disease (AD), and Parkinson's disease (PD) (Mizrahi et al., 2003; for reviews, see Mattson and Shea, 2002; Shea and Rogers, 2002a; Shea et al., 2002a). These deleterious effects arise at least in part from the increase in oxidative stress that accompanies folate deficiency. Folate deficiency increases neuronal oxidative stress by increasing levels of the neurotoxin homocysteine—levels of which are related to the progression and severity of AD (Postiglione et al., 2001)—by decreasing endogenous antioxidants, by depleting overall cellular methylation reactions, and by inducing DNA damage and depleting energy reserves (Ho et al., 2002, 2003; Kruman et al., 2000, 2002; Shea and Rogers, 2002b). Folate deprivation also potentiates the deleterious impact of certain other risk factors for AD, including  $\beta$ -amyloid, glutamate, and metal neurotoxicity, and apolipoprotein E (ApoE) deficiency (Ho et al., 2001, 2002, 2003; Kruman et al., 2000, 2002; Mihalick et al., 2003; Shea and Rogers, 2002b; Shea et al., 2002b, 2002c; White et al., 2001) and sensitizes dopaminergic neurons to environmental neurotoxins in a mouse model of PD (Duan et al., 2002).

The relative impact of the above deleterious consequences of folate deprivation is unclear. However, one likely major impact of folate deprivation is increased generation of homocysteine (HC), because folate is a necessary cofactor for the enzyme (5,10-methylene tetrahydrofolate reductase) that mediates conversion of HC to methionine (Fiskerstrand et al., 1997; Pietrzik and Bronstrup, 1998). Elevated HC levels are also associated with neuropsychiatric disorders, including AD (Allen et al., 1998; Bell et al., 19992; Clarke et al., 1998; Miller, 2000). Direct addition of HC is toxic to cultured neurons and neuronal cells as well as the brain (Duan et al., 2000; Kruman et al., 2000; Ho et al., 2001, 2002), and potentiates neurotoxicity of other neurotoxins (Duan et al., 2002; Ho et al., 2001, 2002; White et al., 2001).

We have previously demonstrated that 3-deazaadenosine (DZA), a potent inhibitor of S-adenosyl homocysteine (SAH) hydrolase (Duerre, 1988; Jeong et al., 1999), the enzyme that prevents conversion of SAH to HC and prevents the accumulation of HC that normally accompanies folate deprivation. When differentiated human neuroblastoma cells were cultured in the absence of folate, HC levels increased approx 10-fold within 2 h (Ho et al., 2003); however, supplementation with DZA completely prevented this increase and, moreover, reduced HC levels in folate-deprived neuroblastoma cells below those present in the presence of folate (Ho et al., 2003). These data obtained in neuronal cell culture also confirm that increased HC can be derived directly from neuronal cells rather than surrounding vasculature (Blom, 2000) and confirm the efficacy of DZA on neuronal cells.

We tested herein whether or not dietary supplementation with DZA could alleviate the consequences of folate deprivation and increase HC generation in a mouse model for age-related oxidative stress. Prior studies have demonstrated that ApoE<sup>-/-</sup> mice demonstrate increased susceptibility to oxidative stress and that this is potentiated by dietary folate deficiency. Dietary deficiency in vitamin E alone did not increase oxidative damage, but augmented the increase observed following folate deprivation (Shea and Rogers, 2002b). Moreover, the combined impact of dietary deficiencies in folate and vitamin E, inclusion of excess iron as a pro-oxidant, and a genetic deficiency in oxidative buffering capacity (lack of ApoE function) induced a significant increase in reactive oxygen species in brain tissue under conditions where no single insult no combination of any two of these insults invoked a significant increase in reactive oxidative species in brain tissue (Shea and Rogers, 2002b). The combined deleterious effect of these insults was reflected in diminished cognitive performance in maze trials (Mihalick et al., 2003). These data highlight that a key genetic deficiency placing an individual at risk for AD might remain latent pending an age-related decline in nutrition (*reviewed in* Mattson and Shea, 2002; Shea et al., 2002a). To determine the relative impact of increased HC generation under these conditions, we supplemented the diet of normal and ApoE<sup>-/-</sup> mice under the above conditions with DZA. We demonstrate herein that inclusion of DZA in the diet prevents the increase in oxidative damage

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to brain tissue and, furthermore, maintains cognitive performance of genetically compromised mice despite withholding of dietary folate.

## Materials and Methods

### Mouse Strains and Diet

Male and female normal and ApoE<sup>-/-</sup> mice between 9 and 12 mo of age were maintained on a basal chow diet (AIN-76; Purina/Mother Hubbard, Inc.) (Shea and Rogers, 2002b; Shea et al., 2002b), with chow and drinking water ad libitum for 1 mo under ambient lighting. ApoE<sup>-/-</sup> mice exhibit increased oxidative stress and therefore represent a useful model for the impact of oxidative stress on neurodegeneration (e.g., Huang et al., 2000; Ramassamy et al., 1999, 2002; Shea and Rogers, 2002b; Shea et al., 2002b, 2002c; Veinsbergs et al., 2000). For some groups, this basal diet was supplemented with folic acid (2 mg/kg total diet wet weight) and vitamin E (1 g/kg total diet wet weight); this was defined as the "complete diet." Additional groups did not receive vitamin supplementation and their diet was instead supplemented with iron (50 g/500 g total diet); as a pro-oxidant (Shea and Rogers, 2002b; Shea et al., 2002b, 2000c); this was defined as the "deficient diet." Additional groups of ApoE<sup>-/-</sup> mice received DZA mixed into the above deficient diet at a final concentration of 0.04 g/kg diet (Walker et al., 1999). Cages (three to four mice each) were provided with an excess of chow each day, which was weighed prior to dispensing and the remainder weighed the following day, which allowed calculation of the average amount of DZA consumed; both mouse strains consumed identical amounts of food regardless of diet (Mihalick et al., 2003).

### Y-Maze Tests

Mice maintained as described earlier were subjected to standard Y-maze tests (Corcoran et al., 2002; King et al., 1999) as described previously (Mihalick et al., 2003). Mice were tested within the same room in which they were housed during the middle of the light cycle over a 2 h total period. Placement of the maze within the room and the location of the observer and recorder were identical for each trial. The pattern of exploration of the maze was recorded over 5-min intervals and the percent alternations

determined, which was defined as the frequency in which mice visited each of the 3 arms during any three-arm visitation sequence (Mihalick et al., 2003 and references therein). Notably, these same mice were subjected to maze trials and then sacrificed the following day for analyses of oxidative species and HC (diagnosed in a later subsection), providing an experimental link between alleviation of oxidative damage and cognitive performance following supplementation with DZA.

### TBAR Analyses

Thiobarbituric acid-reactive substances (TBARs) were quantified in total-brain homogenates as an index of end-point oxidative damage as utilized previously for the ApoE<sup>-/-</sup> mouse central nervous system as well as in AD (Butterfield and Lauderback, 2002; Lovell et al., 1995; Ramassamy et al., 2002; Shea and Rogers, 2002b; Shea et al., 2002a). Briefly, brain tissue homogenates (50 µg total protein) were mixed with 1 µM copper sulfate in 5 mM HEPES (total volume 400 µL). Samples then received 1 mL of a 0.375% TBA/15% trichloroacetic acid in 0.25 N HCl, incubated for 30 min at 90°C, and were clarified by centrifugation (1500 rpm for 10 min). The resulting supernatants were aspirated and fluorescence quantified in a fluorescent spectrophotometer (excitation 520 nm, emission 553 nm) by comparison with a standard curve of tetramethoxypropane in HCl.

### Quantification of HC

To monitor HC accumulation in brain tissue, 1 g of cortex from normal mice maintained on the complete or deficient diet was incubated for 5 min with 0.025% trypsin, triturated gently, centrifuged, resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% serum containing or lacking folate or 10 µM DZA, and dispersed into culture plates. The medium was decanted 48 h later, clarified by centrifugation, and subjected to high-performance liquid chromatography (HPLC) analyses for HC as described previously (Ho et al., 2003). Briefly, the medium was also utilized for analysis of HCY along with cellular lysates, as HCY is normally exported from cells (Christensen et al., 1991). Aliquots of medium or lysates (100 µL) were combined with 30 µL of 30 µM cystamine (as an internal standard) and 10 µL of tricarboxyethylphosphine (100 mg/mL in 0.05 M HCl). Samples

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were vortexed, incubated at room temperature for 10 min, and then centrifuged at 10,000g for 10 min. Eighty microliters of the resulting supernatant were combined with 160  $\mu$ L of 2 M boric acid containing 4 mM EDTA (pH 10.5), followed by 80  $\mu$ L of 1.0 mg/mL SBDF (7-fluorbenzo-2-oxa-1,3-diazide-4-sulfonate) in the same buffer. Samples were incubated for 1 h at 60°C and then injected (50  $\mu$ L) into a Hewlett-Packard model 1090 HPLC equipped with a model 1046A fluorescence detector and a Hewlett-Packard 4.6  $\times$  60-mm high-speed analytical column packed with 3  $\mu$ M ODS (C18) Hypersil silica. The isocratic mobile phase consisted of 2 vol of methanol/98 vol of 0.1 M phosphate buffer (pH 2.0). HCY and total glutathione concentrations were then determined by comparison of peak height ratios to the cystamine internal standard.

### Statistical Analyses

All analyses are derived from 3 independent experiments, with 3–4 normal and 3–4 ApoE<sup>-/-</sup> mice under each dietary condition for each experiment, for a total of 9–12 mice of each strain under each condition. Statistical analyses were carried out with Student's *t*-test and analysis of variance (ANOVA).

## Results

### DZA Reduces Oxidative Species Following Folate and Vitamin E Deprivation in Brain Tissue

To examine the efficacy of dietary supplementation with DZA, we maintained normal and ApoE<sup>-/-</sup> mice for 1 mo under the complete (supplemented with vitamins) or deficient (lacking vitamins and containing excess iron as a pro-oxidant) diet while additional groups of ApoE<sup>-/-</sup> mice received the deficient diet supplemented with DZA. ApoE<sup>-/-</sup> mice on the deficient diet consumed an average  $10.8 \pm 1.1$  g chow/d; based on our incorporation of 0.04 g DZA/kg chow, these mice therefore consumed an average of  $0.43 \pm 0.04$  mg DZA/d. No change in body weight was observed in this or prior studies (Shea et al., 2002b) from maintenance on either diet (not shown).

As shown previously (Shea and Rogers, 2002b; Shea et al., 2002b), brain tissue of normal mice exhibited no significant difference in TBARs when main-

tained on the deficient versus the complete diet; brain tissue of ApoE<sup>-/-</sup> mice also did not differ statistically from normal mice. By contrast, ApoE<sup>-/-</sup> mice routinely display increased TBARs when maintained on the deficient diet ( $p < 0.05$  vs the complete diet (see also Shea and Rogers, 2002b; Shea et al., 2002b, 2002c). However, supplementation of the deficient diet with DZA prevented the increase in TBARs normally observed in ApoE<sup>-/-</sup> mice (Fig. 1); values obtained in ApoE<sup>-/-</sup> mice on the deficient diet when supplemented with DZA were statistically identical ( $p < 0.60$ ) to those of normal mice.

### DZA Improves Cognitive Performance Following Folate and Vitamin E Deprivation

Because the deficient diet has been shown to affect cognitive performance (Mihalick et al., 2003), we next examined whether or not supplementation with DZA could compensate for this cognitive impairment. Normal mice demonstrated an identical exploratory behavior on either diet, as indicated by percent alternations in the Y-maze on either diet, as did ApoE<sup>-/-</sup> mice on the complete diet (Fig. 2). As demonstrated previously (Mihalick et al., 2003), ApoE<sup>-/-</sup> mice displayed reduced performance when maintained on the deficient diet (Fig. 2;  $p \leq 0.005$  vs all other groups). However, supplementation with DZA prevented this decrease; ApoE<sup>-/-</sup> mice maintained on the deficient diet supplemented with DZA exhibited statistically identical ( $p < 0.21$ ) percent alternations as compared to normal mice on either diet or ApoE<sup>-/-</sup> mice receiving a complete diet.

### DZA Reduces HC Levels in Brain Tissue

We have previously demonstrated that folate deprivation under the conditions utilized herein increases plasma HC levels in normal mice by 43% (Shea and Rogers, 2002b), and DZA has been reported to inhibit HC accumulation within plasma (Bowron and Stansbie, 2003). We set out herein to confirm whether or not DZA reduced HC production within brain tissue. We were unable to detect HC levels above baseline by HPLC analyses of homogenates of brain tissue of either mouse strain in the presence or absence of folate; this is likely a result of the rapid export of HC from tissues

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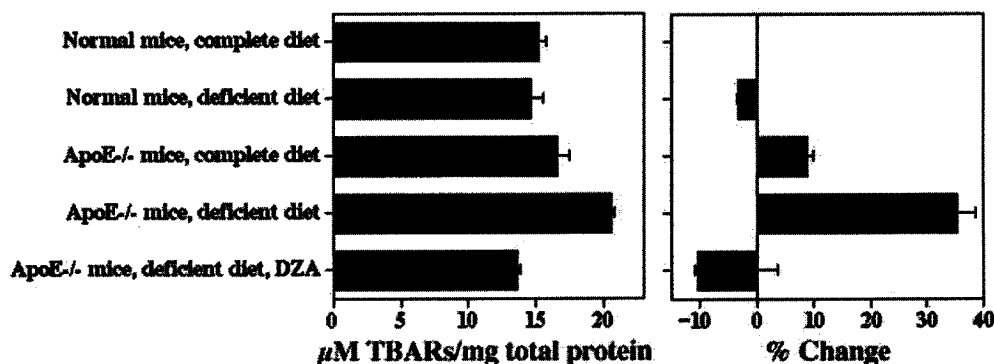


Fig. 1. Supplementation DZA is neuroprotective against oxidative stress. Normal and ApoE<sup>-/-</sup> mice received dietary regimens as indicated for 1 mo, after which total brain tissue was harvested and analyzed for TBARs as described in the Materials and Methods section. Values represent mean  $\pm$  SEM  $\mu\text{mol}$  TBARs/mg total protein, compiled from three independent experiments, with  $n = 3-4$  mice for each diet/experiment (for a total of 9-12 mice). Note that DZA prevents the increase in TBARs in ApoE<sup>-/-</sup> mice maintained on the deficient diet.

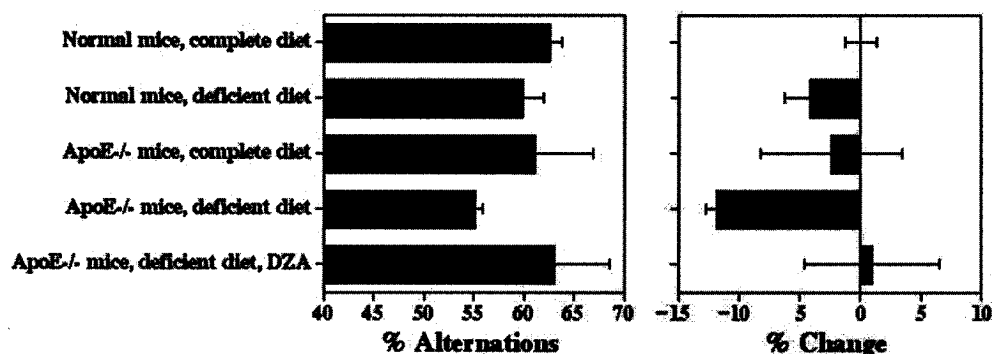


Fig. 2. Supplementation with DZA prevents memory impairment in ApoE<sup>-/-</sup> mice as a result of vitamin deficiency and oxidative challenge. Normal and ApoE<sup>-/-</sup> mice were maintained on the indicated diets and then subjected to the Y-maze test and the percent alternations determined. Values on the leftmost graph present the mean  $\pm$  SEM for percent alternations; for simplicity of interpretation, values on the rightmost graph present the percent change in performance for each condition relative to normal mice receiving the complete diet. Values are derived from compiled from three independent experiments, with  $n = 3-4$  mice for each diet/experiment (for a total of 9-12 mice). Note that ApoE<sup>-/-</sup> mice demonstrate impaired performance on the deficient diet and that supplementation with DZA prevented this impairment.

(Christensen et al., 1991), coupled with its continuous systemic elimination via liver and kidney metabolism and clearance (Tapiero et al., 2001). To monitor HC accumulation in brain tissue, we therefore dispersed 1 g of cortex from normal mice that had received or been deprived of folate for 1 mo into 1 mL of culture medium containing or lacking folate or 10  $\mu\text{M}$  DZA, respectively, and then assayed HC within clarified culture medium 48 h later, as

previously carried out for primary brain cultures; this approach allows reproducible analyses of HC levels because (1) all detectable HC was exported (with no detectable levels; therefore,  $<1$  nM was retained within brain cells), and, once exported, HC was not subject to further metabolism and therefore accumulated within culture medium (Ho et al., 2003). A  $26 \pm .02\%$  increase in HC was observed in cultures from brain tissue that had undergone folate

Table 1  
DZA Reduces HC Export From Primary Cultures of Mouse Brain

Diet	DZA	HC ( $\mu\text{M}$ )	% Reduction in HC
Complete	–	5.75 $\pm$ 0.96	
	+	2.60 $\pm$ 0.49	55 $\pm$ 5.3%
Deficient	–	7.25 $\pm$ 0.14	
	+	3.20 $\pm$ 0.04	57 $\pm$ 0.6%

*Note:* Brain tissue (1 g) from normal mice maintained on the complete and deficient diets were dispersed into 1 mL of culture medium containing or lacking folate or 10  $\mu\text{M}$  DZA for 48 h, after which medium was clarified and HC quantified by HPLC as described in the Materials and Methods section.

Note that folate deprivation increased HC accumulation, and that inclusion of DZA within culture medium not only prevented this increase, but lowered the baseline [HC] of brain tissue under both conditions.

deprivation demonstrated (Table 1), which is in accord with the 43% increase observed in plasma of these mice (Shea and Rogers, 2002b). Inclusion of DZA prevented this increase and, moreover, reduced HC levels in the presence or absence of folate by approx 50% (Table 1).

## Discussion

The findings of the present study confirm the notion that dietary supplementation with a pharmacological inhibitor, in this case DZA, can diminish or prevent oxidative damage and resultant cognitive impairment associated with dietary and/or genetic deficiencies, specifically deficiencies in dietary intake of folate and vitamin E, coupled with diminished ApoE activity.

Although we have determined the average amount of DZA consumed per day, we have no further information regarding the systemic concentration of DZA or that within brain tissue resulting from this feeding regimen, nor have we, in fact, demonstrated that DZA can cross the blood–brain barrier. However, DZA is a nucleoside analog and similar nucleoside analogs such as AZT have been shown to enter the nervous system and are in current use for human immunodeficiency virus (HIV) therapies (Masereeuw et al., 1994; Mayers et al., 1995). Effective transfer of DZA across the blood–brain barrier is further supported by our observation of a reduction in TBAR-reactive species in brain tissue.

Because DZA prevented the increase in HC that normally accompanies folate deprivation (Ho et al.,

2003; Shea and Rogers, 2002b), these data are consistent with the notion that HC accumulation is a major neurotoxic consequence of folate deprivation and that DZA provided neuroprotection by prevention of HC accumulation. However, by inhibiting conversion of SAH to HC, DZA also fosters an increase in both SAH and its upstream precursor S-adenosyl methionine (SAM), which mediates overall transmethylation reactions, including methylation of DNA (Wu and Santi, 1987; Wainfan et al., 1989; Mudd and Cantoni, 1991). In addition to preventing HC accumulation, DZA may therefore provide neuroprotection in part by maintaining SAM levels. Prevention by DZA of the increase in reactive oxygen species that normally accompanies folate deprivation as shown herein and in culture (Ho et al., 2003) could be derived from reduced generation of HC, by maintenance of SAM and resultant transmethylation reactions, or both. In this regard, HC accumulation has the additional deleterious effect of inhibiting the forward progression of the methionine cycle and, in doing so, reducing levels of SAM (Cantoni, 1986; Endersen et al., 1994, 1996). The deleterious impact of HC-mediated depletion of SAM was underscored by a 50% reduction in HC-mediated apoptosis when cultured neuronal cells were cotreated with SAM (Ho et al., 2002). DZA could therefore maintain critical SAM levels both by inhibiting SAH and by preventing HC accumulation, inhibiting HC-induced depletion of SAM. Such possibilities could be addressed in future studies by supplementation of the deficient diet utilized herein with various combinations of HC, SAM, and DZA. DZA also has anti-inflammatory effects

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(Krenitsky et al., 1986; Medzihradsky et al., 1992), which may provide an additional mechanism by which DZA may reduce neurodegeneration (Launer, 2003; Naccari, 2003; Sun et al., 2003). Although additional studies are therefore warranted to determine the full range of mechanisms by which DZA provides neuroprotection, the importance of the present study is that DZA is capable of preventing oxidative damage and cognitive impairment following folate deficiency in a mouse model of age-related neurodegeneration (Veinsbergs et al., 2000, Huang et al., 2000; Ramassamy et al., 1999, 2000, 2002; Shea and Rogers, 2002b, Shea et al., 2002b, 2002c).

The neuroprotective effects of DZA as demonstrated herein are particularly relevant for AD because the epsilon 4 allele (E4) of the apolipoprotein E gene (*ApoE*) is linked with an increase in and an earlier age of onset of sporadic and familial AD (Growdon, 2001; Rebeck et al., 2002). Oxidative damage in the brain is elevated in AD patients, and the extent of this damage correlates with the presence of the E4 allele (Ramassamy et al., 1999). Moreover, folate deprivation has been shown to impair multiple aspects of learning and memory in humans (Hassing et al., 1999) as well as in experimental animals (Crowe and Ross, 1997; Lalonde et al., 1993; Mihalick et al., 2003). The current study using *ApoE*<sup>-/-</sup> mice is limited in that it provides information only regarding the consequences of lack of *ApoE* function. The extent of oxidative damage and cognitive impairment in our model system exhibits a gene-dosage effect, with *ApoE*<sup>+/-</sup> mice demonstrating less impairment than *ApoE*<sup>-/-</sup> mice (Shea et al., 2004). Nevertheless, it remains unclear whether the full association of *ApoE*4 with AD is derived from the diminished function(s) of *ApoE*4 relative to the other isoforms, or derives from the absence of protective effects provided by *ApoE*3 and/or *ApoE*2, or derives from the actual presence of *ApoE*4 (for reviews, see Rebeck et al., 2002; Teter et al., 2002). Of interest, therefore, would be to examine the impact of folate deficiency, and the efficacy of DZA, on mice expressing various *ApoE* isoforms.

The deficient diet utilized herein lacked both folate and vitamin E. Our prior studies demonstrated that vitamin E deprivation alone for 1 mo did not increase oxidative damage as assayed by TBARs, but it did potentiate the increased oxidative damage resulting from folate deprivation (Shea and Rogers, 2002b). The neuroprotective effect of

DZA as demonstrated herein also encompassed any consequences of vitamin E deprivation. In this regard, longer periods of vitamin E deprivation did result in cognitive deficits in *ApoE*<sup>-/-</sup> mice (Veinsbergs et al., 2000); whether or not DZA alone would be effective following long-term folate and vitamin E deprivation or whether additional supplementation would be required remains unclear. Nevertheless, because both folate deprivation and HC accumulation induce neurodegeneration in AD as well as potentiate the neurotoxicity of other risk factors for AD (Mattson and Shea, 2002), these findings suggest that DZA or similar agents should be considered as part of a combinatorial therapeutic approach for AD.

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