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Permalink

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Journal

Brain research, 957(2)

ISSN

0006-8993

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Publication Date

2002-12-01

DOI

10.1016/s0006-8993(02)03551-5

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Peer reviewed

Research report

Reversal of biochemical and behavioral parameters of brain aging by melatonin and acetyl L-carnitine

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Accepted 26 June 2002

Abstract

The potential utility of dietary supplementation in order to prevent some of the oxidative and inflammatory changes occurring in the brain with age, has been studied. The cerebral cortex of 27-month-old male B6C3F1 mice had elevated levels of nitric oxide synthase 1 (EC 1.14.13.39) (nNOS) and peptide nitrotyrosine relative to cortices of younger (4-month-old) animals. After 25-month-old mice received basal diet together with 300 mg/l acetyl L-carnitine in the drinking water for 8 weeks, these levels were fully restored to those found in younger animals. A partial restoration was found when old animals received basal diet supplemented with 200 ppm melatonin in the diet. Levels of mRNA (messenger RNA) for nNOS were unchanged following these treatments implying translational regulation of nNOS activity. Behavioral indices indicative of exploratory behavior were also depressed in aged animals. Dietary supplementation with melatonin or acetyl L-carnitine partially reversed these changes. These findings suggest that dietary supplementation cannot merely arrest but indeed reverse some age-related increases in markers of oxidative and inflammatory events occurring with the cortex.
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Theme: Neural basis of behavior

Topic: Aging

Keywords: Melatonin; Acetyl L-carnitine; Nitric oxide synthase; Nitrotyrosine; Mouse; Aging

1. Introduction

There are various reports of age-related increases in free radical-associated damage in mammalian brain [8,27]. One of the major species in effecting such damage is thought to be the very reactive peroxynitrite anion, which is derived from nitric oxide [13]. This anion can modify protein structure by nitrosylation of various polypeptide amino acid residues, notably tyrosine and cysteine. Consequently, in this report we have searched for changes in cortical levels of nitric oxide synthase (NOS) that may occur in the aging mouse. In addition to immunoblot analysis of both inducible NOS (iNOS) and neuronal NOS (nNOS), we

have looked for any changes taking place at the transcriptional level by quantitation of the corresponding mRNAs. The presence of nitrotyrosine residues within protein has also been assayed.

Several laboratories have studied the possibility of mitigation of age-related deficits in CNS function by administration of a range of pharmacological agents, vitamins or other nutritional components. The potential value of two agents in mitigation of changes associated with aging, melatonin and acetyl L-carnitine, has been described. The potential value of melatonin is generally related to its ability to reduce free radical-associated damage, especially that due to reactive nitrogen species [33,37]. There is less consensus on the source of acetyl L-carnitine's effects on brain metabolism [31]. They may be based on the up-regulation of mitochondrial efficacy, which may concurrently increase pro-oxidant events [16].

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However, acetyl L-carnitine has also been reported as having anti-oxidant properties [18]. Another possibility is that this agent may act as an acetyl donor and thus assist in maintaining acetylcholine levels [32]. In the current study, evidence for the utility of the dietary presence of these agents, in attenuating changes in NO-related parameters that occur with aging, has been sought. An attempt has also been made to correlate these age-associated changes in such parameters, and their nutritional modulation, with alterations in behavioral indices.

2. Materials and methods

2.1. Animal treatment

Virgin male B6C3F1 mice, a hybrid between C57BL/6 and C3H from Harlan Labs (Indianapolis, IN), aged 4 months (young group) and 27 months (old group), were housed five per cage and were maintained on a 12-h light/dark cycle in a temperature-controlled ($22 \pm 1^\circ\text{C}$) room. Food and water were provided ad lib to five animals per group. The pelleted basal diet (#101101, Dyets, Bethlehem, PA) consisted of 50% sucrose and 26% casein (w/w) as well as a minimal salt and vitamin mix. For one group of mice this was supplemented with 200 ppm (w/w) melatonin (Sigma, St. Louis, MO) for 8 weeks. For a second group the basal diet was supplemented with 300 mg/l acetyl L-carnitine (Sigma) in drinking water for 8 weeks. Other groups were fed unsupplemented diet.

2.2. RNA extraction

Mice were killed by cervical dislocation; cerebral cortices were excised quickly, immediately frozen on dry ice and stored at -70°C . Total RNA was extracted using the Tri Reagent Kit (Molecular Research Center, Cincinnati, OH), following the manufacturer's protocol. RNA concentrations were determined by absorption at 260 nm wavelength. Purity was monitored by measuring the ratio of absorbance at 260 nm to that at 280 nm.

2.3. Northern blot analysis

Aliquots of total RNA (10 μg each, as determined from absorbance at 260 nm wavelength and verified by gel ethidium bromide fluorescence intensity) were denatured with formaldehyde and formamide, electrophoresed on 1.2% agarose gel containing 6% formaldehyde and transferred onto nylon Zeta-probe blotting membranes (Bio-Rad Laboratories, Hercules, CA). Ethidium bromide fluorescence standardization was selected in preference to use of the housekeeping genes actin- γ or glyceraldehyde phosphate dehydrogenase because expression levels of these genes were responsive to varying age and to melatonin supplementation (data not shown). Membrane-bound RNA

was then hybridized with one of the cDNA probes labeled with [^{32}P]dCTP using the RTS Radprime System (Life Technologies, Rockville, MD) to yield a specific activity of approximately 10^9 cpm/ μg . The membranes were autoradiographed for periods varying from 8 h to 7 days at -70°C on X-ray film (X-OMAT AR, Kodak, Rochester, NY). Probe specificity was checked by verifying that the size (in kbp) of the mRNA transcript matched the size given by the probe manufacturer. Splenic RNA from a 14-month-old mouse served as a positive control for iNOS. A densitometer (Eagle Eye image-processor combined with DNA Scan signal analysis software, Stratagene, San Diego, CA) was used to quantify the signals as area-integrated optical density.

2.4. Western blot analysis

Brain tissues were prepared for measurements of neuronal NOS (nNOS), inducible NOS (iNOS) and nitrotyrosine protein abundance by Western blot analysis using anti-nNOS, anti-iNOS and anti-nitrotyrosine monoclonal antibodies. The procedures for all antibodies were performed in a manner that was identical to that described in our previous studies [45]. Briefly, brain tissues were homogenized at 0°C (20%, w/v) in lysis buffer containing 1% sodium dodecyl sulfate, 1 mM sodium vanadate, 10 mM Tris, pH 7.4, 10 μM pepstatin, 13 μM leupeptin and 1 mM phenylmethylsulphonyl fluoride. The homogenate was centrifuged at $12\,000\times g$ for 15 min at 15°C in order to remove tissue debris without precipitating plasma membrane fragments. Protein content was determined using a Bio-Rad kit (Bio-Rad, Hercules, CA). Tissue supernatants containing 50 μg protein were then size-fractionated on 4–12% Tris–glycine gel (Invitrogen, Carlsbad, CA) at 120 V for 3 h. In preliminary experiments, we found that the given protein concentrations were within the linear range of detection for our Western blot technique. After electrophoresis, proteins were transferred onto Hybond ECL membranes (Amersham, Little Chalfont, Bucks, UK) at 400 mA for 120 min with the use of the Novex transfer system. The membrane was prehybridized in 10 ml buffer A (10 mmol/l Tris–hydrochloride, pH 7.5, 100 mmol/l NaCl, 0.1% Tween 20, and 10% nonfat milk powder) for 1 h and then hybridized for an additional 1-h period in the same buffer containing 10 μl of the anti-nNOS monoclonal antibody (1:1000), the anti-iNOS monoclonal antibody (1:1000), or the anti-nitrotyrosine antibody (1:1000). The membrane was then washed for 30 min in a shaking bath, with the wash buffer (Buffer A without nonfat milk) changed every 5 min before 1 h of incubation in buffer A plus goat anti-mouse IgG–horseradish peroxidase at a final titer of 1:1000. Experiments were performed at room temperature. The washes were repeated before the membrane was developed with a light-emitting nonradioactive method using ECL reagent (Amersham). The membrane was then subjected to autoluminography for 1–5 min. The

autoluminographs were scanned with a laser densitometer (model PD1211, Molecular Dynamics, Sunnyvale, CA) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain before prehybridization. This step verified the uniformity of protein load and transfer efficiency across the test samples. Lysates from a rat pituitary cell line and from mouse macrophages were used as positive controls for nNOS and iNOS, respectively. A blot for nNOS is shown in Fig. 1.

2.5. Measurement of locomotor activity

During the week prior to sacrifice, mice were tested for open field locomotor activity by using a Digiscan Animal Activity Monitor (Accuscan, Columbus, OH). The testing apparatus consisted of an empty plastic cage (40×40×30 cm) with 16 photocell detectors along two perpendicular sides of the box and 16 light-emitting diodes (LEDs) along the other two sides. Another set of eight LEDs and detectors were mounted 7.5 cm above the floor to detect vertical rearing activity. Total distance traveled was measured rather than horizontal activity as this is a more accurate indicator of ambulatory activity. Advantages of the optical beam technique for measuring activity include: (1) Two indices reflecting exploratory activity were emphasized: (i) vertical rearing behavior and (ii) the difference between the time spent at cage margins and that spent in the central area of the test chamber. The test subjects are unaware of the invisible infrared light beams so that behavior is unaffected by the monitoring instrument. (2) The Digiscan is insensitive to activity outside the narrow range of optical beams, unlike proximity or vibration type sensors. (3) The output, collected and printed with a Digiscan Analyser, is digital in nature and very repeatable. The apparatus was cleansed with detergent to remove residual odors.

Testing was conducted between 07:00 and 17:00 h; starting times were randomized among groups. Each animal was tested for 30 min at the same time of day on each of two successive days; the sum of the two values for each parameter was used. The animals were introduced into the cage for the first time on day 1.

Fig. 1. Representative Western blot images of neuronal nitric oxide synthase protein derived from cortex of 4-month-old (young, Y) and 27-month-old (old, O) mice. One group of animals of either age was treated with melatonin (M) while the control group (C) received basal diet. Each lane was derived from a single animal.

2.6. Materials

Rat nNOS and murine iNOS cDNAs were purchased from Cayman Chemical (Ann Arbor, MI); murine glyceraldehyde phosphate dehydrogenase cDNA was purchased from Ambion (Austin, TX). Anti-nNOS and anti-iNOS monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY); monoclonal anti-nitrotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Unless otherwise noted, all other chemicals were obtained from Sigma.

2.7. Statistical analyses

For mRNA expression levels and behavioral data, differences between groups were assessed by one-way ANOVA followed by Gabriel's Test as computed using the Clinstat program [7]. The acceptance level of significance was $P < 0.05$ using a two-tailed distribution.

For protein levels, ANOVA was used, with the data presented as mean $\pm$ S.E.M. Values with $P < 0.05$ were considered significant.

3. Results

The levels of nNOS protein in mouse cortex were derived by Western blotting. These were markedly increased in the aged 27-month-old control animals in comparison to younger 4-month-old control animals, and this was accompanied by a corresponding major elevation of protein nitrotyrosine content (Figs. 2 and 3); changes in both parameters were statistically significant. nNOS protein levels increased from 870 ± 130 units in the younger untreated animals to 1910 ± 60 units in the old untreated

Fig. 2. Densitometric quantitation of cortical nNOS protein. Values $\pm$ S.E. are for 4-month (young) and 27-month (old) mice receiving control diet, acetyl L-carnitine or melatonin supplementation ($n = 4$ animals in each group; $F = 19.8$ ($P < 0.0001$), one-way ANOVA). Means of groups bearing unlike letters differ significantly by Gabriel's test ($P < 0.05$). Values are in arbitrary densitometric units.

Fig. 3. Densitometric quantitation of nitrotyrosine residue-containing protein. Values $\pm$ S.E. are for 4-month (young) and 27-month (old) mice receiving control diet, acetyl L-carnitine or melatonin supplementation ($n=4$ animals in each group; $F=61.5$ ($P<0.0001$), one-way ANOVA). Means of groups bearing unlike letters differ significantly by Gabriel's test ($P<0.05$). Values are in arbitrary densitometric units.

animals; corresponding protein nitrotyrosine levels increased from 280 ± 30 to 780 ± 10 units.

Levels of cortical nNOS mRNA were quantitated with Northern blotting. Levels were not significantly altered with either age or treatment (Table 1). The corresponding levels of both iNOS protein and iNOS mRNA were undetectably low (data not shown).

These parameters were assayed in mice where acetyl L-carnitine was present in the drinking water (300 mg/l) for the preceding 8 weeks (Figs. 2 and 3). The average daily intake of acetyl L-carnitine was 113 mg/kg for young mice and 100 mg/kg for old mice; the difference is accounted for by the greater weight of the old animals, and is likely to be insignificant in view of the uncertainty in the measurement of water consumed. However both nNOS and nitrotyrosine values were dramatically reduced in the old group of mice receiving this treatment, in comparison with the old untreated group (old untreated $\pm$ S.E. units versus ALCAR $\pm$ S.E. units: nNOS, 1900 ± 60 vs. 1090 ± 100 ; nitrotyrosine, 780 ± 10 vs. 310 ± 10). In fact dietary acetyl L-carnitine of aged mice caused these levels to be statistically indistinguishable from corresponding values found for the untreated younger group (nNOS,

Table 1
Cortical levels of nNOS mRNA in 4- and 27-month-old mice after 8 weeks of treatments with melatonin or acetyl L-carnitine

Treatment group	nNOS mRNA level
Young control	1.310 ± 0.230
Old control	1.486 ± 0.261
Young melatonin	1.297 ± 0.192
Old melatonin	1.578 ± 0.257
Young acetyl L-carnitine	1.630 ± 0.391
Old acetyl L-carnitine	1.155 ± 0.116

Values are arbitrary units of optical density and represent mean $\pm$ S.E. ($n=4$). All values did not differ significantly ($P<0.05$).

870 ± 130 units; nitrotyrosine, 280 ± 30 units). Thus treatment could be said to have completely restored the cortical nNOS and nitrotyrosine profiles of aged mice to those found in mice that were 21 months younger. In contrast, treatment of the younger group of mice with acetyl L-carnitine resulted in a significant increase in nNOS and nitrotyrosine concentrations (young untreated $\pm$ S.E. units versus ALCAR $\pm$ S.E. units: nNOS, 870 ± 130 vs. 1450 ± 40 ; nitrotyrosine, 280 ± 30 vs. 480 ± 40).

A parallel study was conducted using dietarily administered melatonin (200 ppm, Figs. 2 and 3). Mean daily intake of melatonin was 28.5 mg/kg for old mice and 22.9 mg/kg for young mice. Melatonin significantly depressed levels of nNOS in old mice from 1910 ± 60 units to levels statistically indistinguishable from those found in untreated young animals (younger untreated, 870 ± 130 units versus old melatonin, 860 ± 110 units). In contrast, melatonin left nNOS levels little changed at 920 ± 80 units in younger animals.

As in the case of nNOS, melatonin also significantly depressed levels of nitrotyrosine in old mice from 780 ± 10 units in the untreated animals to 520 ± 10 units in the melatonin-treated animals. This level remained significantly higher than that of the young controls (280 ± 30 units). However, melatonin also significantly increased the levels of nitrotyrosine in young untreated mice to 530 ± 10 units in the melatonin-treated animals. These responses after melatonin treatment resulted in levels of both nNOS and nitrotyrosine being very similar for treated animals of either age.

One behavioral index relating to exploratory behavior, time spent at cage margins relative to time in center (margin time–center time), was significantly depressed in untreated old animals compared to the young (Fig. 4). Two

Fig. 4. Time that mice spent time at test cage margin, less the time spent in the center of the area. Values represent time in seconds determined during a 1-h testing period. Value represents mean $\pm$ S.E. of values derived from five to seven animals (young control and young melatonin, $n=7$; old control, $n=4$; young acetyl L-carnitine, $n=6$; old melatonin and old acetyl L-carnitine, $n=5$; $F=4.56$ ($P=0.004$), one-way ANOVA). Means of groups bearing unlike letters differ significantly by Gabriel's test ($P<0.05$).

Fig. 5. Mean weight of mice from various groups during the experimental feeding period. Each group consisted of five to seven animals (young control (□) and young melatonin (◇), $n=7$; old control (■) and young acetyl L-carnitine (△), $n=6$; old melatonin (◆) and old acetyl L-carnitine (▴), $n=5$).

other indices, the number of vertical rearing movements and the rate of movement (speed) during horizontal motion were significantly lower in old than in young mice, and these parameters were not modulated by either dietary treatment (data not shown).

Dietary supplementation of younger mice with either melatonin or acetyl L-carnitine had no significant effect on vertical rearing or margin time–center time. However, treatment of older animals with either agent resulted in a partial restoration of values for margin time–center time, to levels closer to those found for younger animals (Fig. 4). Melatonin significantly raised this exploratory parameter to the level found in young animals (time $\pm$ S.E., s: old untreated, 1300 ± 100 ; old melatonin, 1800 ± 250 ; young untreated, 1920 ± 120). Acetyl L-carnitine raised the test time to 1580 ± 90 s, an intermediate value, statistically indistinguishable from the values for untreated animals of either age.

While younger mice continued to gain weight throughout the study, the weight of old mice remained unchanged. There was no detectable effect of either melatonin or acetyl L-carnitine treatment upon the body weights of treated mice (Fig. 5). This implies no major systemic effects on overall animal wellbeing.

4. Discussion

4.1. Age-related changes in untreated animals

During aging, there was a marked increase in both cortical nNOS, the enzyme responsible for most nitric oxide production under basal conditions, and the extent to which tyrosine residues within proteins were nitrosylated. In prior studies, both nNOS and iNOS have been found to be elevated with age, most relevantly in rat cortex [44].

Cortex may thus differ from other brain regions with respect to age-induced nNOS elevation, since nNOS has been measured as unchanged by age in the rat hypothalamus [12,46] and in the rat gracile nucleus [26]. Nitrosative stress has been implicated in both aging and in the pathogenesis of several neurodegenerative diseases [10]. The role of chronic inflammation in such diseases is becoming increasingly recognized. It has been proposed that iNOS, which is readily induced by inflammatory stimuli, may play a major role in aging of many organ systems, including the brain [27]. Finding of elevated iNOS and nitrotyrosine in aged rhesus monkeys [38] supports this. However, there is also evidence suggesting that activation of neuroimmune pathways can activate constitutive nNOS. Thus, administration of a systemic endotoxin, lipopolysaccharide (LPS), can stimulate production of nNOS in the CNS [43]. During human aging, 3-nitrotyrosine content of cerebrospinal fluid increases with age and this increase is even more pronounced in Alzheimer's disease [42]. Age-related increases in mRNA for nNOS have also been described [47]. In our study neither iNOS protein nor mRNA levels of iNOS were detectable in aged mouse cortex which had not been subjected to an inflammatory challenge.

The relative role of various NOS subclasses during aging may in part be species-related. Humans are prone to a variety of neurodegenerative diseases, which possess an extended inflammatory component, and this may be reflected by a chronic up-regulation of iNOS. Lipopolysaccharide stimulates rat [15], but not human [22] iNOS in cultured microglia. Failure of rodent CNS iNOS levels to increase with age may represent a deficiency. When a stimulus, evoking appearance of iNOS mRNA and excess nitric oxide (NO) production in rats is removed, NO levels rapidly return to basal levels [25]. It may be that extended low-level immune responses are difficult to reproduce in rodents. Thus, the primary enzyme responsible for prolonged NO production in this case may be nNOS. Although this enzyme is constitutively present in unstressed animals, it is also capable of upregulation and nNOS appears to be present to a greater extent in older animals.

4.2. Acetyl L-carnitine

Acetyl L-carnitine is a source of acetyl groups available for acetylcholine synthesis and is selectively taken up by the primate brain [20]. It is also involved in mitochondrial metabolism [16] and may have antioxidant properties [18]. In addition, pre-treatment with acetyl L-carnitine can be protective against the oxidative damage incurred with age [23] or consequent to ischemic insult followed by reperfusion [11]. Although in some studies acetyl L-carnitine has failed to slow the progression of neurodegenerative disease [41] or to modify neurochemical parameters significantly [9], acetyl L-carnitine can improve both spatial and temporal memory in aged rats [23]. There is also evidence that

acetyl L-carnitine may be of limited value in treatment of depressive disorders in the elderly [6,14] and of cerebellar ataxia [39].

Our results show that while acetyl L-carnitine can reverse age-related increases in nitrotyrosine and nNOS protein levels in older animals, it also tends to raise these levels in younger animals. This is paralleled by a report that glycerophosphocholine levels in rat brain (which rise with age) are increased by acetyl L-carnitine supplementation in young but lowered in old animals [4]. Another example of ALCAR acting in opposite directions in animals of differing age relates to the mitochondrial respiratory control ratio in rat skeletal muscle. This ratio declines with age, and acetyl L-carnitine lowers it in young and raises it in old animals [17].

The precise mechanism of action by which acetyl L-carnitine may be neuroprotective in relation to aging, is not known. However, long-term feeding studies reveal that it can prevent age-dependent increases in both cholesterol and sphingomyelin [5].

4.3. Melatonin

Melatonin has been described as neuroprotective and this is reported to occur by antioxidant means [21,28,29,48]. However, melatonin is a fairly poor inhibitor of lipid peroxidation, requiring higher than physiological concentrations for any free radical scavenging properties to become apparent [24]. Melatonin is an unusual neuroprotectant in that it has endocrine properties. The mechanisms underlying the biological actions of melatonin upon chronologically related changes, are thus unlikely to be by way of simple antioxidant effects. In fact melatonin may act primarily by enhancing gene expression of antioxidant enzymes [2]. In line with this concept is our finding that melatonin not only diminishes the enhanced production of cortical cytokine mRNAs that typifies aged animals [36], but also prevents age-related changes in complex IV of the mitochondrial respiratory chain [35].

The present finding that melatonin and acetyl L-carnitine can reverse age-related changes in nNOS protein but not nNOS mRNA implies that they act largely at the translational level. The reported antioxidant effects of these compounds are thus likely to be secondary rather than involving direct quenching of active oxygen species.

4.4. Correlation with behavior

The finding that dietary treatment of old mice with acetyl L-carnitine or melatonin, reduced levels of nNOS and protein nitrosylation, in concert with alteration of the behavioral profile toward that present in younger animals, implies that these agents may effect a broad reversal of age-related changes in brain biochemistry and behavior.

Acetyl L-carnitine has previously been reported as beneficial in improving spatial retention in aged rats [40]

and in preventing age-related increases of cortical lipofuscin [1]. This compound also prevented the age-related loss of rearing activity in rat [19]. Melatonin supplementation can also inhibit the appearance of age-related behavioral changes [3,30]. The absence of significant effects of either of these agents upon the behavioral indices studied in younger animals, suggests that their administration only has pronounced effects when a deficit associated with senescence is present.

Previously we found that nitrotyrosine levels in aorta, heart and kidney are elevated in rats fed a combined high fat/high sucrose diet [34], although we did not determine if the effect was due to sucrose alone. Moreover, these increased levels remained virtually unchanged between the 2-month and 2-year time points of the study. We also found that renal total NOS activities were unchanged at the 2-year time point with respect to feeding a standard diet. Therefore, any possible increases in nitrotyrosine induced by dietary sucrose in the present study would be expected to affect young and old animals equally; thus it is unlikely that dietary sucrose contributed significantly to any of the differences in cortical nitrotyrosine or nNOS levels observed between young and old animal groups. The fact that nitrotyrosine levels in non-CNS tissues remain relatively unchanged in our previous study suggests that the age-related change in nitrosative stress in neural cortex may differ substantially from that in other tissues.

This report of the concurrent reversal of age-related trends associated with aging by two dietary supplements, does not address the issue of the relation between the biochemical and behavioral changes found. Many other unexamined cellular events may occur with age and be reversed by such treatments and so which of these is a critical behavioral determinant is unknown. Nevertheless, the relation between elevated NO-related parameters and the decline in behavioral parameters found in aged mice, together with concurrent pharmacological reversal of these indices, suggests a possible connection between active nitrogen species and depression of neurological function.

Acknowledgements

This work was supported in part by grants from the National Institutes of Health (ES 7992, AG 16794 and AG 14882).

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