



## Basic nutritional investigation

## Short-term blueberry-enriched diet prevents and reverses object recognition memory loss in aging rats

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## ABSTRACT

**Objective:** Previously, 4 mo of a blueberry-enriched (BB) antioxidant diet prevented impaired object recognition memory in aging rats. Experiment 1 determined whether 1- and 2-mo BB diets would have a similar effect and whether the benefits would disappear promptly after terminating the diets. Experiment 2 determined whether a 1-mo BB diet could subsequently reverse existing object memory impairment in aging rats.

**Methods:** In experiment 1, Fischer-344 rats were maintained on an appropriate control diet or on 1 or 2 mo of the BB diet before testing object memory at 19 mo postnatally. In experiment 2, rats were tested for object recognition memory at 19 mo and again at 20 mo after 1 mo of maintenance on a 2% BB or control diet.

**Results:** In experiment 1, the control group performed no better than chance, whereas the 1- and 2-mo BB diet groups performed similarly and significantly better than controls. The 2-mo BB-diet group, but not the 1-mo group, maintained its performance over a subsequent month on a standard laboratory diet. In experiment 2, the 19-mo-old rats performed near chance. At 20 mo of age, the rats subsequently maintained on the BB diet significantly increased their object memory scores, whereas the control diet group exhibited a non-significant decline. The change in object memory scores differed significantly between the two diet groups.

**Conclusion:** These results suggest that a considerable degree of age-related object memory decline can be prevented and reversed by brief maintenance on BB diets.

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## Introduction

Oxidative stress, reflecting the accumulation of oxygen-containing free radicals, increases with aging and may play a key role in age-related functional deficits of the brain [1–8] and other organs, such as the heart [9]. Blueberries are one of the foods with the greatest ability to neutralize oxygen-containing free radicals [10]. In rodent models of brain aging, dietary blueberry supplementation impeded the development of impairments in neurochemistry, synaptic transmission, and behavior [11–18].

Aging human beings tend to be impaired in visual object recognition memory [19]. A non-spatial object recognition memory task tests a rat's memory for previously explored objects

[20]. The task, which requires no deprivation or punishment, is based on rats' innate tendency to preferentially explore novel versus familiar objects. Task performance depends on intact hippocampal function [20]. Two studies found that aging impaired memory for visual object recognition [21,22], although another study detected no consistent age-related trend [23]. A previous study in this laboratory found that middle-aged (19-mo-old), but not young, Fischer-344 rats had highly impaired memory for this visual object recognition task in a manner dependent on retention interval [24]. The aging rats were not impaired when there was only a 30-s delay between object familiarization and testing. However, with a 1-h delay, aging rats performed no better than chance. In contrast, aging rats fed a diet supplemented with lyophilized blueberries (2% by weight) for 4 mo (age 15–19 mo) performed as well as young rats.

The active form (p65) of nuclear factor- $\kappa$ B, a sensitive indicator of oxidative stress and inflammation [25], was elevated in brains of aging rats compared with young rats [24]. The 4-mo

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blueberry-supplemented diet largely prevented the elevation of nuclear factor- $\kappa$ B levels in aging rats. Nuclear factor- $\kappa$ B levels, averaged across all evaluated brain regions, correlated negatively and significantly with object memory performance. Also, levels in the hippocampus alone and the cerebellum alone each correlated negatively and significantly with performance. Taken together, these results are consistent with the hypothesis that blueberry supplementation improved object memory through decreasing oxidative stress.

In the present study, experiment 1 evaluated the effect of changing the duration of the same blueberry-supplemented diet on the prevention of object memory impairment in 19-mo-old postnatal rats. It also determined whether the benefits of the blueberry-supplemented diet would be lost rapidly or would persist for a time after returning to a normal rodent diet.

Experiment 2 addressed an issue arising from a surprising result of experiment 1: the impairment of object memory in aging rats was prevented by feeding an antioxidant-enriched diet containing 2% blueberries (BB) for as briefly as 1 mo (18 to 19 mo postnatally). It seemed doubtful that the entire memory impairment developed during that single month. Therefore, it was hypothesized that the diet might have *reversed* a degree of pre-existing impairment. Experiment 2 determined whether 1 mo of the antioxidant diet could *reverse* a memory impairment that had been demonstrated in a group of 19-mo-old Fischer-344 rats.

## Materials and methods

### Subjects

The subjects were 35 male Fischer-344 rats that were 19 mo old at the initial evaluation of object memory. The Fischer-344 rats are selected as test subjects because their size changes very little as they age. By the age of 19 mo, although about 5 mo below their average lifespan, Fischer-344 rats are greatly impaired in spatial memory and a variety of motor tasks [26] and in object memory [24]. The rats were handled for 5 min/d over a 2-wk period to habituate them to human contact. All procedures were approved by the University of Houston–Clear Lake institutional care and use committee in accordance with federal and university guidelines.

### Diets

The base diet was NIH-31 (Harlan Teklad Rodent Chow, Madison, WI, USA) rodent chow in the form of large pellets. This diet is formulated to be nutritionally equivalent to a balanced human diet, with proportional amounts of antioxidants such as fat-soluble vitamins and vitamin C, so as not to artificially handicap the performance of the aging animals on the object recognition (memory) tasks. The 2% BB diet was prepared by homogenizing Tif-blue cultivar of *Vaccinium ashei* Reade (U.S. Highbush Blueberry Council, Folsom, CA, USA) in water, centrifuging the homogenate, and lyophilizing the supernatant to a powder. Successive batches of this preparation were tested and found to be consistent for polyphenolic content and oxygen radical absorbance capacity. This is a relevant approach to standardization, because polyphenolic compounds are the probable beneficial active ingredients in blueberries and neutralization of reactive oxygen-containing free radicals is one major hypothesized mechanism of action [1,10]. The powder was milled into the NIH-31 rodent chow. The control diet was supplemented with 2% dried corn by weight to make it isocaloric with the BB diet.

### Apparatus

The apparatus was a black Plexiglas arena (93 × 93 × 61 cm) with an open top. The stimulus objects were ceramic figurines one to two times the rats' size. The ceramic figurines were weighted down with pebbles so the rats could not move them.

### Training and familiarization

All subjects were habituated to the Plexiglas arena for 5 min/d for 5 consecutive days. On test days, object recognition memory was evaluated by the following procedure, modified from that of Clark et al. [20]. Each rat was allowed to explore the empty arena for 1 min, and then the rat was returned to its home cage. Immediately after this brief re-habituation, two identical stimulus objects

were placed in symmetrical locations in the arena. The rat was allowed to explore and become familiar with each object for a total of 30 s of object exploration or until 10 min of time had elapsed. The object exploration time was recorded when the rat's nose was within 1 cm of the object while the rat's vibrissae (whiskers) were moving. Rats touching the objects with the front paws or standing on the object was also recorded as object exploration time. After familiarization, the rats were removed from the arena for a period of 1 h. This interval between object exposure and object memory testing previously resulted in no better than chance performance in aging rats [24].

### Retention trial

After the 1-h delay period, test subjects were returned for 30 s to the Plexiglas apparatus containing one previously explored object (the "familiar object") and a new or "novel" object not previously explored. The rats were scored separately on the number of seconds spent exploring each object. The dependent variable, termed *object recognition memory score*, was the time that the subject spent exploring the novel object as a percentage of the subject's total object exploration time spent with either object. For example, if a rat explored the objects for 20 s and 12 s of that time was spent with the novel object, its score would be 60%.

### Experiment 1 procedure: Effect of 1- and 2-mo BB diets on preventing impaired object memory

The subjects were 21 male Fischer-344 rats that were 17 mo of age at the beginning of the diets. For 2 mo, group A was fed a control diet supplemented with dried corn to be isocaloric with the 2% BB diet. Group B was fed the control diet for the first month and the BB diet for the second month. Group C was fed the BB diet for the 2 mo. All rats were tested for object recognition memory at 19 mo.

After this experiment, all rats were placed on a normal balanced rodent diet (Harlan Teklad Rodent Chow). To determine the loss or retention of any benefits from the previous diets, rats were retested for object recognition memory 2 and 4 wk later. Before each retest, subjects were handled and re-habituated to the apparatus for 2 d. Each retest involved new sets of familiar and novel objects, so that there would not be any carryover effect from previous trials. The overall research design is summarized in Figure 1 (top).

### Experiment 2 procedure: Reversal of impaired object memory

The subjects were 14 male Fischer-344 rats. They were tested for object recognition memory at 19 mo of age. Over the subsequent month, seven rats were maintained on a 2% BB diet (group D). Seven rats were maintained for 1 mo on the control diet previously described (group E). All rats were handled and habituated in the apparatus for 5 d before retesting for object recognition at 20 mo of age. A different pair of stimulus objects was used to prevent any carryover effect from the previous test. At 20 mo of age, an object memory score was obtained for each rat, as was a change score (the subject's object memory score after the diet at 20 mo minus the same subject's object memory score before the diet at 19 mo). The experimental design is summarized in Figure 1 (bottom).

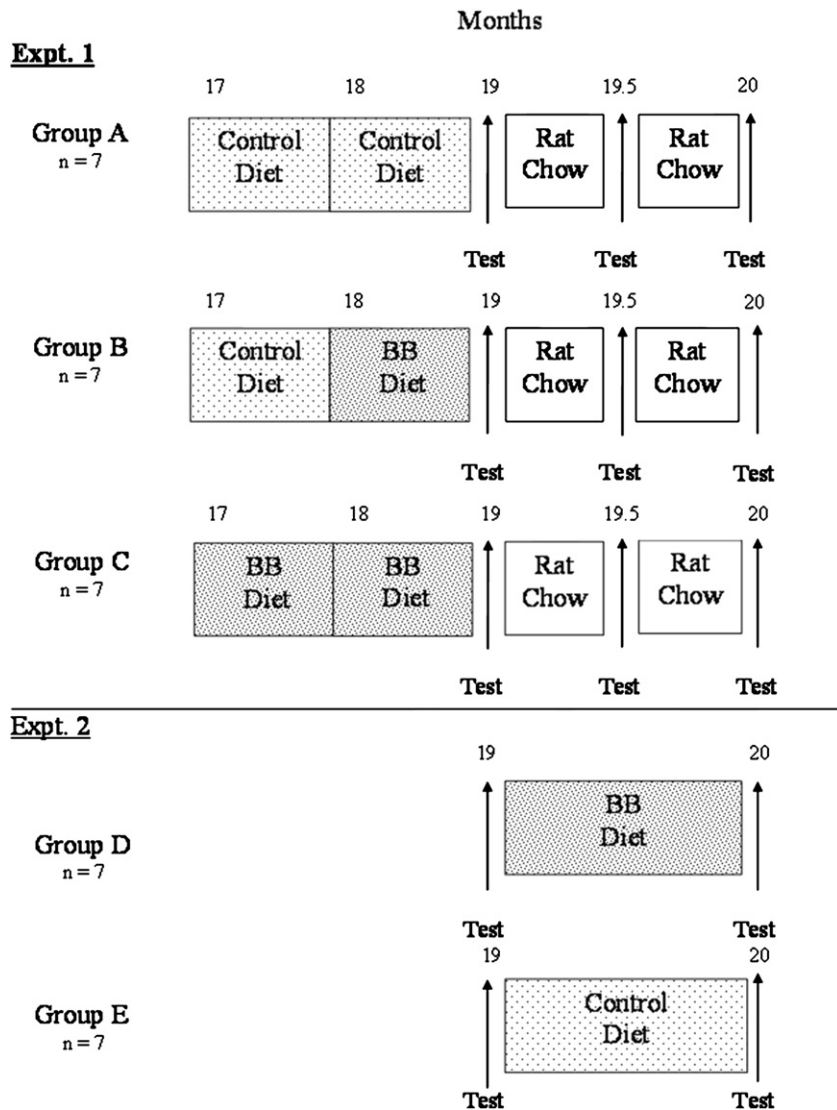
### Statistical analysis

Memory scores in the two diet groups were compared by independent-groups *t* test. One-sample *t* tests were used to compare memory scores from each group with chance performance (50%). One-sample *t* tests were also used to detect whether each group's memory scores changed significantly over time.

## Results

### Experiment 1: Effect of brief BB diets on subsequent object memory performance

Object memory recognition scores (percentage of exploration time spent with the novel object) at 19 mo of age are shown in Figure 2A. As in previous studies, the aging control diet rats performed no better than chance (50%). However, the 1-mo and 2-mo BB diet groups performed significantly better than the control diet group ( $P = 0.037$  and  $P = 0.047$ , respectively). They spent approximately 70% of the exploration time with the novel object. Corresponding data from the previous study with 4-mo diets are shown in Figure 2B. The control diet performance in both experiments was almost identical. Likewise, the performance of rats on the BB diets for 1 and 2 mo in the present study



**Fig. 1.** Design of experiment 1: effects and delayed aftereffects of 1- and 2-mo BB diets on prevention of object recognition memory impairment. Design of experiment 2: effect of a subsequent 1-mo BB diet on existing impairment of object memory. BB, 2% blueberry-enriched.

was almost identical to the performance of rats on the BB diet for 4 mo in the previous study.

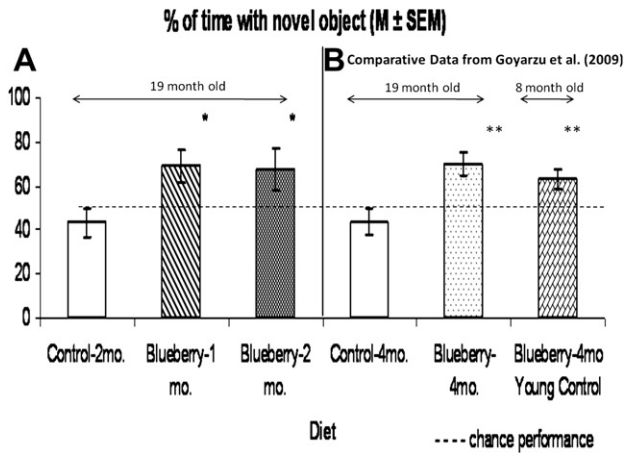
As shown in Figure 3, the 1-mo and 2-mo BB diet groups did differ in terms of retaining the benefits to memory function. The performance of the 2-mo group remained nearly constant, whereas the performance of the 1-mo group declined at 2 wk after the diet and was identical to the control group at 4 wk after the diet. According to one-sample *t* tests, the control group failed to perform significantly better than chance (50%) at the end of the diet ( $t_6 = -0.96$ , NS), 2 wk later ( $t_6 = 0.79$ , NS), and 4 wk later ( $t_6 = 1.18$ , NS). The 1-mo BB diet group performed significantly better than chance at the end of the diet ( $t_5 = 2.65$ ,  $P < 0.05$ ). However, its performance was not significantly better than chance 2 wk after diet termination ( $t_4 = 1.40$ , NS) and 4 wk later ( $t_6 = 0.61$ , NS). At the end of its diet, the 2-mo BB diet group approached significantly better than chance performance ( $t_5 = 1.76$ ,  $0.05 < P < 0.10$ ). Its performance was significantly better than chance 2 wk later ( $t_6 = 2.33$ ,  $P < 0.05$ ) and 4 wk later ( $t_4 = 2.60$ ,  $P < 0.05$ ). It should be noted that the degrees of freedom may vary between trials, because animals that failed to

explore the objects sufficiently ( $\geq 8$  s) during the familiarization trial had their retention trial scores disqualified for that trial.

One-sample *t* tests were used to evaluate each treatment group's change in object memory scores (percentage of time exploring the novel object) from the end of the diet to 4 wk later. The analyses excluded data from any subject that failed to explore the objects sufficiently during the familiarization trials. The 1-mo diet group displayed a significant decrease in its object memory scores over the 4 wk ( $t_5 = -3.05$ ,  $P < 0.02$ ). There was no significant change in the control diet group ( $t_6 = 1.30$ , NS) or the 2-mo diet group ( $t_4 = -0.46$ , NS).

#### Experiment 2: Subsequent effect of a BB diet on impaired object memory

In the 19-mo pretest, the time spent exploring the novel versus the familiar object was near chance (53% for the subsequently control diet-fed group versus 49% for the subsequently BB-fed group), a non-significant difference ( $t_{12} = 0.27$ , NS). As shown in Fig. 4, aging rats subsequently maintained on a 2% BB

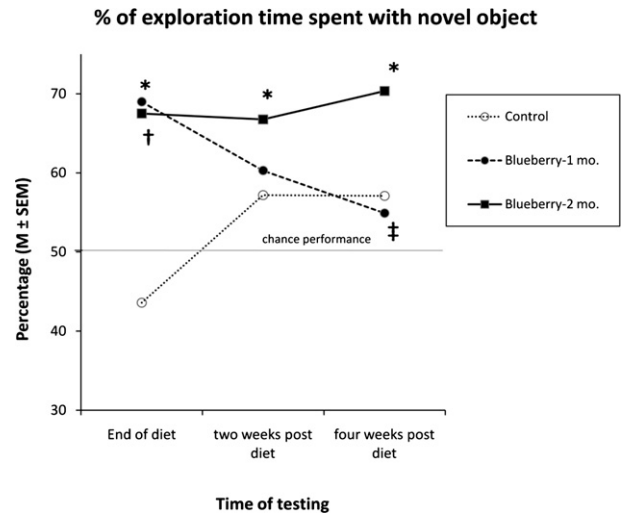


**Fig. 2.** Effects of 2% blueberry-enriched and control diets on object recognition memory (percentage of exploration time spent with a novel object) tested at 19 mo of age. The object memory score was the percentage of total exploration time (mean  $\pm$  SEM) spent with a novel object versus a familiar object explored 1 h before testing. (A) Data from the present study. Rats received the control diet for 2 mo before testing (white bar), the control diet for 1 mo, followed by the 2% blueberry-enriched diet for 1 mo (striped bar) or for 2 mo (dark bar). (B) Data from the previous 4-mo diet study [18] shown for purposes of comparison. The two leftmost bars show the performance of aging in 19-mo postnatal rats maintained for 4 mo before testing on the control diet (open bar) and the blueberry-enriched diet (dotted bar). The rightmost bar shows memory scores from young (8-mo postnatal) rats that had been maintained for 4 mo on the control diet. \*  $P < 0.05$  versus 2-mo control diet (present study). \*\*  $P < 0.01$  versus aging rats on the 4-mo control diet (previous study).

diet for 30 d significantly improved their object recognition memory scores, according to the one-sample  $t$  test ( $t_6 = 2.18$ ,  $P = 0.036$ ). In contrast, the control diet group actually showed a non-significant decline in object memory scores from 19 to 20 mo ( $t_6 = -1.47$ , NS). The change over 1 mo differed significantly between the experimental (BB) and control groups ( $t_{12} = 2.52$ ,  $P = 0.013$ ). The 20-mo-old rats on the BB diet spent 63% of their exploration time with the novel object, similar to young (8-mo-old) rats in the previous study [24]. The  $10.1 \pm 1.2$  g (mean  $\pm$  standard error of the mean) weight gain for the BB diet group was very similar to the  $11.2 \pm 1.3$  g weight gain for the control diet group, suggesting that differences in food consumption or weight did not account for dietary effects on memory. The difference in weight gain was not significant ( $t_{12} = 0.62$ , NS).

## Discussion

The performance of the aging rats on the control diet in the present study was almost identical to that of the aging control diet rats in the previous study [24]. In each study, control group performance was no better than chance. The 1 mo and 2 mo of a BB diet prevented memory impairment in rats at 19 mo of age. The performance of rats that had been maintained on 1- and 2-mo BB diets was almost identical to that of rats maintained for 4 mo on the BB diet in the previous study (Fig. 2). That performance was closely similar to that of young rats. Therefore, 1-, 2-, and 4-mo diets substantially reversed the age-related object memory impairment found in 19-mo-old rats. This illustrates a surprisingly prompt and powerful effect of an antioxidant dietary intervention. It would be of interest in future studies to determine even shorter diet durations that produce threshold or suboptimal effects. The use of suboptimal durations would avoid a “ceiling effect” in assessing the benefits of adding other dietary



**Fig. 3.** Mean object memory recognition memory scores (percentage of rats) at 2 and 4 wk immediately after termination of a 1-mo blueberry diet, a 2-mo blueberry diet, or the control diet and 2 and 4 mo afterward. The diets were terminated at 19 mo postnatally. †  $P < 0.05$  and ‡  $0.05 < P < 0.10$  versus chance performance. ‡  $P < 0.05$  versus zero (no change from performance at termination of the diet).

supplements to the blueberries. Such additional supplements might include plum juice [27], curcumin (from turmeric) [28], or a number of other nutrients that have demonstrated beneficial cognitive effects in aged rodents.

An interesting result in the present study is that enhanced object memory performance persisted virtually unchanged at 2 and 4 wk after termination of the 2-mo BB diet. In contrast, performance declined steadily at 2 and 4 wk after termination of the 1-mo BB diet. It appears that, although optimal levels of blueberry nutrients may be initially achieved after a relatively short period, longer-term consumption may be important for maintaining the benefits. There remains the question, Why is there such a difference in the aftereffects of the 1- or 2-mo diets? One possible explanation is a “threshold hypothesis.” This hypothesis assumes there is a threshold concentration of antioxidants, particularly longer-lasting fat-soluble antioxidants, needed to maintain alleviation of memory impairment. The 2-mo diet might have produced a larger surplus of antioxidant nutrients over the threshold, whereas the 1-mo diet might have produced only a scant surplus above the threshold. Then, as the antioxidant nutrients are metabolized, the 1-mo diet might soon lose its ability to prevent memory impairment, whereas this loss of effectiveness might hypothetically take much longer after the 2-mo diet.

Another explanation might be a “secondary antioxidant hypothesis.” Blueberry-supplemented diets can activate the powerful endogenous antioxidant defenses by chemically reducing oxidized (and thus inactivated) glutathione [29]. It is thus reasonable to suppose that antioxidant anthocyanins in the blueberries produce an aftereffect by reactivating or “recharging” the brain’s endogenous antioxidants, notably glutathione. Perhaps the more prolonged diet was more effective at activating the longer-acting endogenous antioxidant compounds. This hypothesis could be tested in future research by measuring reduced glutathione in brain tissue and nuclear factor- $\kappa$ B (an indicator of oxidative stress) at the end of 1-mo and 2-mo blueberry-enriched diets and at weekly time points thereafter. Blueberry supplementation in aging rats can also increase brain



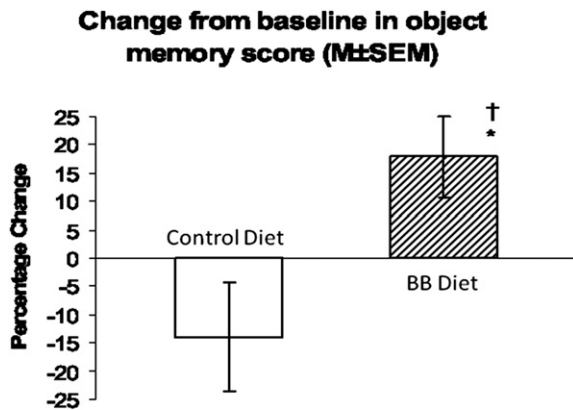


Fig. 4. Change in object recognition memory scores from 19 to 20 mo in Fischer-344 rats maintained during that 1-mo interval on a BB diet or an isocaloric control diet. \*  $P = 0.013$  versus change in control diet group. †  $P = 0.036$  versus zero (no change). BB, 2% blueberry-enriched.

neurogenesis [17]. A greater effect on neurogenesis might also be responsible for the greater aftereffects of the longer blueberry diet.

Unless the rats' memory impairment developed entirely from 18 to 19 mo of age, an improbably sudden decline, the 1-mo BB diet must have reversed some pre-existing decline in object memory. Experiment 2 directly tested this hypothesis. Rats displayed near-random object memory performance at 19 mo of age. They were then placed on BB or control diets and re-evaluated for changes 1 mo later. Object memory improved significantly in the BB-fed group, whereas the control diet group showed no improvement.

The bulk of the published work on the benefits of blueberries in aging has relied on rodent models [12]. The present study is encouraging in terms of potential human application. First, the present results suggest that even a relatively brief blueberry diet might produce measurable benefits. Second, the benefits of several months of the diet might be maintained for a considerable period after the diet is interrupted. Third, blueberry supplementation might possibly reverse some degree of memory impairment that has already developed. This raises the possibility that this sort of nutritional intervention might still be beneficial even after certain memory deficiencies have become evident.

The present results might help inform the design of future human clinical trials of antioxidant or anti-inflammatory diet supplementation. For example, an impairment-reversal study might have certain advantages over an impairment-prevention study. In a prevention study, one would have to wait a number of years for widely differing degrees of memory impairment to develop at widely differing times in various subjects. In a reversal study, the investigators could purposely recruit subjects who already exhibit a selected degree of impairment. This might greatly decrease random variability and the necessary duration of the study.

## Conclusions

In aging rats, a considerable degree of age-related object memory decline can be reversed and prevented by maintenance for 1 mo on a blueberry-enriched diet. However, a somewhat longer blueberry-enriched diet (2 mo as opposed to 1 mo) resulted in a more prolonged benefit after diet termination.

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