

# Melatonin treatment in old mice enables a more youthful response to LPS in the brain

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## Abstract

Melatonin modulates the expression of a number of genes related to inflammation and immunity. Declining levels of melatonin with age may thus relate to some of the changes in immune function that occur with age. mRNA expression levels in murine CNS were measured using oligonucleotide microarrays in order to determine whether a dietary melatonin supplement may modify age-related changes in the response to an inflammatory challenge. CB6F1 male mice were fed 40-ppm melatonin for 9 weeks prior to sacrifice at 26.5 months of age, and compared with age-matched untreated controls and 4.5-month-old controls. A subset of both young and old animals was injected *i.p.* with lipopolysaccharide (LPS). After 3 h, total RNA was extracted from whole brain (excluding brain stem and cerebellum), and individual samples were hybridized to Affymetrix Mouse 430-2.0 arrays. Data were analyzed in Dchip and GeneSpring. Melatonin treatment markedly altered the response in gene expression of older animals subjected to an LPS challenge. These changes in general, caused the response to more closely resemble that of young animals subjected to the same LPS challenge. Thus melatonin treatment effects a major shift in the response of the CNS to an inflammatory challenge, causing a transition to a more youthful mRNA expression profile.

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## 1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a short-lived indole produced by the pineal gland during the dark phase of the photoperiod. This neurohormone possesses a number of important biological activities including its action as an endogenous synchronizer. Many studies have identified a regulatory role for melatonin in the immune system (Nelson et al., 1995). Melatonin may also attenuate some aspects of aging (Pierpaoli and Regelson, 1994; Sharman et al., 2004). In aged mice, melatonin treatment can rejuvenate the involuted thymus and partially restore peripheral immune functions (Tian et al., 2003). A greater proportion of old mice than of young succumbs to an *i.p.* injection of LPS (Saito et

al., 2003). Melatonin also increases the survival rate of young mice injected with an otherwise lethal dose of LPS (Carrillo-Vico et al., 2005). The age-related decline in amplitude of circadian melatonin secretion is especially pronounced in aged demented subjects and the decline of its nocturnal peak is correlated with the severity of cognitive impairment (Magri et al., 2004).

The biological activity of melatonin is brought about in part by two high affinity G protein coupled receptors (GPCRs) Mtnr1a and Mtnr1b, by quinone 2 NAD(P)H dehydrogenase (NQO2), and by nuclear receptors RZR/ROR. Inflammatory cytokine expression is also modulated by melatonin; for example, IL-2 production by Jurkat cells from human leukemia, is enhanced by way of a nuclear receptor-mediated mechanism (Guerrero et al., 2000). Melatonin also reduces IL-6 secretion in amyloid  $\beta$  peptide-treated brain slices, in a concentration dependent manner (Clapp-Lilly et al., 2001). Inflammatory processes in the CNS are increased with normal aging (Joseph et al., 2005), and elevated astroglial activation

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in aging in mouse brain is evidenced by increased transcription of GFAP (Goss et al., 1991). In addition rat microglia also increase basal gene expression of IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 cytokines with aging (Yu et al., 2002). We have previously reported the influence of low doses of melatonin in mouse brain on the effect of LPS upon genes relating to immune function (Sharman et al., 2002; Bondy et al., 2005). The current study examined the global changes of cortical gene expression after acute LPS treatment following extended administration of dietary melatonin. Conditional tree analysis demonstrated that melatonin had significant but minor effects on gene expression in the unchallenged old mice. However melatonin-treated old animals responded to an LPS challenge in a manner resembling the corresponding response in young rather than that of old mice receiving basal diet. Thus melatonin treatment effected a major shift in the response of the CNS to the inflammatory challenge of LPS in a direction which was closer to the corresponding response of younger animals.

## 2. Materials and methods

### 2.1. Animal treatment

Male CB6F1 mice, a hybrid between C57BL/6JM and BALB/cJF from Harlan Labs (Indianapolis, IN), aged 4.5 months (young group) and 26.5 months (old group), were housed two to four per cage and were maintained on a 12-h light/dark cycle in a temperature controlled ( $22 \pm 1$  °C) room. The CB6F1 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, while maintaining genetic similarity to the published C57BL/6 mouse genome sequence ref. Food and water were provided *ad libitum*. Young (YC, 2.4 months old) and old (OC, 24.3 months old) control animals were fed a pelleted minimal basal diet (AIN-93M, Dyets #100900, Dyets Inc., Bethlehem, PA) consisting of 10% sucrose and 14% casein (w/w) as well as a minimal salt and vitamin mix. The basal diet of a cohort of the 24-month-old animals (OM) was supplemented with 40-ppm (w/w) melatonin (Sigma, St. Louis, MO) for 9.3 weeks. 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. Three hours prior to sacrifice, half of each group of mice was injected intraperitoneally with 100  $\mu$ l of 300  $\mu$ g/ml of *E. coli* lipopolysaccharide (LPS, Sigma L4005), the other half with 100  $\mu$ l of 0.9% saline.

### 2.2. 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. Cerebellum and brain stem were removed; total RNA was extracted from the remaining tissue using the TRI Reagent kit (Molecular Research Center Inc., Cincinnati, OH), following the manufacturer's protocol. Aliquots of the total RNA were further purified on an RNeasy column (Qiagen Inc., Valencia, CA) to yield a 260 nm to 280 nm absorbance ratio of  $\geq 1.9$ . RNA concentrations were determined by absorption at 260 nm.

### 2.3. cRNA preparation and micro-array hybridization

Total RNA from each of three animals per treatment group was individually prepared for analysis on a Mouse 430 2.0 GeneChip (Affymetrix, Santa Clara, CA). First strand cDNA was synthesized from 20  $\mu$ g of total RNA from each sample using Superscript Choice System (Invitrogen Life Technologies, Carlsbad, CA) and an HPLC purified primer encoding poly(dT) and T7 RNA polymerase promoter sequence (Integrated DNA Technologies, Coralville, IA). In vitro transcription reactions were carried out using Enzo High Yield RNA Transcript labeling kit (Affymetrix and Enzo Life Sciences, Farmingdale, NY). cRNA was extracted using phase lock gels (Eppendorf), ethanol precipitated, purified using RNeasy spin columns (Qiagen, Valencia, CA) and fragmented. Biotin labeled cRNA was hybridized to high-density oligonucleotide micro-arrays, Mouse 430 2.0 GeneChips (Affymetrix) and each GeneChip was read on a Hewlett Packard GeneArray Scanner. The background, noise, housekeeper gene levels and 3'/5' ratio values of all chips were within the quality control limits set by Affymetrix (Supplemental Table A). Micro-array data are being submitted to GEO.

### 2.4. Micro-array analysis

Image files were processed using robust multi-array average (RMA) (Irizarry et al., 2003) and expression data were imported into GeneSpring (Agilent Technologies). The data were converted from  $\log_2$  to linear values; then a per-gene normalization was performed with a median cutoff value of 0.01 for normalization. The cross-gene error model was applied with replicates. To exclude poorly performing probe sets a signal intensity filter was then used to exclude the 5% of probe sets with the lowest expression levels in all conditions. A *t*-test filter was also used to exclude all probe sets with a differential expression *t*-test value of less than 0.2. An expression restriction was applied on control signal values with the lower cutoff value determined by the CGEM average value of base/proportional ratio for each experiment. Following these filters a collection of 26,951 probe sets remained and is referred to as the 'working list'. This list was further filtered using a one-way ANOVA with a *p* value cut off of 0.05 to include only those probe sets with a significant change in expression between any two conditions, 'significantly changed list' (16,661 probe sets). This list was further modified to include only those probe sets with both a

statistically significant change and fold-change of greater than 1.5 following LPS treatment for the three animal groups, YC, OC and OM, the ‘LPS major changes list’. This list is described as the *LPS modulated list* and includes 503 probe sets (Supplemental Table B). These lists were used to generate a variety of conditional trees in GeneSpring using a Standard Correlation. The *LPS modulated list* was also used to generate the gene tree.

### 2.5. Semi-quantitative RT-PCR

Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis was used to confirm array data by a more precise quantitation of the expression levels of selected mRNA transcripts. Equal amounts of total RNA from each sample were amplified using the OneStep RT-PCR kit (Qiagen, Valencia, CA) and a Perkin Elmer GeneAmp 2400 PCR System (Perkin Elmer, Norwalk, CT). Separation and quantitation of the PCR products were performed by capillary electrophoresis with an Agilent 2100 Bioanalyzer. An aliquot of 0.3  $\mu$ l 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 a number of 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. The number of thermal PCR cycles ranged from 25 to 35 and was adjusted as necessary to yield an amount of product appropriate for loading onto the capillary electrophoresis column. Expression levels for each sample were normalized to values of young untreated mice, calculated relative to expression of the *Mthfd1* (methylenetetrahydrofolate dehydrogenase 1) gene used as a control.

### 2.6. Quantitative real-time RT-PCR

Quantitative (real-time) polymerase chain reaction (qRT-PCR) analysis was used to measure the expression levels of

selected mRNA transcripts and for  $\beta$ -actin. Reactions were carried out on a LightCycler instrument (Roche Diagnostics, Indianapolis, IN) using the QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Valencia, CA) according to the manufacturers’ directions. Product fluorescence was detected at the end of the elongation cycle at 72 °C. Melting curves all exhibited a single peak at a temperature characteristic of the primer pair used and none of the primer pairs produced amplicons in the absence of sample or reverse transcriptase. Expression levels for each sample were normalized to values of the corresponding untreated young control group, and were calculated as the average of two determinations, relative to  $\beta$ -actin expression. Using  $\log_2$ -transformed data where appropriate, differences between the six treatment groups were assessed by one-way Analysis of Variance; this was followed by Gabriel’s multiple comparisons procedure test as computed using the Clinstat program (Bland, 2000). In all cases, the acceptance level of significance was  $p < 0.05$  using a two-tailed distribution.

### 3. Results

Condition trees built using either the ‘working list’ of 26,951 probe sets or ‘significantly changed list,’ of 16,661 probe sets, displayed the same relationships between different treatment conditions (Fig. 1). Prior to LPS treatment, the OC and OM groups bore a closer similarity to each other than to the young control group, YC. However after LPS treatment the expression pattern of OM was altered so as to more closely resemble that of YC rather than that of OC. This trend was further enhanced when the condition tree was restricted to those probe sets that were significantly changed by LPS. Thus, although melatonin treatment of old animals unchallenged with LPS did not broadly alter basal gene expression levels, LPS treatment resulted in a response more closely resembling that of the young animal.

The *LPS-modulated list* of those probe sets ( $n=503$ ) which were significantly altered by  $<1.5$  following LPS treatment, was grouped using the gene tree algorithm according to similar

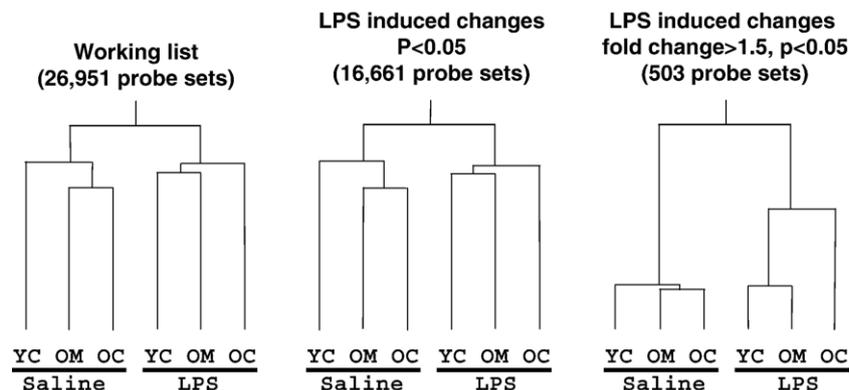


Fig. 1. Gene expression relationships between groups of animals: a condition tree built using (a) the ‘working list’ of 26951 probe sets, (b) ‘significantly changed list,’ of 16,661 probe sets, and (c) ‘LPS major changes list’ of 503 probe sets, showing the degree of identity in relationships between different treatment groups. The Y-axis represents the estimated divergence in gene expression patterns, so that the longer each line is before bifurcation, the more remote the extent of relationship. YC=young control, OC=old control, OM=old melatonin.

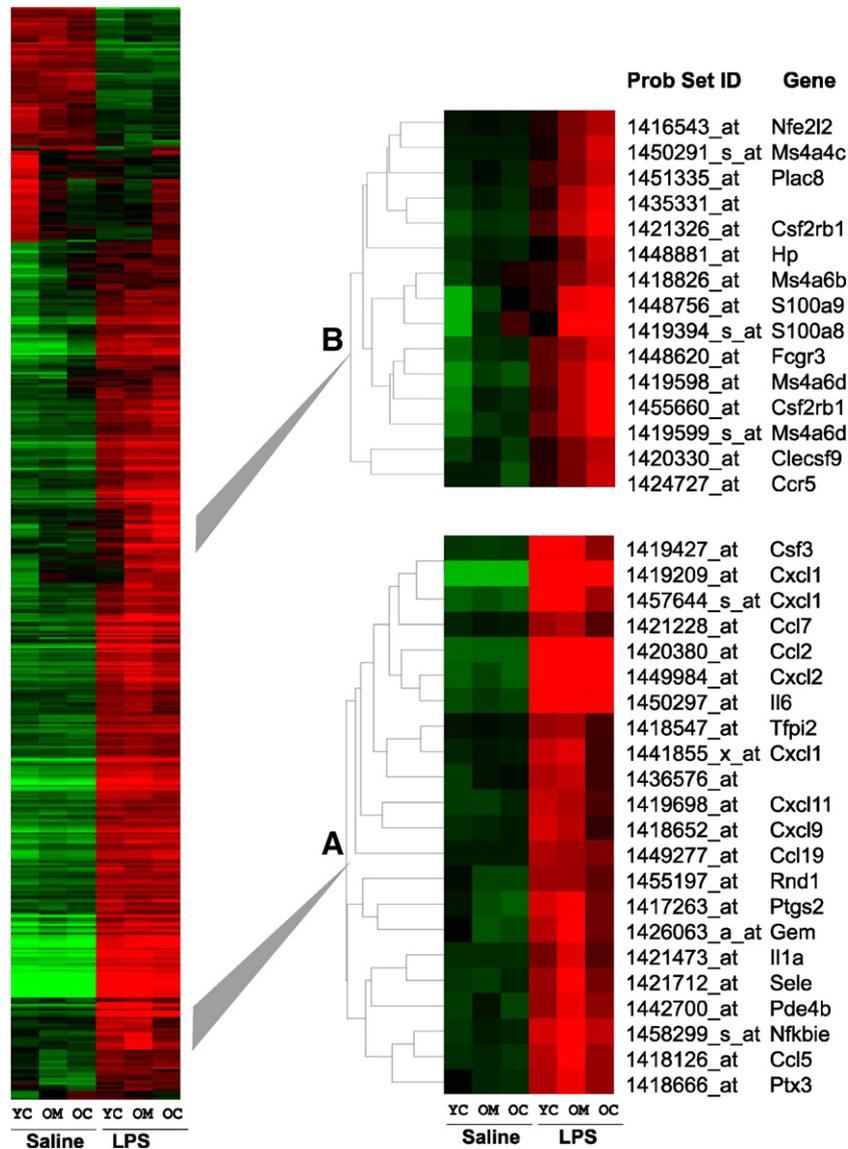


Fig. 2. Comparative intensity of gene expression: a *heat map* of the ‘LPS major changes list’ of 503 probe sets ( $>1.5$  fold change,  $p < 0.05$ ) grouped using the gene tree algorithm according to similar expression patterns. Two gene clusters *A* and *B* whose response to LPS was altered by melatonin in the old animal are expanded and identified. Relative gene expression is represented graphically by coloring each cell corresponding to a particular gene (vertical axis) from an animal of a given age or treatment (horizontal axis). Genes with similar expression patterns are collected and displayed together in clusters. Cells with log ratios of 0.0 (genes unchanged) are colored black, increasingly positive log ratios (increased expression) with reds of increasing brightness, and increasingly negative log ratios (decreased expression) with greens of increasing brightness. YC=young control, OC=old control, OM=old melatonin.

expression patterns. This is expressed in the form of a ‘heat map’ where increasing intensities of green reflect low expression levels and red reflects high expression levels (Fig. 2). Absence of color implies an ‘average level’ of expression. A number of gene clusters, whose response to LPS was altered by melatonin in the old animal, were identified from the gene tree constructed using the LPS-modulated list. One such cluster, *A*, comprised several cytokines, chemokines, and inflammation-related genes, all of which responded to LPS with major increases in expression (Fig. 3).

Responsive genes were catalogued into four classes.

(1) *Genes whose expression increased greatly in young but not old mice after LPS treatment and where a robust*

*response was re-established in old mice following melatonin treatment.*

This group, Fig. 3A (cluster *A*) included interleukin (IL)-6, chemokine (CXC-motif) ligand 1 (Cxcl1), and prostaglandin-endoperoxide synthase 2 (Ptgs2 or COX2). Increases were lower in OC compared to YC, but were elevated in the OM group. The OM group response thus resembled that of the YC group more closely than did the challenged OC group.

(2) *Genes whose expression in old mice responded weakly to LPS and where this reaction was enhanced by melatonin.*

The effects of age and melatonin on the LPS-induced response of two cluster *A* genes, NF $\kappa$ B inhibitor  $\epsilon$  (Nfkbie,

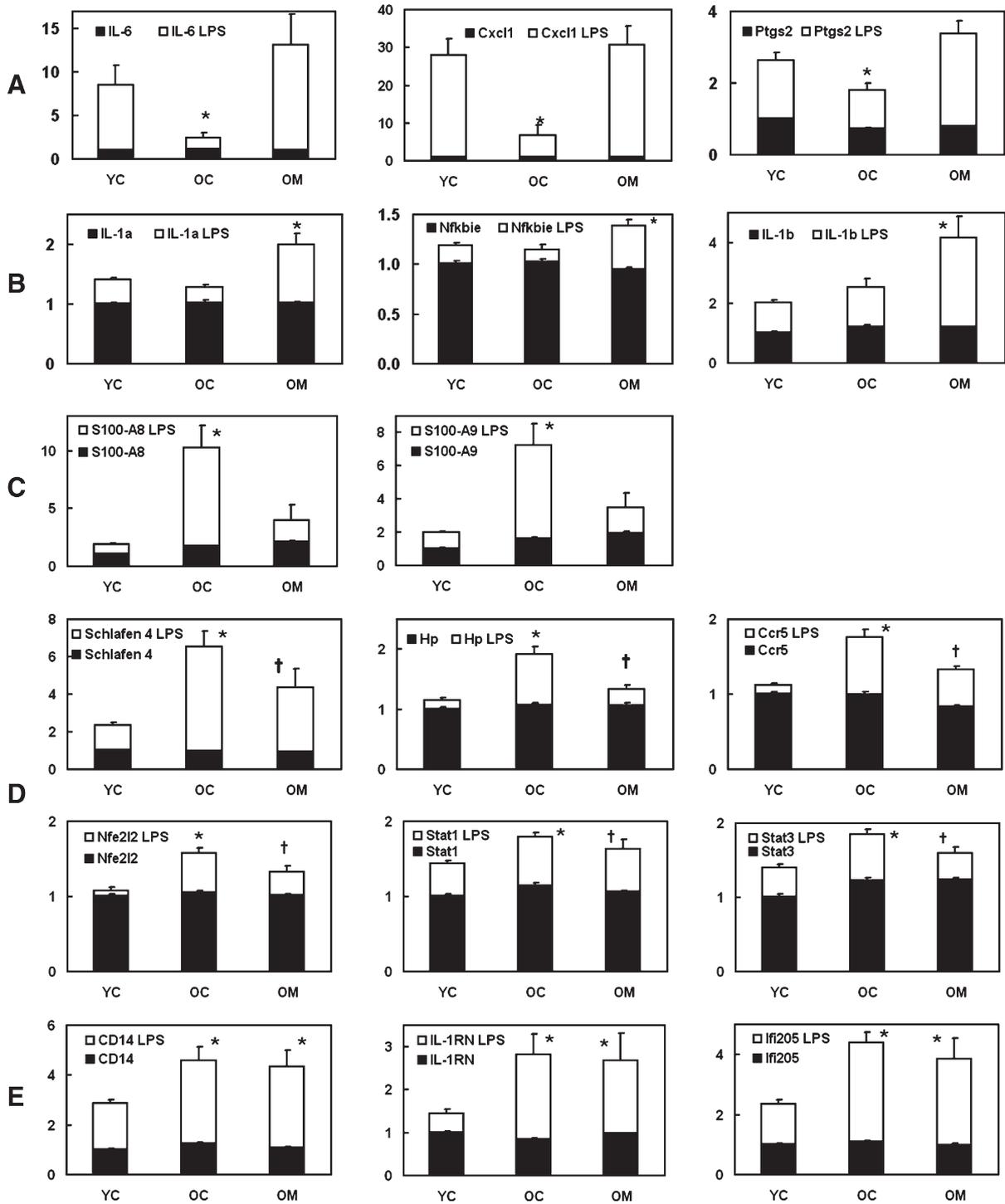


Fig. 3. Gene sets illustrating various response patterns to age and treatment: microarray analysis of cortical levels of several mRNAs in 4.5- and 26.5-month-old mice. Expression levels, relative to young controls, of selected genes in the three groups of mice are compared, with or without LPS challenge. One subset of each group received 40-ppm dietary melatonin for the preceding 9.3 weeks. YC=young control, OC=old control, OM=old melatonin. Each value is mean±S.E. of values from three individual mice. Black bars represent basal values while white bars show the increment gained after LPS injection. Basal levels of all profiles shown were unchanged with age. (A) LPS response depressed with age and restored to young level by melatonin. (B) LPS response unchanged with age, and LPS response in aged increased by melatonin. (C) LPS response increased with age and restored to young level by melatonin. (D) LPS response increased with age and further increased with melatonin. (E) LPS response increased with age not altered by melatonin. Statistically significant differences between mean levels for the six treatment groups (Gabriel's multiple comparisons test,  $p < 0.05$ ): \* = from YC LPS-treated; † = from both YC LPS- and OC LPS-treated.

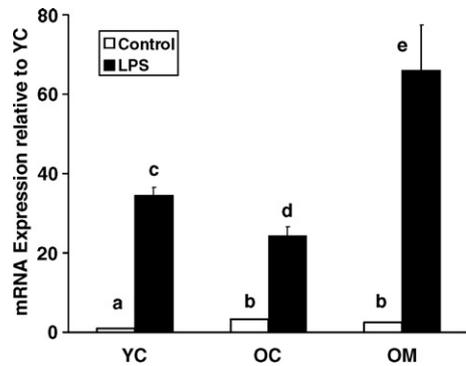


Fig. 4. RT-PCR analysis of cortical levels of mRNA for TNF- $\alpha$  in 4.5- and 26.5-month-old mice. Relative expression levels of selected genes in the three groups of mice are compared, with or without LPS challenge. One subset of each group received 40-ppm dietary melatonin for the preceding 9.3 weeks. YC=young control, OC=old control, OM=old melatonin. Each value is mean  $\pm$  S.E. of values from 3 individual mice. Differences between mean levels marked with different letters are statistically significant ( $p < 0.05$ ).

IL-6 $\epsilon$ ) and IL-1 $\alpha$ , and two additional cytokine genes IL-1 $\beta$  (Fig. 3B) and TNF- $\alpha$  (Fig. 4) were similar: age had little effect, aside from modestly lowering the TNF- $\alpha$  and IL-1 $\beta$  responses, but melatonin substantially amplified the LPS-induced expression of all four genes in old animals.

(3) *Genes whose response to LPS was low in young mice, higher in old mice, and where melatonin attenuated this elevated response of aged animals.*

The expression of a second cluster of genes (within cluster B, Fig. 2) was increased in OC more than YC following LPS treatment. In a number of cases, responses were decreased by melatonin treatment (Fig. 3C–D). Dramatic increases in expression following LPS injection were found for the calgranulins S100A8 and S100A9, members of the calcium binding S-100 protein family (Fig. 3C). These were reduced by melatonin supplementation so that they were not statistically different from those in young. Schlafen 4, a gene involved in T cell development (Tannenbaum et al., 1993), also evinced increased LPS-induced expression in old mice (Fig. 3D). Less pronounced changes with the same pattern were measured for the chemokine receptor Ccr5, haptoglobin (Hp), and the transcription factor Nfe2l2 (nuclear respiratory factor 2, Nrf-2) (Fig. 3D). Also in this group were the genes for Stat1 and Stat3, the Signal Transducer and Activator of Transcription factors through which IL-6 exerts its effects on acute phase response genes.

(4) *Genes whose LPS-induced expression was greater in old animals than in young but which were unaffected by melatonin.*

This group comprised CD14, IL-1 receptor antagonist (Il1rn), interferon-activated gene 205 (Ifi205/p205) and the IL-1 receptor antagonist (IL1-RN) (Fig. 3E). CD14 is a cell-activating receptor for LPS (Wright et al., 1990).

We measured the expression of a subset of genes with quantitative RT-PCR (Table 1). In all cases, the expression patterns generally agreed with the microarray data, and the

changes in expression measured by qRT-PCR were often much larger than those measured on the microarrays. This difference in expression ratios has been noted previously (Jenson et al., 2003). The fact that the expression patterns derived from qRT-PCR were often much larger than those measured on the microarrays, suggests that results from microarrays are more prone to fail to detect differences (Type I errors), rather than to report false positives (Type II errors). In contrast to the microarray data (not shown), RT-PCR analysis of TNF- $\alpha$  showed a dramatic response to LPS (Fig. 4). Previously we observed a similar discrepancy with the TNF- $\alpha$  probe set on the Affymetrix MG-U74Av2 mouse genechip (unpublished data).

#### 4. Discussion

Following administration of LPS, mice respond with a time-dependent pattern of gene expression changes that evolves over a period of up to 72 h (Saban et al., 2001; Utsuyama and Hirokawa, 2002; Zheng et al., 2006). In this exploratory study, a relatively early time point of 3 h after LPS injection was selected to allow enough time for the expression of early-responding genes such as IL-6 and TNF- $\alpha$  to increase, but before more downstream genes could respond. These LPS-related changes at the 3-h time point can be interpreted in either of two ways: (1) the time course of the response may remain unchanged, but the magnitude of the gene expression response may be amplified or reduced, or (2) the magnitude of the overall response may remain unchanged, but the time course of the response may be accelerated or slowed, resulting in a differing expression at the 3-h time point.

Many of the gene changes described here are consistent with the observations that injection of LPS is more lethal for old mice than for young (Saito et al., 2003), and that survival is increased by melatonin, at least in young animals (Carrillo-Vico et al., 2005). The issue as to how gene expression patterns may be consistent with, or in some cases contradictory to, the mortality data is now discussed.

##### 4.1. S100A8 and S100A9

S100A8 (calgranulin A) is expressed by activated microglial cells (Engel et al., 2000) and as such is a marker of chronic inflammation. S100A9 (calgranulin B) is expressed in Alzheimer's disease but not in normal human brains (Shepherd et al., 2006). In peripheral tissues, chemokine calprotectin (the heterodimer of S100A8 and S100A9) attracts neutrophils to sites of infection or inflammation and possesses antimicrobial activity as a consequence of its zinc-binding capacity (Stríž and Trebichavský, 2004). There is evidence that excessive S100A8/A9 protein levels are harmful. In humans, high synovial fluid levels S100A8/A9 are measured in rheumatoid arthritis; concentration increases with disease severity and resistance to treatment (Frosch et al., 2000). S100A8 mRNA expression is greatly induced

Table 1

Confirmation of microarray mRNA expression patterns by quantitative real-time RT-PCR (qRT-PCR) or semi-quantitative RT-PCR

Gene name	Gene ID	YC		YC LPS		OC LPS		OM LPS	
		$\mu$ Array	qRT-PCR						
IL-1 $\alpha$	16175	1.00 $\pm$ 0.03	1.00 $\pm$ 0.01	1.41 $\pm$ 0.04	8.91 $\pm$ 1.54	1.28 $\pm$ 0.04	5.00 $\pm$ 0.24	2.00 $\pm$ 0.18	14.7 $\pm$ 1.5
IL-1 $\beta$	16176	1.00 $\pm$ 0.05	1.00 $\pm$ 0.17	2.01 $\pm$ 0.08	4.58 $\pm$ 0.48	2.54 $\pm$ 0.28	5.47 $\pm$ 1.36	4.19 $\pm$ 0.71	13.7 $\pm$ 5.3
IL-6	16193	1.00 $\pm$ 0.07	1.00 $\pm$ 0.40	8.57 $\pm$ 2.22	10.6 $\pm$ 5.18	2.46 $\pm$ 0.63	2.62 $\pm$ 0.94	13.2 $\pm$ 3.5	12.0 $\pm$ 3.4
IL1RN	16181	1.00 $\pm$ 0.04	1.00 $\pm$ 0.43	1.44 $\pm$ 0.10	3.91 $\pm$ 0.32	2.83 $\pm$ 0.47	7.62 $\pm$ 1.68	2.69 $\pm$ 0.64	7.24 $\pm$ 2.89
NFE2L2	18024	1.00 $\pm$ 0.04	1.00 $\pm$ 0.24	1.08 $\pm$ 0.04	1.34 $\pm$ 0.39	1.57 $\pm$ 0.07	1.92 $\pm$ 0.16	1.33 $\pm$ 0.08	1.56 $\pm$ 0.11
S100A8	20201	1.00 $\pm$ 0.06	1.00 $\pm$ 0.30	1.92 $\pm$ 0.08	13.5 $\pm$ 1.0	10.2 $\pm$ 2.0	142.3 $\pm$ 32.	4.02 $\pm$ 1.32	49.8 $\pm$ 23.8
S100A9	20202	1.00 $\pm$ 0.06	1.00 $\pm$ 0.36	1.98 $\pm$ 0.06	13.2 $\pm$ 2.6	7.24 $\pm$ 1.29	100.3 $\pm$ 26.	3.48 $\pm$ 0.88	49.9 $\pm$ 25.8
STAT1	20846	1.00 $\pm$ 0.04	1.00 $\pm$ 0.05	1.44 $\pm$ 0.04	1.92 $\pm$ 0.15	1.80 $\pm$ 0.06	2.09 $\pm$ 0.08	1.64 $\pm$ 0.12	1.95 $\pm$ 0.30
TNF $\alpha$	21926		1.00 $\pm$ 0.01		34.3 $\pm$ 2.1		24.3 $\pm$ 2.3		65.9 $\pm$ 11.5

mRNA expression levels (mean $\pm$ standard error,  $n=3$ ) relative to young control animals (YC) determined with microarray and by qRT-PCR or semi-quantitative RT-PCR (for IL-1 $\alpha$  and TNF $\alpha$ ). Microarray values are given as ratios relative to normalization and RMA levels derived from Genespring. qRT-PCR values are ratios relative to  $\beta$ -actin (housekeeper gene) expression. For all genes measured, RT-PCR corroborated the rank order of magnitude of expression measured on the microarrays. YC=young control group, OC=old control group, OM=old melatonin-supplemented group; YC LPS, OC LPS, OM LPS=YC, OC, or OM groups injected with lipopolysaccharide.

by  $\beta$ -amyloid in microglia cultured from post-mortem human brain tissue (Walker et al., 2006), while S100A9 protein is found in reactive microglia in Alzheimer's disease brains (Akiyama et al., 1994). Excessive levels of calprotectin can induce symptoms of zinc deficiency and reduced resistance to infection (Sampson et al., 2002), common conditions in the elderly. In our study, LPS modestly induced gene expression of both S100A8 and S100A9 in young animals. This induction was greatly augmented in old animals compared to young, whereas the augmentation was substantially reduced in the melatonin-fed animals. Given that moderate induction of S100A8/A9 is a normal response to infection and that high levels of S100A8/A9 are deleterious, these changes are consistent with the greater LPS-induced mortality in aged mice and the reduced mortality associated with melatonin supplementation reported by others.

#### 4.2. Stat3

Stat3 is alternately-spliced to produce  $\alpha$  and  $\beta$  forms; Stat3 $\beta$  plays a critical role in systemic inflammation, and LPS treatment leads to a transient increase in the Stat3 $\beta$ /Stat3 $\alpha$  ratio (Yoo et al., 2002). Humoral factors have been proposed to be involved in Stat3 activation; candidate factors for mediating LPS-induced Stat3 activation in brain are IL-6, Leptin, and IL-10. IL-10 activates Stat3 and is induced in response to LPS (Barsig et al., 1995). Our finding that the elevated response of Stat1 and Stat3 to LPS in aged animals is reduced by melatonin again suggests that melatonin is able to partially reverse age-related changes in inflammatory responsiveness.

#### 4.3. Chemokines

Chemokines are small proteins with the ability to attract immune cells to sites of infection or injury. In the brain, chemokines can also modulate the action of opioids and

cannabinoids and act widely in concert with neurotransmitters and neuropeptides to influence brain function (Adler et al., 2006). In aged animals, chemokines constituted the largest proportion of genes with a reduced response to LPS that was reversed in melatonin-fed mice (Fig. 2, cluster A).

#### 4.4. IL-1, IL1RN, and IL-6

Expression of IL-1 $\beta$  in the hippocampus has been shown to increase in response to LPS and this induction is further elevated with age (Terao et al., 2002). LPS induction of mRNA for IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 also increases in glial cultures with increasing age (Xie et al., 2003).

These reports are in agreement with our results concerning age- and LPS-related increases in both IL-1 $\alpha$  and IL-1 $\beta$ .

The LPS-induced expression of IL-1 receptor antagonist (IL1RN) expression was greater in OM and OC groups than in YC (Fig. 3E). IL1RN is predominantly expressed in macrophages (Svensson et al., 2004) and blocks the functional receptor from activation by either IL-1 $\alpha$  or IL-1 $\beta$ . IL1RN and IL-1 $\beta$  expression following LPS treatment in PBMC do not correlate, suggesting that they are differentially regulated in response to LPS (Fenton et al., 1988). This is supported by our finding that IL-1 $\alpha$  and IL-1 $\beta$  but not IL1RN expression is modulated by melatonin treatment. Thus, melatonin treatment selectively affects induction pathways responding to LPS treatment in CNS. Increased LPS-induced expression of IL-1 by melatonin in old animals may be secondary to pre-existing increased IL1RN levels in old animals.

The chemokine Cxcl1 mRNA response to LPS was substantially attenuated in old animals. This decline may be a factor in accounting for the lessened inflammatory response to acute illness observed in elderly humans (Bruunsgaard et al., 2001). The gene expression of this inflammatory protein was increased to young-animal levels in the melatonin-fed mice, suggesting it as a possible treatment candidate for restoring the immune response to more appropriate levels in the elderly.

#### 4.5. Selective nature of immune modulation by melatonin

Many other genes involved in the immune response that differ in expression between young and old animals, were unaffected by melatonin and this may provide clues as to mechanism of action. Endothelial mRNA expression of CD14 in the brain, undetectable prior to LPS, dramatically increases following LPS treatment reaching maximal expression at 5 h (Singh and Jiang, 2004). LPS forms a complex with CD14 and LPS binding protein; it is then transferred to the endothelial cell LPS receptor complex consisting of toll-like-receptor 4 and MD2 (Dauphinee and Karsan, 2006). This binding of LPS to CD14 on endothelial cells in the brain and consequent initiation of transcription of proinflammatory cytokines was recently proposed as the mechanism underlying the central effects of peripherally administered LPS (Singh and Jiang, 2004). Since LPS-induced CD14 expression was elevated in old animals and unaltered by melatonin, the initial events following LPS induction may be unaltered by melatonin in old animals. It may be that melatonin acts by modulating cerebral signal transduction downstream to receptor activation by LPS.

#### 4.6. Immune response in the aging brain

The current study reveals that many age-related changes in response to LPS in the brain are reversed by melatonin treatment. These changes include the major regulatory cytokines of the acute phase response: IL-6, TNF- $\alpha$  and IL-1. The robust response of IL-6 to LPS in young animals was essentially abolished in the old and was completely restored by melatonin. This is in agreement with our previous study using a different mouse strain and shorter feeding time (Sharman et al., 2002). Since IL-6 is one of the major controllers of the acute phase response, this implies that melatonin mediates a return to a more effective IL-6 triggering of immune defense genes, downstream to and regulated by this cytokine.

For the genes discussed here, we observed substantially less expression associated with age or melatonin in the absence of an LPS challenge. This suggests that the efficiency of the transcription process under resting conditions is maintained in the aged animal. In contrast, the reduced expression of some genes – and the excessive expression of others – in response to LPS in old animals suggest that the regulation of gene expression in response to a stressful challenge can be greatly modified by age on occasion. This could partially explain the lowered ability of aged animals to withstand such stresses. Moreover, the restoration of some immune-related genes by melatonin, to young-animal expression levels, may play a role in the improved immune function associated with melatonin supplementation.

Age-related increases of both the message and the proteins of inflammatory cytokines within the CNS, together with an enhanced response to LPS have been reported (Terao

et al., 2002; Xie et al., 2003), and the current study reveals the breadth of these changes.

Astrocytes and microglia are the immune-regulatory and inflammatory cells of the central nervous system (Aloisi, 2001; Dong and Benveniste, 2001). They are activated in response to injury or infection resulting in the production of a host of pro-inflammatory mediators including cytokines such as tumor necrosis factor, TNF- $\alpha$ , and interleukins IL-1 and IL-6. Gene expression of these mediators in the brain is induced by LPS (Ban et al., 1992; Gatti and Bartfai, 1993; Sharman et al., 2002). Increases in activation of astrocytes (increased GFAP expression) and microglia occur in the aged brains in numerous species. Microglia may be the main cell type expressing increased IL-6 levels (Ye and Johnson, 1999) and IL-1 $\alpha$  and IL-1 $\beta$  mRNA levels also increase in microglia with aging (Yu et al., 2002). Immune response genes are expressed at high levels in hippocampus, and IL-1, IL-6 and TNF- $\alpha$  effect the induction of hippocampal long-term potentiation (Edagawa et al., 2005; Balschun et al., 2004; Butler et al., 2004). This suggests a distinctive role for these factors in the CNS.

As a result of the association of brain aging with excess inflammation, a range of anti-inflammatory agents has been proposed to be of utility especially in the treatment of Alzheimer's disease. We propose melatonin as a candidate agent, especially as it is both very non-toxic and inexpensive. Standard oral doses of melatonin (0.1–10 mg) result in 400–500 times increased serum concentrations after about 2 h with no serious side effects (Dollins et al., 1994).

We have used microarray analysis of whole brain mRNA to identify robust changes in response to LPS. We compared the response of mice at two different ages to LPS at a single 3-h time point. Consequently, significant gene expression changes within discrete brain regions and at different time points following LPS administration may occur and these would not be detected in this analysis of the cerebral cortex. The goal of this report is to provide a framework for more detailed research on this subject. In order to elucidate the functional significance of the gene expression changes reported here, future experiments are planned that will utilize immunohistochemical analysis of corresponding proteins from more discrete brain regions at varying time points. These will permit precise anatomical localization of changes observed and more definition of their temporal evolution.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2006.09.005.

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