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Dietary melatonin attenuates age-related changes in morphology and in levels of key proteins in globus pallidus of mouse brain.

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Abstract: The ability of melatonin treatment of aged animals to partially restore the pattern of gene expression characterizing the younger animal has been frequently reported. The current study examines the effect of melatonin upon age-related changes of some key proteins relevant to the aging process. These include glial fibrillary acidic protein (GFAP), NF-κB, and protein disulfide isomerase (PDI). In addition, overall neuronal status was examined by Nissl staining and evidence of apoptosis was sought with TUNEL staining. Some age-related changes were in an upward direction (GFAP, NF-κB and the extent of apoptosis as judged by the TUNEL method), while others were depressed with age (PDI and intensity of Nissl staining). However, in either case, melatonin treatment of aged mice invariably altered these parameters so that they came to more closely resemble the levels found in younger animals. The extent of this reversal to a more youthful profile, ranged from complete (for NF-κB) to very minor (for Nissl staining and PDI). Overall, these findings are in accord with prior data on the effect of melatonin on cortical gene expression and confirm the value of melatonin as a means of retarding events associated with senescence.

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November 1, 2012

Dear Sirs,

We are submitting this manuscript entitled "Dietary melatonin attenuates age-related changes in morphology and in levels of key proteins, in mouse brain" for your consideration as a publication in **Brain Research.**

This article is original work and has not been previously submitted to any other journal.

There is no conflict of interest between any of the authors and the substance of this article.

Each author listed has materially participated in the research and article preparation. The precise contributions made are as follows:

Jun Zhou: overall leadership, organization and integration of the project in both the USA and China. Also quantitation of microphotography.
Wang-Jiang-gang: statistical analysis.
Yang Fengzhen, Zhou Li: immunohistochemistry, and microscopy
Wen Puyan and Luo Hao: Tissue dehydration, paraffin embedding and slicing, and Nissl staining
Li Wenwen and Song Zhi, Experimental design and interpretation of results.
Edward Sharman: Responsible for the design and preparation of melatonin diets and their application to animals.
Stephen Bondy: Manuscript preparation, development of overall experimental concept and literature survey.

Thank you for your attending to this matter.

Sincerely yours,

Stephen Bondy, Ph. D.
Professor

Highlights

- The aging mouse brain shows progressively increased levels of indices of cell loss, glial activation, and inflammation. These include glial-fibrilllary acidic protein, NF-kB activation and elevated apoptosis.

- Manifestations reflecting cellular well-being, namely intensity of Nissl staining and levels of protein disulfide isomerase were diminished with age.

- Treatment with dietary melatonin for an extended period altered all these parameters to a greater of lesser extent but always in a direction so that they came to more closely resemble the levels found in younger animals.

- It is concluded that extended treatment with melatonin may retard the rate of onset of some of the deleterious features associated with aging. Some characteristics of younger animals that diminish with aging may also be prolonged by such a dietary protocol.

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Dietary melatonin attenuates age-related changes in morphology and in levels of key proteins in mouse brain

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Abstract

The ability of melatonin treatment of aged animals to partially restore the pattern of gene expression characterizing the younger animal has been frequently reported. The current study examines the effect of melatonin upon age-related changes of some key proteins relevant to the aging process. These include glial fibrillary acidic protein (GFAP), NF-κB, and protein disulfide isomerase (PDI). In addition, overall neuronal status was examined by Nissl staining and evidence of apoptosis was sought with TUNEL staining. Some age-related changes were in an upward direction (GFAP, NF-κB and the extent of apoptosis as judged by the TUNEL method), while others were depressed with age (PDI and intensity of Nissl staining). However, in either case, melatonin treatment of aged mice invariably altered these parameters so that they came to more closely resemble the levels found in younger animals. The extent of this reversal to a more youthful profile, ranged from complete (for NF-κB) to very minor (for Nissl staining and PDI). Overall, these findings are in accord with prior data on the effect of melatonin on cortical gene expression and confirm the value of melatonin as a means of retarding events associated with senescence.

Keywords: Brain aging; melatonin; inflammation; apoptosis; protein folding

1. Introduction

Aging is characterized by a progressive deterioration of physiological functions and metabolic processes. In the aging brain, increased neuroinflammation is evidenced by higher steady-state levels of inflammatory cytokines and decreases in anti-inflammatory molecules (Sparkman and Johnson, 2008). Elevated inflammatory responses in the aged brain can lead to increased neuronal death (Finch and Morgan, 2007, Marchalant et al., 2008) and this may form a platform enabling the progression of neurodegenerative disease (Teeling and Perry, 2009).

Melatonin (N-acetyl-5-methoxytryptamine) was discovered as a hormone of the pineal gland, but it is meanwhile known to be also synthesized in various other organs, tissues, and cells (Pandi-Perumal et al., 2006, Hardeland, 2008). Since melatonin levels decline sharply with age, it has been hypothesized that the reduction of melatonin levels with age contributes to the aging process (Hardeland, 2012). The decline in melatonin production in aged individuals has been suggested as one of the primary contributing factors for the development of age-associated neurodegenerative diseases (Bondy and Sharman, 2010, Pandi-Perumal, 2012). Melatonin is a multifunctional signaling molecule that has a variety of important functions. Numerous studies have shown that melatonin is efficient in preventing cell damage under both acute and chronic states. These include sepsis, asphyxia in newborns, neurodegenerative diseases, cancer and inflammation (Sharman and Bondy, 2010, Sharman et al., 2011, Hardeland et al., 2011, Boga et al., 2012).

Abbreviations: GFAP =glial fibrillary acidic protein, PDI = Protein disulfide isomerase

This suggests a potential therapeutic use for melatonin in different fields of medicine. We have previously shown that during senescence, there is a selective increase in mRNA levels of many genes associated with immune function. Such genes include those for several inflammatory cytokines. These age-related increases can be attenuated or even reversed by extended treatment with dietary melatonin (Bondy and Sharman, 2007, Sharman et al., 2008).

The current study sought to extend this work by examination of some proteins of the signaling pathways associated with the inflammatory cascade. It was found that age related changes in GFAP and NFkB activity, were attenuated by melatonin and that several morphological events associated with senescence were also attenuated by prior melatonin administration. Protein disulfide isomerase (PDI), is thought to be for reconstructing misfolded protein in endoplasmic reticulum lumen and since PDI has been identified as a link between misfolded protein and neuron apoptosis, this role may be important in protection against several neurodegenerative diseases (Hoffstrom et al., 2010, Andreu et al., 2012). Levels of PDI and intensity of Nissl staining in aged mice were found to be enhanced by treatment with dietary melatonin.

2. Results

Treatment of mice with melatonin in the diet did not alter rates of food consumption or accretion of body weight in either young or old mice. In accordance with several prior reports, GFAP levels were increased with age. GFAP was expressed in cortex and the hippocampus area of brain of all groups of mice (Fig. 1a). However, in the globus pallidus,

GFAP stain was reduced staining after melatonin treatment (Fig. 1b). The proportion of astrocytes expressing GFAP can be considered as a marker of astroglial activation. Quantitative analysis revealed that GFAP levels were markedly elevated with age and that this was significantly attenuated by melatonin treatment (Figs 1c). NF-κB levels were also elevated in aged mice and were completely restored to younger levels in older animals treated with melatonin (Figs. 2a,b)

Protein disulfide isomerase (PDI) was visualized using immunohistochemical procedures (Fig. 3a) and staining intensity was quantitated (Fig. 3b). PDI was strongly reduced in cortex with aging and this was reversed to a minor extent by the melatonin treatment.

Nissl staining was used to visualize the regional density of cortical neurons, and was reduced in the globus pallidus of older mice relative to young animals. This reduction was partially but not completely restored by melatonin treatment (Figs. 4 a, b).

The terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP end labeling (TUNEL) method, labels fragmented DNA, which is a characteristic of the apoptotic cells. Little evidence of apoptotic cells was seen in the YC and YM groups, but TUNEL-positive cells were found in the cortex and hippocampus in both groups of older mice (Fig. 5a). Quantitative analysis revealed that apoptotic cells were more prevalent in the OC group than in the OM group (Fig. 5b).

3. Discussion

Aging involves a series of multifaceted and complex degradation processes whose overall effect is to gradually impair cellular and ultimately organismic efficiency. In the nervous

system, these include behavioral, and neuroendocrine changes, leading to progressive loss of ability to adapt effectively to the environment, and increased susceptibility to disease. Melatonin is an indole neuroendocrine hormone secreted by the pineal gland. It was first isolated from bovine pineal gland and characterized by Lerner and his colleagues (Lerner et al., 1958). The amphiphilic nature of melatonin allows it to pass readily through hall biological membranes (Bonnefont-Rousselot and Collin, 2010). Levels of melaton in plasma and in brain decline markedly with senescence (Lahiri et al., 2004). However, application of exogenous melatonin by way of the diet can increase cortical levels of free unconjugated melatonin (Lahiri et al., 2004). This may extend life expectancy, postpone aging, reducing the incidence of age-related diseases (Bondy and Sharman 2010, Tan et al., 2010). The beneficial effects of melatonin have been variously attributed to its properties as an antioxidant, a modulator of apoptosis and a positive regulator of immune functions (Bondy and Sharman 2010, Korkmaz et al., 2012). Melatonin treatment can partially reverse many of the increases in expression of genes related to inflammation that take place with aging (Sharman et al.,2007). This leads to a more youthful response to an inflammatory challenge (Perreau et al., 2007). This influence on gene expression may underlie the many reported attributes of melatonin.

We observed an effect of melatonin treatment on reducing basal GFAP levels in medial globus pallidus. This is in accord with our prior finding that GFAP mRNA expression is also elevated with age (Sharman et al., 2008). Not only is GFAP elevated with age but also its increase in response to brain injury is accentuated and prolonged. This may contribute to

the worse cognitive outcome encountered following traumatic brain injury in the elderly (Sandhir et al., 2008).

The age-related increase in NF-κB immunoreactivity that occurred despite the presence of an exogenous stimulus, was attenuated by melatonin treatment. In nervous tissue, NF-κB is not merely a pro-inflammatory species but also has an important role in regulation of neurogenesis and short-term spatial memory (Mattson, 2005)) and in neuroprotection from stressors (Yang et al., 2007). Since neurogenesis and short-term spatial memory are not improved with aging, inhibition of an age-related elevation of NF-κB is likely to be beneficial rather than detrimental.

The mammalian PDI (protein disulfide-isomerase) family encompasses several highly divergent proteins that are involved in the processing and maturation of secretory proteins in the endoplasmic reticulum by catalyzing the rearrangement of disulfide bonds. Most secretory and membrane proteins contain disulfide bonds that are required for their functions (Vekitch et al., 2012). Protein disulfide bond formation in the endoplasmic reticulum (ER) is also critical for a formation of the correct tertiary structure of proteins, enabling normal protein folding (Lipton et al., 2007, Uehara et al., 2006). Such chaperone activities can have important implications for neurodegenerative processes (Andreu et al., 2012). PDI may help protect the brain tissue by inhibition of pro-inflammatory events (Zhou et al., 2008). The dramatic age-related decrease in PDI that we observed may be a factor underlying the excess inflammation and accretion of proteinaceous deposits found in the senescent brain. Melatonin effect a limited but significant amelioration of this decline, and this may have therapeutic relevance.

Melatonin was able to dramatically reduce the extent of apoptosis in aged mice to levels characteristic of younger mice. Apoptosis is one of the more critical processes implicated in aging and neurodegenerative pathologies, (Caballero and Coto-Montes, 2012). *Melatonin can readily enter subcellular structures, cells and body fluids due to its lipophilic and hydrophilic dual-polarity molecular character. This penetrance capability may account for its ability to xxx* Overall, these findings give further evidence of the potentially protective role of melatonin in retarding the progression of several adverse events associated with senescence.

4. Experimental Procedures

4.1 Animal Treatment

Male B6C3F1 mice, a hybrid between C57BL/6 and C3H from Harlan Labs (Indianapolis, IN), aged 5.5 months (young group) and 23.4 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 ± 1°C) room. The B6C3F1 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. Food and water were provided ad libitum. Young (YC) and old (OC) control animals were fed a pelleted minimal basal diet (AIN-93 M, #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 two similarly aged cohorts (YM and OM, respectively) was supplemented with 40-ppm (w/w) melatonin (Sigma, St. Louis, MO) for 9.3 weeks. Twelve mice were used in each experimental group. 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.

4.2 Tissue preparation

Mice were deeply anesthetized with an overdose of pentobarbital (150 mg/kg, i.p.) and then transcardially perfused with cold phosphate-buffered saline (PBS). After dissection, one half of the brain fixed overnight with 4% p-formaldehyde in PBS, pH 7.4. Thereafter, fixed tissue was stored in PBS/0.02% sodium azide (NaN_3) at 4°C until use. Finally, the fixed brain tissue was further processed by dehydration in a series of graded ethanol solutions prior to paraffin embedding. The blocks were cut in serial 5um thick sections and mounted on adhered slides pretreated with Vectabond reagent (Vector Laboratories, Burlinghame, CA, USA) in a water bath at 45°C. Tissue slides were stored at 4°C before being stained.

4.3 Immunohistochemistry

Sections were heated for 25 m at 56°C and then deparaffinized in a xylene bath followed by rehydration using a graded series of ethanol concentrations. Immunohistochemistry was then carried out after incubation for 20 m at 80°C with antigen unmasking solution (Vector Laboratories, Burlinghame, CA, USA). Endogenous peroxidase in tissue was blocked by treatment with 3% H2O2 in PBS, for 20 m at 25°C. Non-specific background staining was blocked by a 2-h incubation in 2% bovine serum albumin with 0.3% Triton X-100. Sections were then incubated with primary antibodies (GFAP 1:1000 Dako Inc., Carpentaria, CA, USA), NFkB 1:200, PDI 1:500 Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, Sections were then rinsed in PBS with 0.1% Triton X-100, and incubated with biotinylated

secondary antibody (Vector Laboratories) and streptavidin–horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) for 1h at 25°C. Finally, the sections were incubated for 2–5 min with diamino-benzidine (Vector Laboratories). Sections dehydrated in a series of graded ethanol, cleared with xylene, and then cover-slipped with DePeX (BHD; Biomedical Specialties, Santa Monica, CA, USA). As controls, sections were incubated in parallel without primary antibody in order to ascertain that these sections failed to develop specific staining.

Sections were cut with a microtome at a range of 8 um, and put on microscope slides and after a rapid rehydration were placed in 1% Methylene Blue for 30 minutes. Then the slides were dehydrated, put in xylene for 5 minutes, covered with Entellan resinous embedding agent (Merck, Whitehouse Station, NJ), cover-slipped and then images were captured with a Nikon Eclipse 200 photomicroscope.

4.4 Nissl Staining

Nissl staining of formaldehyde-fixed section was used to highlight important structural features of neurons. This was caried out with Nissl stain solution consisting of 20mg each of cresyl violet acetate, toluidine and thionine per 100 ml solution. 0.3 ml glacial acetic acid was added to each 100 ml Nissl stain solution immediately prior to use. The Nissl substance (rough endoplasmic reticulum) appeared dark blue due to the staining of ribosomal RNA, giving the cytoplasm a mottled appearance.

4.5 TUNEL staining

After deparaffinization, the sections were rinsed with 0.1 M PBS, pH 7.4, for 10 min at room temperature, treated with 0.3% Triton X-100 in 0.1 M PBS for 15 min at room temperature,

and rinsed in 0.1 M PBS. The sections were treated with proteinase K (Sigma) 2 µg/ml in 0.1 M PBS for 15 min at room temperature. The sections were washed in 0.1 M for 5 min at room temperature, and then incubated in 0.25% acetic anhydride (Sigma) in water for 30 min. After a wash in sterile dH2O for 5 min, the sections were incubated with prehybridization solution (0.14M Na cacodylate, 1mM cobalt chloride, 0.03Tris-HCl, pH 7.2) for 10 min at RT. The prehybridization solution was then replaced with hybridization solution (prehybridization solution plus reaction mixture: 40U/ml deoxynucleotidyl transferase enzyme, 0.2mg/ml BSA and 0.5nM/ml digoxigenin-11-dUTP) for 2 hours at 37°C. Sections were washed twice for 20 min in 2 x SSC, then washed two more times in 1 x SSC for 15 min and 0.5 x SSC for 15 min at room temperature. Next, sections were washed in 0.1 M Tris for 15 min, pH 7.5, incubated in anti-digoxygenin (DIG)-11 (Roche, Nutley, NJ, USA) at 1:1000 in Tris buffer with 2% BSA for 1.5h, then washed 3 times for 15 m in Tris at room temperature. The sections were next immersed in Tris buffer with 0.1M NaCl and 0.05M MgCl2, pH 9.5,for 15 min, and incubated in Tris buffer in the presence of 4□g/ml nitroblue tetrazolium (NBT) and 1.75 □g/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche) for color development. Sections were rinsed several times in 0.01M Tris buffer with 1 mM EDTA and then mounted on glass slides with water-soluble mounting medium.

4.6 Image Quantitation and Statistical analysis

Immunostaining was observed under a Nikon Eclipse 80i microscope (Nikon, Japan) and images acquired with a Nikon DS high-resolution digital color camera (1280x1024 pixel) using NIS-Elements AR 3.0 software. Digital images were analyzed using Ver.3.00 analysis program (Nikon). Percentage of immunostained area (field area of immunostaining/total

image area x100) was determined for all the markers studied by averaging several images per section that cover all or most of the region of study. All experiments were repeated at least twice, with n= 10 animals per group per marker. All quantitative comparisons were performed on sections processed at the same time.

Single ANOVA statistical analysis was used to assess the significance of the differences in anti-GFAP, NF-κB, PDI and TUNEL staining reactivity among the animal groups. A one-tailed value of $p<0.05$ was accepted as significant.

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Figure legends

Fig 1a. Brain sections immunostained for GFAP. YC = young controls, YM = young melatonin-treated, OC = old control, OM = old melatonin-treated. a) Magnification = X40, GP = medial globus pallidus., Magnification = X40

Fig. 1b. Enlarged aspect of globus pallidus. Magnification = X200. Arrows indicate activated astrocytes.

Fig. 1c. Quantitation of effect of melatonin treatment on GFAP and staining in globus pallidus of 4 and 24 month-old mice. Bars indicate mean of 11-12 animals, ± S.E.M. *: Value for mice treated with melatonin differs from value for corresponding untreated control of same age. #: Old control value differs from that of the young control ($p<0.05$).

Fig. 2a. Immunostaining of the globus pallidus for NF-κB. YC = young control, YM = young melatonin , OC = old control, OM = old melatonin. Magnification = X200

Fig. 2b. Quantitation of effect of melatonin treatment on NFkB staining in globus pallidus of 4 and 24 month-old mice. Bars indicate mean of 11-12 animals, ± S.E.M. *: Value for mice treated with melatonin differs from value for corresponding untreated control of same age. #: Old control value differs from that of the young control ($p<0.05$).

Fig. 3a. Effect of melatonin treatment on protein disulfide isomerase (PDI) immunostaining of globus pallidus. Magnification = X200.

Fig 3b. Quantitation in globus pallidus of young and old animals. YC = young control, YM = young melatonin , OC = old control, OM = old melatonin. Bars indicate mean of 11-12 animals, ± S.E.M. *: Value for mice treated with melatonin differs from value for corresponding untreated control of same age. #: Old control value differs from that of the young control ($p<0.05$).

Fig. 4a. Effect of melatonin treatment on Nissl staining of globus pallidus sections of 4 and 24 month old mice. YC = young control, YM = young melatonin , OC = old control, OM = old melatonin. Magnification = X200.

Fig. 4b. Quantitation of Nissl staining. Bars indicate mean of 11-12 animals, ± S.E.M. *: Value for mice treated with melatonin differs from value for corresponding untreated control of same age. #: Old control value differs from that of the young control ($p<0.05$).

Fig 5a. Effect of melatonin treatment on extent of apoptosis in cortex of 4 and 24 month-old mice as determined by TUNEL staining. Arrows indicate cells with positive TUNEL stain. YC = young control, YM = young melatonin , OC = old control, OM = old melatonin.

Fig. 5b. Quantitation of TUNEL staining. Bars indicate mean of 11-12 animals, ± S.E.M. *: Value for mice treated with melatonin differs from value for corresponding untreated control of same age. #: Old control value differs from that of the young control ($p<0.05$).

Figure 1

Figure 1a

X40

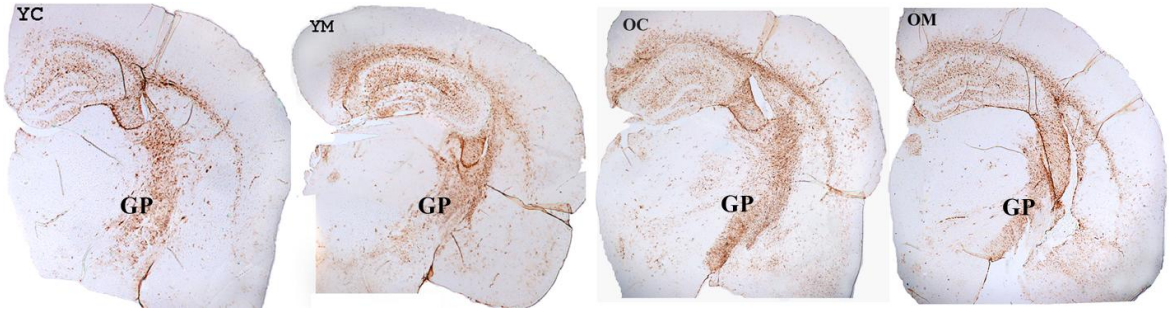

Figure 1b
X200

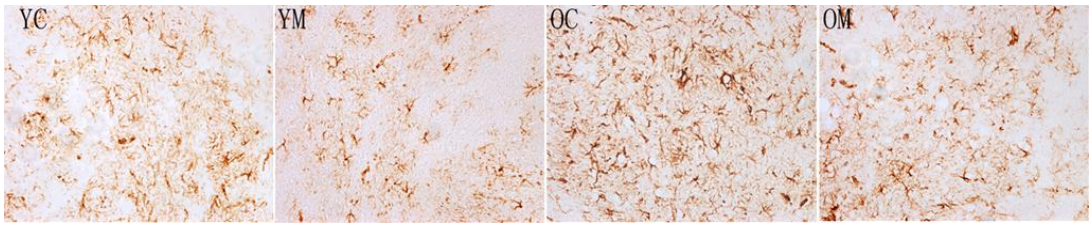

Figure 1c

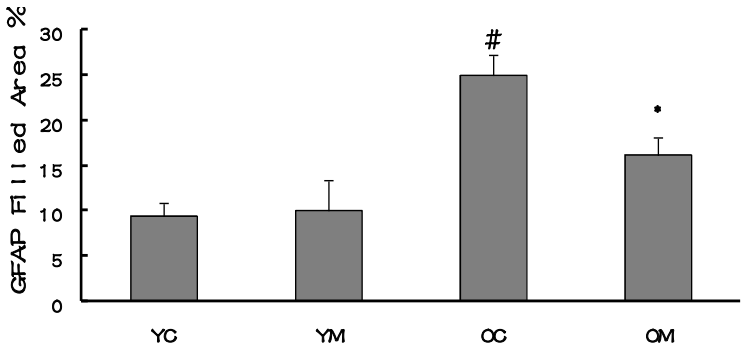

Figure 2

Figure 2a

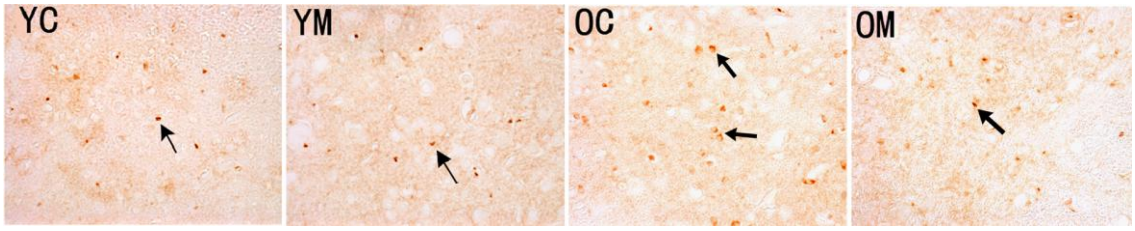

Figure 2b

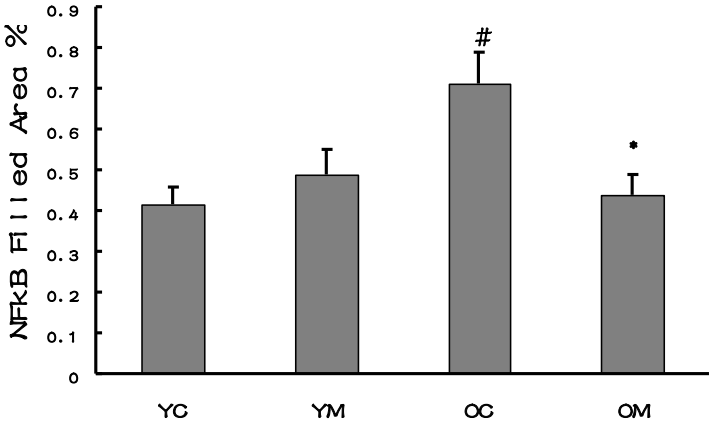

Figure 3

Figure 3a

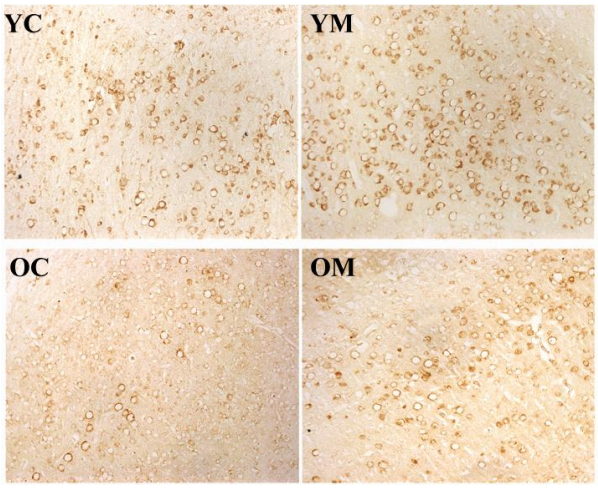

Figure 3b

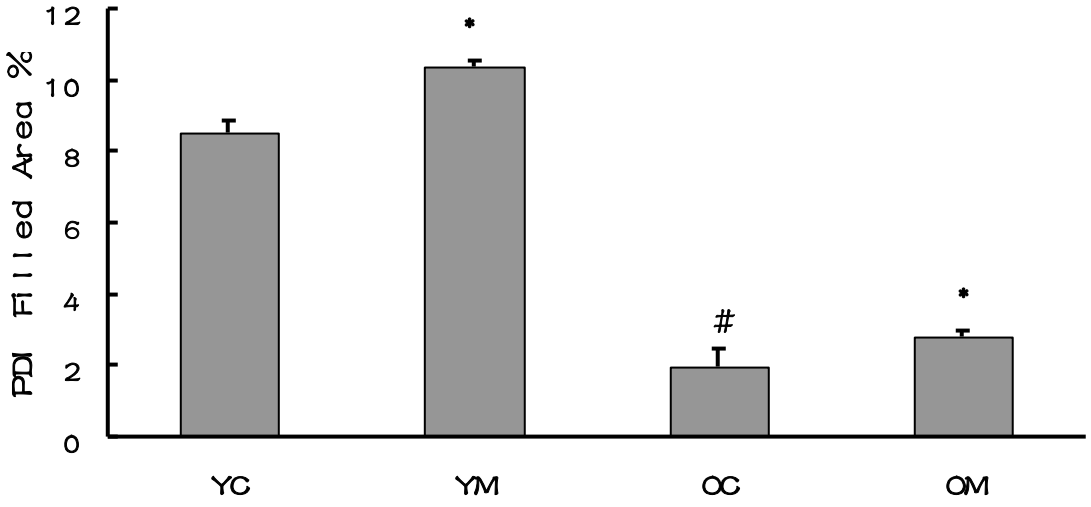

Figure 4

Figure 4a

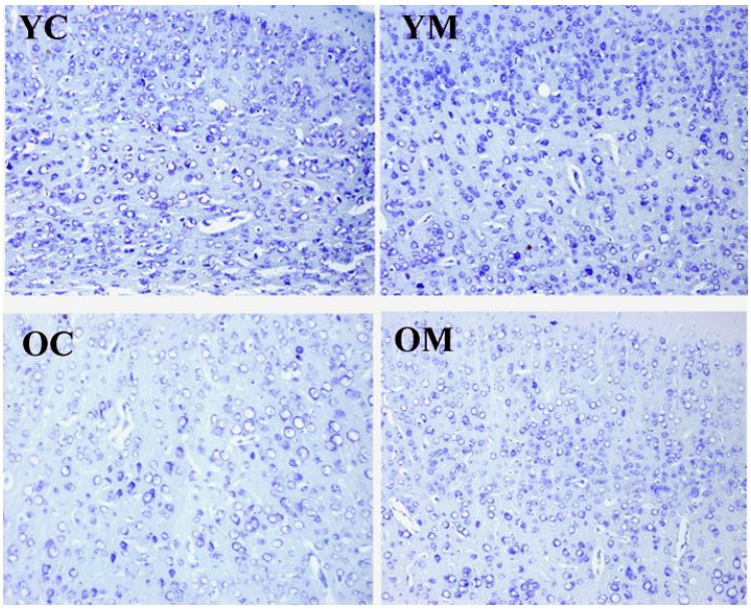

Figure 4b

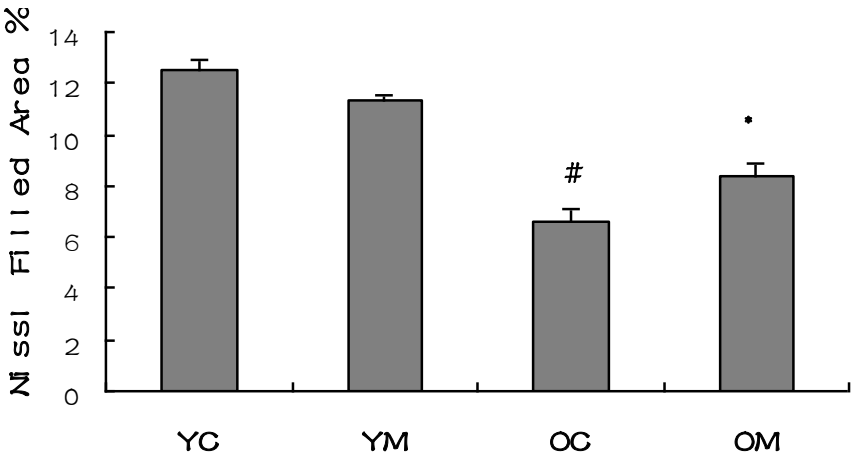

Figure 5

Figure 4a

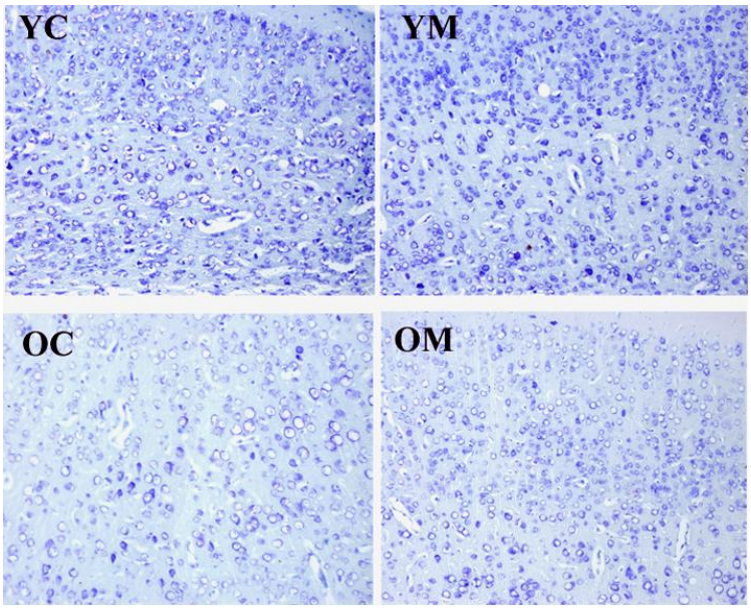

Figure 4b

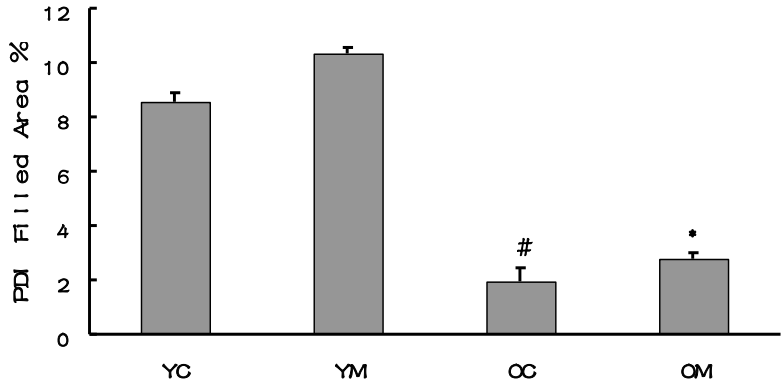

Figure 5a

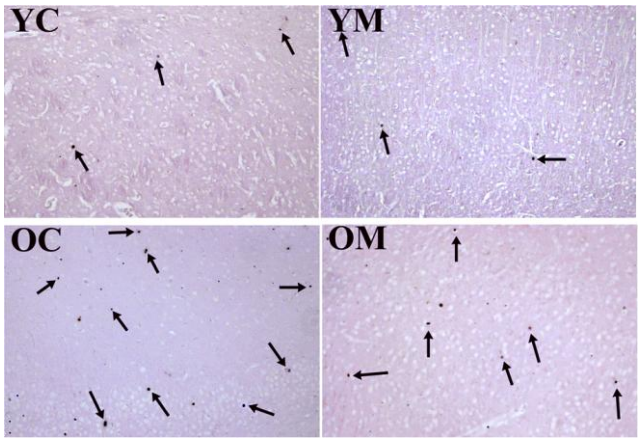

Figure 5b