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Age-related changes in murine CNS mRNA gene expression are modulated by dietary melatonin

Abstract: Brain cellular functions decline with normal aging, accompanied by a changing profile of gene expression. Gene array analysis was used to quantitatively estimate messenger RNA (mRNA) expression levels in the cerebral cortex of both young (4-month) and old (27-month) B6C3F1 male mice. A stringent degree of significance was obtained by using multiple gene chips. Out of 12,423 mRNA levels, only 25 changed significantly with age. Nine of these genes coded for inflammatory proteins, all of which were elevated in aged, relative to younger mice. Melatonin (200 p.p.m.) included in the diet of aged animals for 8 wk elevated serum and cortical melatonin and reversed 13 of the 25 genes altered with age. In no case did melatonin potentiate age-related changes in gene expression. The restoration of a more youthful gene profile to brains of aged animals by melatonin, to a large extent, involves reversal of age-induced elevation of basal inflammatory parameters.

Edward H. Sharman[1], Kaizhi G. Sharman[1], Yuan-Wen Ge[2], Debomoy K. Lahiri[2] and Stephen C. Bondy[1]

[1]Center for Occupational and Environmental Health, Department of Community and Environmental Medicine, University of California, Irvine, CA; [2]Institute of Psychiatric Research, Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN, USA

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Address reprint requests to
Edward H. Sharman PhD, Department of Community and Environmental Medicine, University of California, Irvine, CA 92697-1825, USA.
E-mail: esharman@uci.edu

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Introduction

Aging in animals is frequently associated with a decline in numerous cellular functions, among which are mitochondrial respiration [1], regulation of lysosomal enzyme activities [2], and both mitochondrial and nuclear DNA repair rates [3]. In the brain, a decline in these cellular functions correlates with the cognitive impairment observed in normal aging as well as in age-associated conditions such as senile dementia, Alzheimer disease (AD) and Parkinson disease [4–6]. Parallelling such functional deterioration, age-related changes in gene expression levels have been observed [7–9]. These changes may represent a direct consequence of, or an adaptive response to the functional decline observed in the senescent brain.

In seeking modalities to slow or reverse age-related cognitive decline, one approach is to identify treatments that reverse the age-induced changes in gene expression, so that the gene expression levels in aged animals are restored to a more youthful pattern. Such a reversal has been observed in calorically restricted mice [9]. We have employed a different approach; instead of caloric restriction, we utilized the multifunctional pineal secretory product melatonin [10] as a dietary supplement. Our interest in melatonin and its implications in 'aging' are supported by the following. First, the production of melatonin in the pineal gland declines progressively with age, and in elderly humans the level of melatonin available is much less than that of young individuals [11]. It has been suggested that melatonin may act as a regulator of aging and senescence [12]. We have found that melatonin affects nitric oxide synthase (NOS) in rat cerebellar extracts [13] and amyloid proteins in neuronal cell cultures [14]. The brain deposition of toxic amyloid β-peptide (Aβ) as senile plaques is characteristic of aging and AD.

Secondly, melatonin is known to modulate the expression of numerous genes by a variety of different pathways [15], and so the age-related decline in melatonin levels may be expected to induce concomitant changes in gene expression. In one pathway, the binding of melatonin to nuclear receptor RZR/RORα effects transactivation of a number of inflammatory and stress-related genes [16]. In a second pathway, melatonin alters immune gene expression by modulation of glucocorticoid receptor activity [17]. Modulation of gene expression via melatonin MT1 and MT2 receptors is involved in melatonin regulation of circadian and seasonal rhythms [18]; because the action of these receptors results in the sensitization of the widely utilized signaling enzyme adenylate cyclase, melatonin has the potential to affect gene expression via many other pathways as well.

Thirdly, melatonin may act as an intracellular scavenger of hydroxyl and peroxyl free radicals when administered at pharmacologic doses both in vitro and in vivo [19].

Melatonin has been shown to act as an antioxidant in vivo [20, 21] and its administration reduces the oxidative and neurologic damage in animal models of Huntington disease and Parkinson disease [22]. Thus, melatonin has a protective role for neurons and other cells [23]. Finally, our previous findings suggest that age-related changes in the expression of certain murine central nervous system (CNS) messenger RNA (mRNA) genes can be modulated by dietary melatonin [24–26].

Materials and methods

Materials

Unless otherwise noted, all chemicals were obtained from Sigma (St Louis, MO, USA) in the highest offered purity.

Animal treatment

Male B6C3F1 mice, a hybrid between C57BL/6 and C3H from Harlan Laboratory (Indianapolis, IN, USA), aged 4 months (young group) and 27 months (old group), were housed three or four per cage and were maintained on a 12 hr light/dark cycle in a temperature controlled (22 ± 1°C) room. A hybrid was used in order to take advantage of both the genetic and phenotypic uniformity and the vigor – increased disease resistance, better survival under stress and greater natural longevity – typical of hybrids. The B6C3F1 hybrid was used to optimize genetic similarity to the published mouse genome sequence, which utilized the C57BL/6 strain [27], while maintaining comparability with our previous studies [24–26]. Food and water were provided ad libitum. The control animals were fed a pelleted minimal basal diet (no. 101101, Dyets Inc., Bethlehem, PA, USA) consisting of 50% sucrose and 26% casein (w/w) as well as a minimal salt and vitamin mix. For melatonin-treated mice the basal diet was supplemented with 200-p.p.m. (w/w) melatonin (Sigma) for 8 wk. Thus, there were four treatment groups: young and old control, and young and old melatonin-treated.

Separately caged sentinel animals were maintained in the same room to monitor for the occurrence of infectious disease; no disease was detected during the treatment period. All efforts were made to minimize both the suffering and the number of animals used; all experiments were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine, and conformed to the National Institute of Health guide for the care and use of laboratory animals.

RNA extraction and purification

Mice were killed by cervical dislocation and visually inspected for signs of disease or other pathology; brain tissues were excised quickly, immediately frozen on dry ice and stored at −70°C. Total cortical RNA was extracted using the TRI Reagent kit (Molecular Research Center Inc., Cincinnati, OH, USA), following the manufacturers protocol. Samples from three disease-free animals in each treatment group were combined and aliquots of the pooled total RNA were further purified on an RNeasy column (Qiagen Inc., Valencia, CA, USA) to yield a 260–280 nm absorbance ratio of ≥1.9. RNA concentrations were determined by absorption at 260-nm wavelength. In this way, samples of young control, old control and old melatonin treatment groups were purified (set 1). Later, to assure purification reproducibility, samples from each of all four-treatment groups were purified in a separate operation (set 2).

Gene array processing and analysis

A 20 μg aliquot of total RNA from each sample was processed and applied to a MG-U74Av2 Gene Array chip according to the manufacturer protocol (Affymetrix, Santa Clara, CA, USA). An aliquot from the set 1 young control sample was applied to each of two replicate gene chips; the process was repeated for set 2, resulting in a total of four young control gene chips. A single young melatonin-treated sample was applied to a single gene chip. A single aliquot from the set 1 old control sample and two replicate aliquots from the set 2 old control sample were applied to gene chips, resulting in three old control gene chips. Old melatonin samples were treated similarly, resulting in three old melatonin-treated gene chips. In all, four young control, one young melatonin, three old control and three old melatonin gene chips were prepared and analyzed.

Northern analysis gene expression

Northern analysis was used to measure the expression of mRNA for glial fibrillary acidic protein (GFAP) and β-actin (internal control). The complementary DNA (cDNA) probes for GFAP and β-actin were prepared by one-step reverse-transcription polymerase chain reaction (RT-PCR; Qiagen Inc.). The sense and antisense primers for GFAP were CTCGAATGACTCCTCCACTCC and CATTTGCCGCTCTAGGGAC, respectively. A commercial primer pair (Promega, Madison, WI, USA) was used to prepare the cDNA probe for β-actin. Utilizing a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA), 1.0 μg mouse brain total RNA was subjected to reverse transcription at 50°C for 30 min; initial PCR activation at 95°C for 15 min was followed by 30 thermal cycles each consisting of a deactivation step at 94°C for 1 min, an annealing step at 52°C for 1 min, and an extension step at 72°C for 60 s; thermal cycling was followed by a final extension at 72°C for 10 min.

Melatonin analysis

Levels of melatonin were assayed in serum by a sensitive enzyme-linked immunosorbent assay (ELISA) method after modification from the commercial source (IBL, Hamburg, Germany). The assay is based on the competition principle and microtiter plate separation. Each sample was passed through a C18 reversed-phase column, extracted with methanol, evaporated to dryness, and reconstituted with water. Then each sample was added to the corresponding well coated with the goat-antirabbit antibody against melatonin. An unknown amount of antigen present in the

sample and a fixed amount of enzyme labeled antigen competed for the binding sites of the antibodies coated onto the wells. After incubation for an hour, the wells were washed to stop the competition reaction. Having added the *p*-nitrophenyl phosphate (PNPP) substrate solution, the concentration of antigen was inversely proportional to the optical density measured.

Melatonin standards were used to construct a calibration curve against which the unknown samples were calculated.

Data and statistical analyses

Each processed gene chip was scanned and digitally analyzed according to the manufacturers instruction, using default settings. The resulting data sets corresponding to young control, young melatonin, old control and old melatonin groups were analyzed and compared using the Affymetrix Microarray Suite V5.0 software; classes of genes obtained by use of this software are printed below in *italics*. The mRNA expression levels were obtained as normalized base-2 log values. Background subtraction and scaling were performed so as to provide comparable expression levels between genes for each pair of samples. Default settings were used to classify each gene as *present, absent* or *marginally present*. Pairwise comparisons were made between young control and old control samples as baseline and experimental data sets, respectively. Comparison data sets were selected from the full set of 12,423 genes; for genes with increased expression, only those classified as *present* in the experimental data set were included for further analysis; for genes with decreased expression, genes had to be classified as *present* in the baseline data set for inclusion. In this way, from each pairwise comparison, a set of *c*. 100–300 genes could be identified as having provisionally changed expression levels. Only the genes, which were common to all 12 pairwise comparisons between young control and old control data sets, are presented as having reliable and significant changes in expression. In the same way, 12 pairwise comparisons were made between young control and old melatonin-treated data sets. As negative controls, pairwise comparisons were also made between only the four young control data sets, between only the

Table 1. Genes with significant age-related expression changes for three animal pooled samples of young (4-month-old) compared with old (27-month-old) mouse brain cortices

GenBank number	Gene name	Functional class	Gene expression		Melatonin reversal (%)
			Fold-change	Fold S.E.	
M80423	IgK-C	Immunity	22.4	3.32	59
M18237	Igκ-V	Immunity	4.6	0.40	68
X06454	Cp4	Immunity	2.6	0.09	7
AI553024	Zn finger protein	Transcription factor	2.5	0.28	11
AI845165	PS decarboxylase	Unknown	2.4	0.06	5
M22531	C1qb	Immunity	2.0	0.04	19
AB017349	Ig-V	Immunity	1.8	0.22	103
AF109906	HSP70	Immunity	2.0	0.20	96
AJ007909	Edr	Unknown	1.8	0.09	105
M22998	Glut1	Glucose transport	2.2	0.31	39
AW123269	Anillin	Cell division	1.8	0.07	40
AW047554	CGI-45	Unknown	1.7	0.07	33
AW046181	Sgk	Signal	1.8	0.19	26
X66295	C1qc	Immunity	1.6	0.06	17
X58861	C1qα	Immunity	1.6	0.03	18
X04653	Ly6a	Immunity	1.6	0.09	50
X82648	ApoD	Lipid transport	1.6	0.06	7
X02801	GFAP	Structural	1.5	0.09	(−21)
AI839662	EST R74645	Unknown	1.4	0.03	32
AF096875	Deiodinase	Metabolic regulatory enzyme	1.4	0.07	102
AW125478	Prss11	Putative metabolic regulatory enzyme	1.4	0.03	15
M28845	Krox-24	Memory	0.7	0.04	14
AF064088	TGFβ egr	Transcription factor	0.6	0.02	7
U04827	Fabp7, B-FABP	Intracellular lipid transport	0.5	0.05	43
V00727	c-fos	Signaling: LTP, NFκB, waking	0.5	0.03	4

The fold expression change (displayed along with the corresponding S.E.) is the average of the 12 possible comparisons between four young animal gene chips and three old animal gene chips. Melatonin reversal is the percentage by which melatonin fed to old animals restored young animal gene expression levels; 100% reversal would indicate that the old melatonin gene expression equals the young control gene expression.
Edr, erythroid differentiation regulator; GFAP, glial fibrillary acidic protein; B-FABP, brain fatty acid-binding protein; LTP, long term potentiation; PS, phoshatidylserine.

three old control data sets, and between only the old melatonin-treated data sets.

Expression fold-change was calculated as the power-2 exponential of the $\log_2$ differences; standard errors of the differences between treatment groups were calculated.

For Northern mRNA analyses, differences between groups were assessed by one-way ANOVA; this was followed by Gabriel's test as computed using the Clinstat programme [28]. In all cases, the acceptance level of significance was $P < 0.05$ using a two-tailed distribution.

Results

Twenty-five genes of 12,423 were found to change significantly with age (Table 1). Twenty-one of the 25 increased in expression. Nine of the 21 genes exhibiting increased expression with age were associated with immunity and inflammatory processes while four of the total number of altered genes were related to metabolism.

Gene expression levels for old animals fed melatonin-supplemented diets were measured on a total of three gene chips and were compared with the expression of the same genes in young animals. Melatonin supplementation altered the expression levels of genes in old mice in a direction trending toward the levels of the corresponding genes of young animals in all but one of the 25 age-related genes (Table 1). Thirteen of these mRNA levels were changed in a more youthful direction by more than 20% ($|\log_2(\text{old}) - \log_2(\text{young})| > 0.26$) (Fig. 1). Three of these genes – for deiodinase and erythroid differentiation regulator (Edr) and a nucleotide sequence including the HSP70 gene – were completely restored to the young animal expression level.

Of the 25 genes whose expression varied with age, three were immunoglobulin genes, and four were genes for complement proteins. Melatonin produced significant reversal in all of the immunoglobulin gene levels, but in none of the complement protein genes. The expression levels of the GFAP gene were also measured by Northern analysis of samples of the same animals as used in the gene chip analyses. The Northern analyses, corrected by β-actin expression, confirm the pattern of changes observed with the gene chips [7, 9].

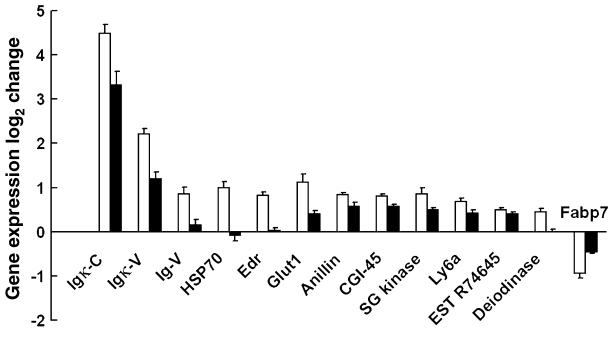

Fig. 1. Age-induced changes in mouse cortex genes showing >20% reversal by melatonin treatment. Graphed values are base-2 logarithms of old to young messenger RNA (mRNA) expression level ratios. □, Old control (27 months) compared with young control (4 months); ■, old melatonin (27 months, 200 p.p.m.) compared with young (4 months). To be included in the figure, the old melatonin to young control gene change had to decline toward the young value by more than 20% of the corresponding old to young gene change $|\Delta\log_2| > 0.26$.

We have also measured levels of melatonin in these animals. In a separate but related study using the B6C3F1 hybrid, dietary melatonin (40 p.p.m.) administered for 11-wk to 27-month-old mice, elevated serum levels from 2.1 ± 0.22 to 39.8 ± 1.1 pg/mL.

Discussion

The age-dependent decline in brain cellular functions correlates with the cognitive impairment observed in normal aging, AD and Parkinson disease [4–6]. These degenerative conditions are associated with increased oxidative damage and inflammation. We have previously found that, within cerebral tissues, dietary melatonin supplementation modulates certain age-related changes [20]. Both plasma and pineal melatonin levels peak in the dark and the diurnal fluctuation plays an important role in the regulation of various neural and endocrine processes that are thus synchronized with the daily photoperiod. In the current study, we sought to discover whether dietary administration of this compound could reverse some of the changes in mRNA content associated with aging. Levels of expression of 12,423 genes were examined with respect to aging and to addition of melatonin. The pooling of RNA samples from several animals was designed to reflect a more typical in vivo profile of genetic expression rather than the idiosyncratic pattern derived from a single animal. Our data suggest that melatonin was indeed able to reverse age-related changes in levels of several genes.

To validate this study, it was necessary to determine whether dietary supplementation with melatonin could increase its level within the nervous system. Whereas we previously measured human plasma melatonin by radioimmunoassay (RIA) [29, 30], now an optimized and highly sensitive nonradioactive ELISA method has been used to measure serum and cortical melatonin. Low but detectable levels of circulating melatonin were observed in B6C3F1 mice and these levels were significantly increased by the dietary supplementation. Since mice were killed during daytime and nocturnal pineal levels of melatonin are increased around sevenfold in C3H mice [31], the levels reached are likely to resemble higher physiological concentrations occurring at night. Dietary melatonin also raised cerebral cortical levels from 2.8 ± 0.3 to 6.4 ± 1.9 pg/mL. Thus, dietary melatonin can both be absorbed in an intact, unconjugated form into the bloodstream, and can also pass across the blood–brain barrier to increase levels within the CNS. Notably, the strain of mouse used (B6C3F1) exhibits normal levels of endogenous melatonin. In contrast, a mutation in the arylalkylamine *N*-acetyltransferase gene of C57BL/6 mice leads to much lower basal levels of melatonin and to disappearance of the nocturnal pulse of melatonin normally present in mammals [32]. However, pulsatile secretion of melatonin is normal in C3H mice [31] – the other parent strain of the B6C3F1 hybrid – as well as in the B6C3F1 hybrid itself [33]. It follows that the effect on changes in levels of certain genes that we observed here is not limited to melatonin-deficient mouse strains and is directly due to melatonin addition.

Genes associated with immunity and inflammation constitute the largest class of genes upregulated with age. Three immunoglobulin genes are upregulated during senescence; all three code for portions of the κ-type light chain. The gene with the largest change (22.4 $\pm$ 3.3-fold) – GenBank M80423 – codes for a constant portion of the κ-chain, while the other two – GenBank M18237 and AB017349 – code for a variable portion. While these latter two genes share noticeable sequence homology, they clearly code for different proteins. For M18237 and AB017349, respectively, there is a considerable difference in the young to old expression change – 4.7 $\pm$ 0.4 versus 1.8 $\pm$ 0.2-fold – and in percentage restoration by melatonin – 68% versus 89%. Some 140 distinct copies of variable κ-type gene segments are reported to occur in the murine genome [34]. Regulation of transcription of immunoglobulin genes is highly complex, involving the interaction of several promoter and enhancer regions, which can bind a variety of regulatory proteins. A great range in transcription rates results from this complex scheme [35]. The fact that only three genes – out of all those coding for the various components of the immunoglobulins – are significantly and consistently upregulated may be an indication of an age-related disregulation of CNS immune system gene expression patterns.

Four complement genes were significantly upregulated with age. This is consistent with the concept that the aged brain is in a relatively inflamed state [36]. This is also in accord with our earlier report of an increase with aging in gene expression of the inflammatory cytokines IL-6 and TNF-α [25]. Melatonin supplementation had little effect on the age-related expression changes of the four complement genes. This is in contrast to immunoglobulin gene expression, which melatonin substantially restored to young animal levels. Among these inflammatory genes, C1q and C4 are also upregulated in AD [37].

Gene expression of brain fatty acid-binding protein (B-FABP, Fabp7) decreased 48% with age. This agrees well with a 60% decrease in cytosolic levels of this protein observed in whole brain homogenate of 25-month-old compared with 4-month-old mice [38].

The age-related changes in gene expression reported using 27-month-old mice are generally consistent with previously reported results for 30-month-old mouse brain neocortex [9]. In both cases, expression of a substantial number of inflammatory and stress-related genes was increased, including complement C4, C1q, GFAP, and apolipoprotein D. Less aged 22-month-old mice did not have a similar increase of inflammatory genes [8]. However, we found the expression of the immediate early stress response gene c-fos to be lowered, whereas its expression was reported to be increased in 30-month-old mice [9].

For data set measurements of the order of 12,000 comparisons, an appreciable number of apparently real values (false positives) could be caused by random experimental fluctuations. This possibility was dealt with in three ways. First, replicates of the samples were run, and only changes found to be significant in all comparisons between young control and old control data sets were accepted; 25 critical genes emerged in this way. Secondly, comparisons between like-treatment chips were performed *viz*., all young control versus young control chips, all old control versus old control chips, and all old melatonin versus old melatonin chips. For each of these critical 25 genes, all resulting changes were averaged. None of the 25 like-treatment fold-changes derived in this manner differed significantly from unity (data not shown). Thirdly, like-treatment comparisons (young control versus young control, old control versus old control and old melatonin versus old melatonin) were calculated for genes determined as *present* on every young control chip; of these 3577 genes, none had a significant fold-change as large as any of the 25 observed genes (data not shown). Thus, the changes in the selected genes represent actual age-dependent changes and are not due to random variations in sensitivity between chips.

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