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Research report

Antioxidant-rich diets improve cerebellar physiology and motor learning in aged rats

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Abstract

The free radical theory of aging predicts that reactive oxygen species are involved in the decline in function associated with aging. The present paper reports that diets supplemented with either spinach, strawberries or blueberries, nutritional sources of antioxidants, reverse age-induced declines in β-adrenergic receptor function in cerebellar Purkinje neurons measured using electrophysiological techniques. In addition the spinach diet improved learning on a runway motor task, previously shown to be modulated by cerebellar norepinephrine. Motor learning is important for adaptation to changes in the environment and is thus critical for rehabilitation following stroke, spinal cord injury, and the onset of some neurodegenerative diseases. These data are the first to indicate that age-related deficits in motor learning and memory can be reversed with nutritional interventions.

Themes: Neural basis of behaviour

Topics: Aging

Keywords: Aging; Cerebellum oxidative Stress; Antioxidants; Motor learning

1. Introduction

Oxidative stress and reactive oxygen species (ROS) are proposed to be major contributors to the aging process and to many neurodegenerative diseases such as Alzheimer's and Parkinson's diseases [1,10]. The free radical theory of aging has gained significant support with the demonstration that the life span of fruit flies is increased by over-expression of superoxide dismutase (SOD), an enzyme involved in free-radical breakdown, and catalase [17]. Moreover, mutation in the gene for SOD has been identified in familial amyotrophic lateral sclerosis [7,18]. A decline in normal antioxidant defense mechanisms is postulated to be a causative factor in aging-related declines in biologic function [1,10,15].

There is clear evidence that consumption of fruits and vegetables is beneficial to health. Much of the evidence supporting the protective role of fruits and vegetables comes from epidemiologic literature. The traditional common diet of the Mediterranean region, which is high in fruits and vegetables, is associated with a significant (17%) reduction in overall mortality in the elderly from these regions [25]. The nature of the protective effects of the specific nutrients found in fruits and vegetables, such as β-carotene, vitamin C, and vitamin E is not yet known. Recent studies with vitamin E indicate that high doses can slow the progression of Alzheimer's disease [19]. With few exceptions, however, a single nutrient is not packaged into a single food, and the combinations of nutrients found in foods might have greater protective effects than each nutrient alone.

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Thus, we examined whether the decreases in antioxidant protection that occur in aging are ameliorated by increasing the dietary intake of fruits and vegetables with high antioxidant activity [5,6]. Consumption of such foods reduces the incidence of cancer [23,24] and ischemic heart disease [13,16]. We previously demonstrated that diets high in antioxidants delay the age-related onset of several behavioral and neurochemical deficits when fed to rats from 6 to 15 months of age [14]. The purpose of the present investigation was to examine whether administration of a high-antioxidant diet at a later age would reverse age-related deficits in behavior and β-adrenergic function in the cerebellum.

The cerebellar noradrenergic system, which shows age-related changes in β-adrenergic function that might underlie age-related deficits in motor learning, was chosen as the model system to examine the effects of the diets. The noradrenergic input to cerebellar Purkinje neurons inhibits spontaneous discharge, enhancing the signal-to-noise ratio for both excitatory and inhibitory neurotransmission, and is thus characterized as a modulatory input [8]. Norepinephrine (NE), applied iontophoretically or via activation of the locus coeruleus, potentiates GABA-induced inhibition of cerebellar Purkinje neurons via the β-adrenergic receptor. In young rats, β-adrenergic potentiation of GABAergic function occurs in 70–80% of the recorded cerebellar Purkinje cells, whereas in aged animals, only 30% of these neurons demonstrate an increased response to GABA during application of the β-adrenergic agonist, ISO [2].

Cerebellar NE is necessary for motor learning. Depletion of cerebellar NE or blockade of cerebellar β-adrenergic receptors impairs the ability of rats to improve performance on a runway task where the rats must learn to walk on varying patterns of pegs that protrude from the runway walls. Aged rats are impaired in learning this task, and this impairment is not the result of a loss in motor coordination, it is an impairment in learning [4]. Moreover, in aged rats the impaired performance is strongly correlated with the loss of β-adrenergic receptor function [2,4].

This study examines the effects of feeding diets enriched in antioxidants on cerebellar motor learning and the concomitant loss of β-adrenergic receptor function as studied using electrophysiological techniques.

2. Materials and methods

2.1. Animals

Forty-six male F344 rats at 18 months of age were obtained from the NIA contract colonies for use in this investigation. Rats were fed either control, blueberry, strawberry, or spinach enriched diets (Table 1). Behavioral tests were begun after the initiation of feeding the modified

Table 1
Diets fed to rats

Ingredient	Diet composition (g/kg)			
	Control	+Strawberry	+Spinach	+Blueberry
Casein, alcohol extracted	189.6	189.6	189.6	189.6
L-Cystine	2.8	2.8	2.8	2.8
Corn starch	450.2	435.4	441.1	431.6
Maltodextrin 10	118.5	118.5	118.5	118.5
Sucrose	94.8	94.8	94.8	94.8
Cellulose, BW200	47.4	47.4	47.4	47.4
Soybean oil	42.7	42.7	42.7	42.7
Salt mix[a]	9.5	9.5	9.5	9.5
$CaPO_4$, dibasic	12.3	12.3	12.3	12.3
$CaCO_3$	5.2	5.2	5.2	5.2
Potassium citrate	15.6	15.6	15.6	15.6
Sodium selenite	0.00009	0.00009	0.00009	0.00009
Vitamin mix (V13401)[b]	9.5	9.5	9.5	9.5
Choline bitartrate	1.9	1.9	1.9	1.9
Strawberry extract[c]	–	14.8	–	–
Spinach extract[c]	–	–	9.1	–
Blueberry extract[c]	–	–	–	18.6
Total	1000	1000	1000	1000

[a] Salt mix contains (in g/kg salt mix): NaC1, 259; MgO, 41.9; $MgS0_4 \cdot 7H_20$, 257.6; $CrKSO_4 \cdot 12H_2O$, 1.925; $CuCO_4$, 1.05; KI, 0.035; Fe citrate, 21.0; $MnCO_4$, 12.25; $ZnCO_4$, 5.6; sucrose, 399.64.

[b] Vitamin mix contains (in g/kg vitamin mix): Vitamin A palmitate (500 000 I.U./g), 0.8; vitamin D_3 (100 000 I.U./g), 1.0; menadione sodiumbisulfate (62.5% Menadione), 0.08; biotin (1.0%) 2.0; cyancobalamin (0.1%), 1.0; folic acid, 0.2; nicotinic acid 3.0; calcium pantothenate, 1.6; pyridoxine–HCI, 0.7; riboflavin, 0.6, thiamine–HCI, 0.6; sucrose, 988.42.

[c] Freeze dried aqueous extract (1 g fresh weight plus 2 ml H_20) prepared by homogenizing, centrifuging and then freeze drying. The freeze dried extracts were combined with the control diet; the amount of corn starch in the control diet was adjusted accordingly when the fruit and vegetable extracts were added.

diets. All procedures were performed in an AAALAC approved animal care facility and were approved by the local IACUC.

2.2. Motor learning

The runway consisted of a straight section (127 cm length×25 cm height×6 cm width) with a square goal box at either end (25 cm per side) in which a waterspout was located. The floor of the runway was made of a readily changeable arrangement of 38 horizontal aluminum rods. The rods (diameter=4 mm, 2–4 cm long from the inner wall, and an inter-rod distance of 2.5 cm) could be arranged in either a regular (REG) or irregular (IRR) configuration. Four photocells were arranged so that the beams were 3 cm above the tops of the rods and 20 cm apart from each other to measure the running times of by the rat to proceed from one end of the runway to the other. Water delivery (0.3 ml per trial) was used to reinforce the running across the apparatus. Water delivery was associated with a loud tone that served as a conditioned reinforcer. Water was removed from the rat cages 12 h before training. Shaping of water drinking and running was achieved with the rods covered by a piece of plastic. The rats were maintained under these conditions for 1 week or until they were able to run successfully back and forth on the runway. Rats were weighed daily and weight loss did not exceed 15% of original body weight. Animals were given 3 min ad libitum water access after each daily session. Water was delivered at either end after each successful crossing. After shaping of runway performance, training proceeded by gradually removing sections of the plastic plate covering the rods. Data collection began when the rat performed two successful traverses of the entire length of the uncovered rods in less than 1 min. Daily performance was calculated by determining the running time for 20 successive trials/day. Sessions were conducted for 5 days during this phase of training. Testing: after training on the REG pattern, the rats were not exposed to the runway for 2 weeks. Criterion for day 1 of testing on this phase is six successful crossings with no pauses in a daily session. Daily sessions of 20 trials or 30 min were conducted on the IRR pattern until running time reached asymptotic levels. This usually requires 6–8 days for young rats and 10–14 days for aged rats. The rate of acquisition of performance was determined for each group of rats by plotting the average daily performance versus time. The running times per day for individual rats were fit using the nonlinear mixed effects model of Hirst et al. [12]. The exponential decay model was of the form $Y = A + (100 - A)e^{C(X-1)} \pm S$, where Y is the percent initial runtime, X is the days of training, A is the asymptote as X approaches ∞, C is the rate constant describing the rate of improvement in running time and S is the total standard deviation from the model. The decay constant was used as an index of the rate of learning.

2.3. Electrophysiology

Extracellular action potentials from Purkinje neurons were recorded with a 5 M NaCl filled barrel of a four- or five-barrel micropipette, with input resistance ranging between 1 and 3 MΩ. Action potentials were detected on an oscilloscope, separated from background activity, and converted to constant voltage pulses using a window discriminator. The action potentials were then integrated over 1-s intervals using a ratemeter and displayed on a strip chart recorder. Neurotransmitter agonists were applied at the site of recording with microiontophoresis or micropressure ejection from other barrels of the micropipette. For iontophoresis, a constant-current source provided ejection and retaining currents for the drug barrels and automatically passed an equal current of opposite polarity through a balance barrel containing 3 M NaCl to neutralize the tip potential. Uniform pulses of drug were applied at regular intervals. For pressure ejection, the pressure applications, ranging from 1 to 35 p.s.i. (1 p.s.i.= 6894.76 Pa), were controlled by a Medical Systems (Great Neck, NY, USA) pneumatic pump that regulated the magnitude of pressure delivered and the timing of the pressure pulse. Only data from cells that demonstrated no decrease in action potential amplitude during the response to an agonist and that recovered after exposure to drugs were used. The drug-induced responses were quantified using a computer; the data from ratemeter displays were digitized using a graphics tablet and analyzed for drug-induced changes of firing rate as described above.

3. Results

Aged rats were fed modified AIN-93 diets supplemented with strawberry, blueberry, or spinach extracts (Table 1). Each diet extract provided equivalent antioxidant activity (1.36 mmol Trolox, a soluble form of vitamin E, equivalent per kg diet) except for the control diet, which was not supplemented. Food intake and body weight were not different between the groups of rats. The rats were tested on a motor learning test previously shown to be affected by age and sensitive to noradrenergic function [3,4]. The spinach-fed animals reached a better level of performance than did the controls, as demonstrated by a significantly lower asymptote, or of the learning curve. (Fig. 1, Table 2). There was also a significant improvement in the spinach group when comparing the data for the entire learning curve ($P<0.001$; Wald statistic). There was a trend for improvement in the blueberry-fed aged rats that did not reach statistical significance. When the data were examined as actual running times, the strawberry-fed aged rats were significantly faster than control rats on the first day of testing on the irregular task (Table 2). This initial improved performance might explain the lack of improve-

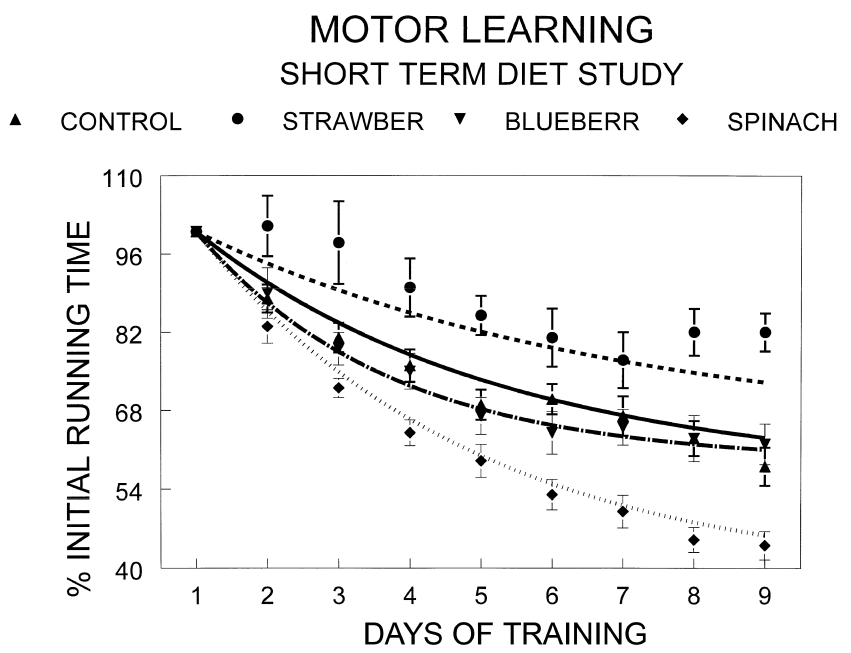

Fig. 1. Learning curves of aged rats on a rod-running motor learning task that involves negotiating a runway of unevenly spaced pegs. The data are represented as percent of running time on the first day of testing where the rat performs at an initial criterion of six crosses. Male Fisher 344 rats ($n=33$) were randomly assigned to one of four groups: control diet (modified AIN-93), or the control diet supplemented with strawberry, spinach, or blueberry extract (Table 1). The behavioral testing was performed at different times for each diet group and a separate group of controls were tested with each diet group, however no difference between control groups was found and the data was pooled. They were fed one of the diets from 18–20 months of age, during which time the behavioral testing was performed. The time to cross the runway decreased with repeated training. Aged rats are impaired in the ability to decrease their running times as compared to young rats (data not shown) and this is reflected in a shallow slope of the learning curve. Addition of spinach to the rat chow significantly improved the rate of learning in the aged rats ($P<0.001$, Wald test chi square). There was also a trend for improved learning in the blueberry-fed group although this did not reach statistical significance. $n=14$ controls, 8 blueberry, 8 spinach and 8 strawberry-fed rats.

ment when normalized values were used in the data analysis, as the rats were already performing significantly better on day 1 and had less room for improvement over time. There was also a trend for the blueberry rats to perform faster on Day 1 although this did not reach significance. The spinach fed rats were not different from controls in terms of actual running times.

The same rats were then examined using extracellular electrophysiologic recordings of cerebellar Purkinje neurons for potentiation of GABAergic inhibition by ISO. Fig. 2 shows an example of data from a control aged rat and a spinach-fed aged rat. In the control animal, ISO did not enhance GABAergic inhibition; however, in the neurons of the spinach-fed rat, ISO enhanced the GABA-induced inhibition from 20 to 95%. When the data were analyzed across groups, there was a significant improvement in all three high-antioxidant diet-fed groups versus controls (Fig. 3).

Glutathione is involved in the response of cells to oxidative stressors [20]. Thus, the effect of the diet on glutathione levels was measured in the cerebellum was examined. Total glutathione levels were increased in the strawberry- and blueberry-fed groups and there was a trend for an increase in the spinach-fed group (Fig. 4).

Table 2
Analysis of percent initial running times by diet group[a]

Diet group	Asymptote (% initial running time)	Decay constant (slope)	Running time (s) (day 1) IRR pattern
Control	56.5±3.1	−0.24±0.04	4.6±0.3
Strawberry	63.72±8.3	−0.17±0.09	2.9±0.1[c]
Blueberry	58.97±3.1	−0.37±0.08	3.8±0.3
Spinach	38.11±3.6[b]	−0.27±0.04	5.4±0.5

[a] Asymptote and decay constant data are derived from fitting the data to a non-linear model as described in Section 2. Asymptotes represent the maximal percentage improvement in running times for each group. Decay constants are the slope of the initial portion of the learning curve and represent the rate of learning of the task.
[b] $P<0.001$ versus control, strawberry and blueberry.
[c] $P<0.01$ versus control and spinach.

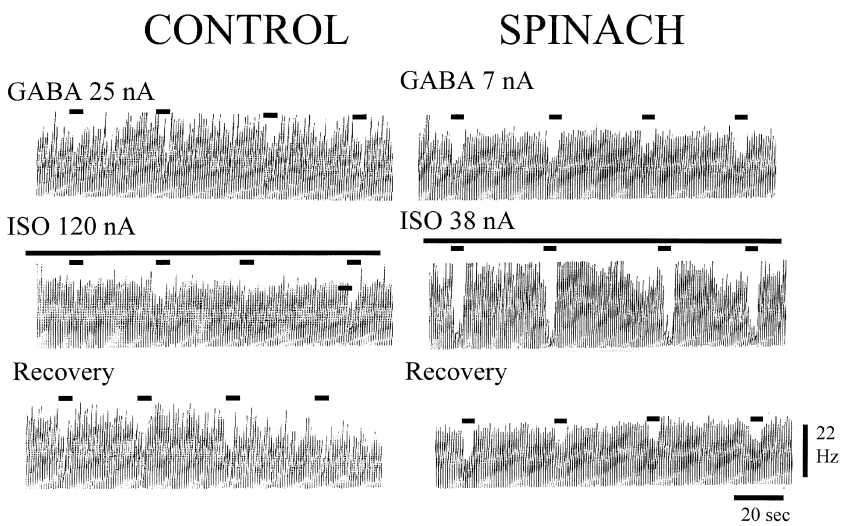

Fig. 2. Ratemeter recordings from Purkinje neurons recorded from an age-matched control (left) and a spinach-fed rat (right) demonstrating ISO potentiation of GABAergic inhibition in the cells of the spinach-fed rat, but not in those of the control rat. Electrophysiologic recordings were performed as described [18]. When GABA is applied (left; bars above the ratemeter) at 25 nA, cell firing rate is inhibited 15%. When ISO is concurrently applied (middle trace), there is no change in the GABA-induced inhibition. On the right side is an example of a Purkinje neuron recorded from a spinach-fed rat. GABA (7 nA; top trace) inhibited the cell firing rate 20%. When ISO is applied concurrently, the GABA-mediated inhibition increases to 95%. Recovery to pre-ISO levels of GABA responses are observed following termination of the ISO application.

4. Discussion

This study is the first to report that diets high in antioxidants can improve performance on a motor learning task and reverse an age-induced decline in cerebellar β-adrenergic receptor function. Thus, nutritional sources of antioxidants are important for improving learning and memory in aged animals and possibly in humans. The effect on learning was observed with a relatively short term feeding regimen, 8 weeks. We have previously observed effects of these diets and other antioxidants in as short a period of time as 2 weeks, thus it is possible that behavioral effects may be observed with even shorter treatments, however, this will need further examination. This investigation did not attempt to delineate which component of the diet was responsible for the observed

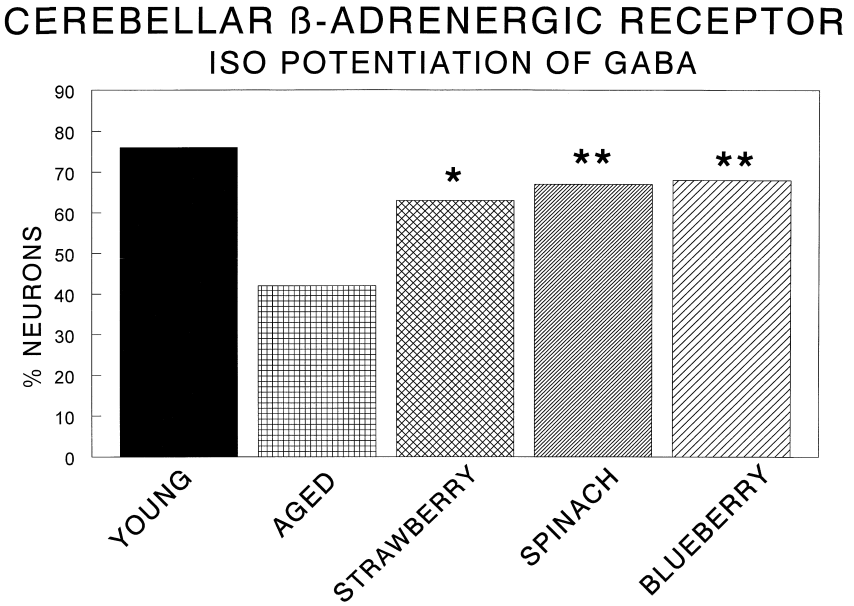

Fig. 3. ISO potentiation of GABAergic inhibition in the cerebellum is observed in a significantly higher percentage of neurons recorded from the blueberry-, strawberry- and spinach-fed groups as compared to controls. Thus, β-adrenergic receptor function in the aged rats fed diets high in antioxidants recover toward what is observed in young rats where 75–80% of the neurons respond to ISO with enhancement of GABAergic inhibition. *$P<0.05$ vs. aged control; **$P<0.001$ vs. aged control, Fisher's exact test ($n=14$ rats, 48 cells, control; 8 rats, 36 cells, blueberry; 8 rats, 40 cells, spinach; 8 rats, 32 cells, strawberry).

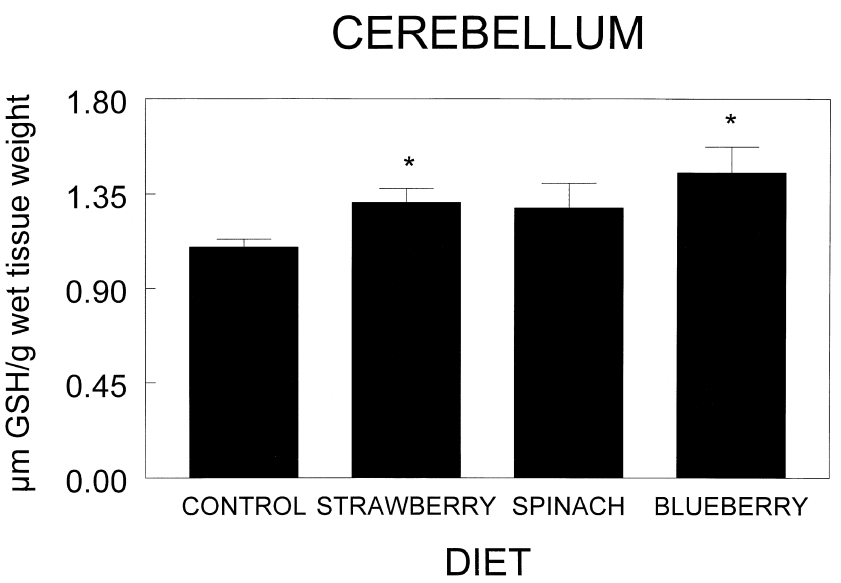

Fig. 4. Graph showing an increase in total glutathione (GSH) levels in the cerebellum of diet-fed groups. There was a significant increase in GSH in the strawberry and blueberry-fed rats (ANOVA [$F=3.235$, $P<0.05$] using a Fisher's LSD post hoc test); $n=9$ controls, 6 strawberry-, 7 spinach-, and 6 blueberry-fed rats.

effects. The diets were added at an equivalent oxygen radical absorbance capacity (ORAC) [5] value. Although all of the diets had roughly equivalent effects in improving cerebellar β-adrenergic receptor function, they were not equivalent in reversing the motor learning deficit. All of the diets improved performance to some degree. The spinach diet, however, was most effective with respect to measures of learning (overall learning curve and percent improvement) on the motor learning task. The strawberry-fed rats were faster than controls initially on the motor learning task, and thus did not have a steep learning curve. The blueberry-fed rats showed a trend towards being faster for the first 3 days of testing compared to controls, and their rate of learning the task also tended to be faster, although this was not statistically significant ($P=0.15$). The improvement in speed during the first few days for the strawberry and blueberry fed rats might be due to improved psychomotor performance as opposed to improved motor learning; however an effect on learning within the first training session would not be detected.

The differences observed between the various diet groups could also be due to differences in the phytochemicals that are present in the diets. Although the diets contained equivalent ORAC, the mixture of phytochemicals was different for each food substance. Further experiments are required to investigate the effects of the various phytochemicals found in these foods.

Previous work indicates a correlation between cerebellar β-adrenergic receptor function and motor learning [2]. All three diets improved the electrophysiologic parameter measured, yet only the spinach diet had a significant effect on the motor learning task. Although it is clear that NE has a role in the learning of motor tasks [9,11,21,22], there might be additional factors important for performance in these tasks. Woodruff-Pak et al. have correlated age-related declines in eyeblink conditioning in rabbits with the number of cerebellar Purkinje neurons [26]. The effect of a loss of cells would be more difficult to reverse than that due to a decline in receptor function, thus early interventions might be more beneficial. Further work is needed to examine this question more closely. In summary, this report demonstrates that nutritional sources of antioxidants reverses age-induced declines in motor learning and cerebellar β-adrenergic receptor function.

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