

# Effect of functional pinealectomy on hippocampal lipid peroxidation, antioxidant enzymes and N-methyl-D-aspartate receptor subunits 2A and 2B in young and old rats

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## **Abstract**

**OBJECTIVES:** To investigate the effects of pinealectomy on lipid peroxidation, antioxidant status and NMDA receptor subunits 2A and 2B concentrations in hippocampus.

**DESIGN:** Forty-eight male Wistar-albino rats were used.

**SETTINGS:** Animals were divided into three groups: 24 h dark throughout the study (highest melatonin release), 24 h light exposure (light-induced functional pinealectomy) and 12 h light/12 h dark exposure (control group). Thereafter, each group was divided into two groups as young and old animals.

**RESULTS:** There was an increase in NR2A and NR2B concentration in DY group compared to all other treatments. CuZn and Mn SOD activities were found to be increased in CO compared to CY group. Continual light exposure for 4 weeks did not change neural CuZn and Mn SOD activities. In old rats, light exposure reduced the activities of both CuZn and Mn SOD relative to those in the young animals. In addition, CuZn and Mn SOD activities were higher in dark exposed rats than in those in the continual light exposed or LD 12:12 rats. GSH-Px activity was found elevated in the DY rats compared to the CY groups. MDA levels were significantly higher in the CO than in the CY group.

**CONCLUSIONS:** NR2A and NR2B receptor concentrations in hippocampus of the rats maintained in dark showed significant increases compared to the control and functional pinealectomy groups but there was no significant increase in lipid peroxidation.

#### ABBREVIATIONS

ROS	– Reactive oxygen species
NMDAR	– N-methyl D-aspartat receptors
CNS	– Central nervous system
NR2A	– NMDA receptor subunit 2A
NR2B	– NMDA receptor subunit 2B
Mn SOD	– Mn superoxide dismutase
CuZn SOD	– CuZn superoxide dismutase
MDA	– Malondialdehyde
CAT	– Catalase
GSH	– Glutathione
GSH-Px	– Glutathione peroxidase
NR1	– NMDA receptor subunit 1
NR2	– NMDA receptor subunit 2
BCIP/NBT	– 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
EGTA	– Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
PVDF	– Polyvinylidene difluoride
CY	– Control young
CO	– Control old
LY	– Light young
LO	– Light old
DY	– Dark young
DO	– Dark old
PBS	– Phosphate buffered saline
EDTA	– Ethylenediaminetetraacetic acid
SDS/PAGE	– Sodium dodecyl sulfate/polyacrylamide gel electrophoresis
TBST	– Tris-buffered saline with Tween 20
BSA	– Bovine serum albumin
LTP	– Long-term potentiation
O <sup>-</sup>	– Superoxide

## Introduction

Of all the organs in the body, the central nervous system (CNS) takes more than its share of oxidative abuse. The major reasons for this are its high utilization of O<sub>2</sub>, its relatively poorly developed antioxidant network, and the fact that it contains large amounts of easily oxidizable fatty acids [1]. Antioxidant defense system becomes insufficient in advanced ages and results in various diseases and symptoms of aging [2]. Melatonin is a secretory product mainly synthesized by the pineal gland and secreted primarily at night, when blood levels reach levels 10 times higher than those present in the daytime [3]. Melatonin is a well-known antioxidant that protects DNA, lipids, and proteins from free radical damage. Melatonin not only scavenges free radicals such as superoxide radicals and hydroxyl radicals, but also activates some antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) [1,3,4]. Furthermore, melatonin has been reported to increase mRNA levels for SOD [5]. Since endogenous melatonin levels fall markedly in advanced ages, the loss of this antioxidant may contribute to the incidence or severity of some age-associated neurodegenerative diseases [6].

The N-methyl-D-aspartate (NMDA) receptor is a member of the group ionotropic glutamate receptors. The NMDA receptor is a heteromeric protein composed of two classes of subunits, NR1 and NR2. Four separate genes encode NR2 subunits, NR2A to NR2D. While NR2 subunits cannot form functional channels when expressed alone, they can alter NMDAR channel prop-

erties when complexed with NR1 subunits [7]. This receptor is involved in a wide variety of processes in the central nervous system (CNS) including synaptogenesis and synaptic plasticity. Additionally, the NMDA receptor has been implicated in excitotoxicity, neurodegenerative disorders and aging [8–11]. Thus, a greater understanding of the modulation of this receptor is likely to be important to the understanding of the physiology and pathophysiology of these processes.

Though there are many reports regarding the effect of melatonin on lipid peroxidation and antioxidant enzymes [1,3–5], the effects of continuous light (low melatonin) and dark (high melatonin) on NMDA receptor concentration in the hippocampus is lacking. Also, it is not known how NMDA receptor concentrations in hippocampi of young and old animals change in response to light and dark. Hence, we have undertaken this study to investigate lipid peroxidation, antioxidant status and NMDA receptor subunits 2A and 2B concentrations in hippocampus of young and old rats exposed to 24h light (functional pinealectomy), 24 h dark and 12h light/12h dark for 4 weeks.

## Material and methods

### *Antibodies and chemicals*

Anti-glutamate receptor NMDAR2A (NR2A), anti-glutamate receptor NMDAR2B (NR2B), monoclonal anti-rabbit IgG alkaline phosphatase conjugate, molecular weight marker kit, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT), leupeptin, aprotinin, sodium orthovanadate, benzamidine, p-nitrophenyl phosphate, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and Immobilon-P polyvinylidene difluoride (PVDF) were purchased from Sigma (St Louis, MO, U.S.A.). All other reagents were of analytical grade or the highest grade available.

### *Experimental procedure*

Forty-eight, male Wistar-albino rats were used in the present experiment. Functional pinealectomy was performed by continuous light exposure [12]. Animals were divided into three groups: 24 h dark throughout the study (highest melatonin release), 24 h light exposure (light-induced functional pinealectomy) and 12 h light/12 h dark exposure (control group). Thereafter, each group was divided into two groups as young and old animals. Finally, there were 6 groups, each containing 8 animals as follows: (1) control young (CY), (2) control old (CO), (3) light young (LY), (4) light old (LO), (5) dark young (DY), (6) dark old (DO). Young rats were three mon of age while old rats were 18–24 mon old. Animals were kept in a room in which humidity and temperature were environmentally controlled. All animals were fed ad libitum with a standard commercial pellet diet and have access to water without restriction for 4 weeks. We hereby declare that the experiments reported comply with the current laws and regulations of the Turkish Republic on the care and handling of experimental animals.

**Table I:** Enzyme activities and MDA levels in hippocampus. Treatment duration was 4 weeks.

	CY	CO	LY	LO	DY	DO
GSH-Px (U/mgprot)	0.16 ± 0.09	0.40 ± 0.04	0.16 ± 0.06	0.38 ± 0.26	1.21 ± 0.73	0.19 ± 0.08
Cu,Zn SOD (U/mgprot)	12.23 ± 1.05	29.16 ± 2.23	10.12 ± 1.87	16.31 ± 1.09	77.87 ± 3.18	58.29 ± 21.89
Mn SOD (U/mgprot)	6.10 ± 1.57	13.84 ± 2.05	5.77 ± 0.64	8.30 ± 0.96	27.38 ± 4.58	11.75 ± 3.31
MDA (nmol/mgprot)	58.76 ± 14.64	93.31 ± 7.31	70.86 ± 9.31	81.95 ± 9.98	65.2 ± 6.5	76.39 ± 8.64

CY: Control young, CO: Control old, LY: Light young, LO: Light old, DY: Dark young, DO: Dark old.

### Biochemical analyses

After sacrificing an animal, the brain was removed and both hippocampi were dissected, washed in ice-cold phosphate buffered saline (PBS) and frozen immediately in deep freezer until further use. One of them was homogenized (1/10, w/v) in a glass-Teflon homogenizer in ice-cold buffer (0.05 M potassium phosphate buffer, pH 7.8). The homogenate was centrifuged at 1,000 xg for 15 min, the supernatant was stored at 4°C and used for the determination of malondialdehyde concentration, and antioxidant enzyme activities.

MDA, an end product of lipid peroxidation, was assayed by the method of Draper and Hadley [13]. Superoxide dismutase activity was measured by the method of Oberley and Spitz [14]. Glutathione peroxidase activity was measured by the method of Paglia and Valentine [15]. Catalase activity was measured by the method of Aebi [16]. Reduced glutathione level was measured by the method of Tietze [17]. Protein in tissue homogenate was assayed by the Lowry method [18].

### Western blot analyses

The other remaining hippocampus (3–4 animals/preparation) was homogenized in ice-cold buffer [50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 25 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM sodium orthovanadate, 10 µM benzamidine and 4 mM p-nitrophenyl phosphate], and an aliquot was taken for protein determination. Equal amounts of protein for each sample (20 µg of protein per lane) were separated by SDS/PAGE on 7.5% minigels, blotted electrophoretically to Immobilon membrane, and incubated in Tris-buffered saline with Tween 20 (TBST) [50 mM Tris-HCl (pH 7.5–8.0), 150 mM NaCl, and 0.1% Tween 20] containing 3% bovine serum albumin for 30 min. Blots were incubated overnight with anti-NR2A (1:3000) or anti-NR2B (1:5000) in 1% BSA. Blots were then subjected to three additional 10-min washings in TBST. Blots were incubated with alkaline phosphatase conjugated monoclonal anti-rabbit IgG (1:10000) in 1% BSA for 1h at room temperature and three additional 10-min washes performed with TBST. The membrane was incubated in 20 mL of fresh reagent solution (BCIP/NBT) until color development. Images of immunoblots were analyzed with a computerized image analysis system (UVIPHOTO MW V.99, Cambridge, UK).

### Statistics

Data are given as mean ± standard deviation. Statistical analysis of data was performed on computer by using SPSS Version 9.0. Kruskal Wallis test was used for comparison of six groups. If a difference was detected by using Kruskal Wallis test, the Bonferroni-corrected Mann-Whitney U test was used to determine which two groups were significantly different.

### Results

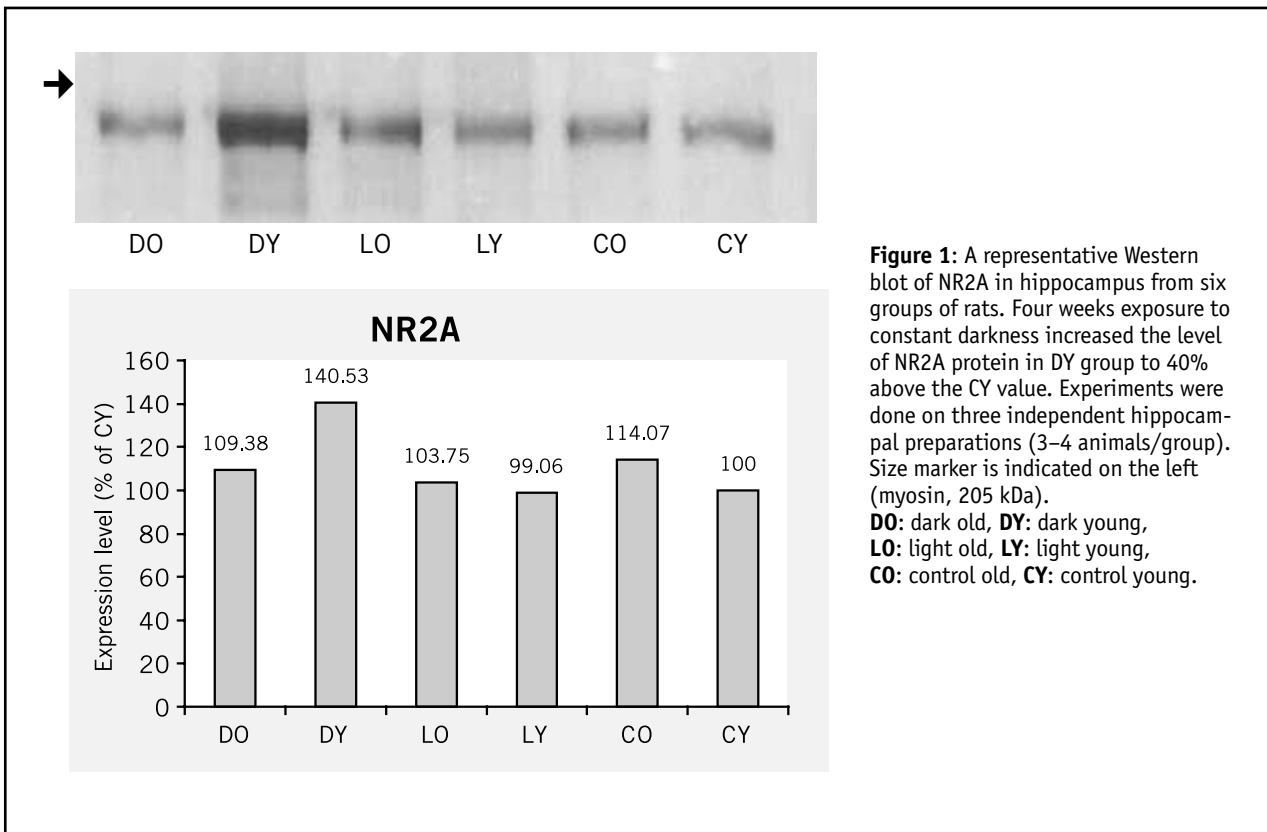
The results of biochemical measurements are shown in Table I. The statistical analyses of biochemical variables are given in Table II.

We unable to quantify GSH level and catalase activity in the hippocampus of the rats although there was measurable level of these parameters in the other part of the brain and also in erythrocytes obtained from the same animals (data not shown). Possibly excessive dilution of the hippocampal homogenate was responsible for this and LY. CuZn and Mn SOD activities were found to be increased in CO compared to CY group. Continual light exposure for 4 weeks did not change neural CuZn and Mn SOD activities. In old rats, light exposure reduced the activities of both CuZn and Mn SOD relative to those in the young animals. In addition, CuZn and Mn SOD activities were higher in dark exposed rats than in those in the continual light exposed or LD 12:12 rats. GSH-Px activity was found elevated in the DY rats compared to the CY groups.

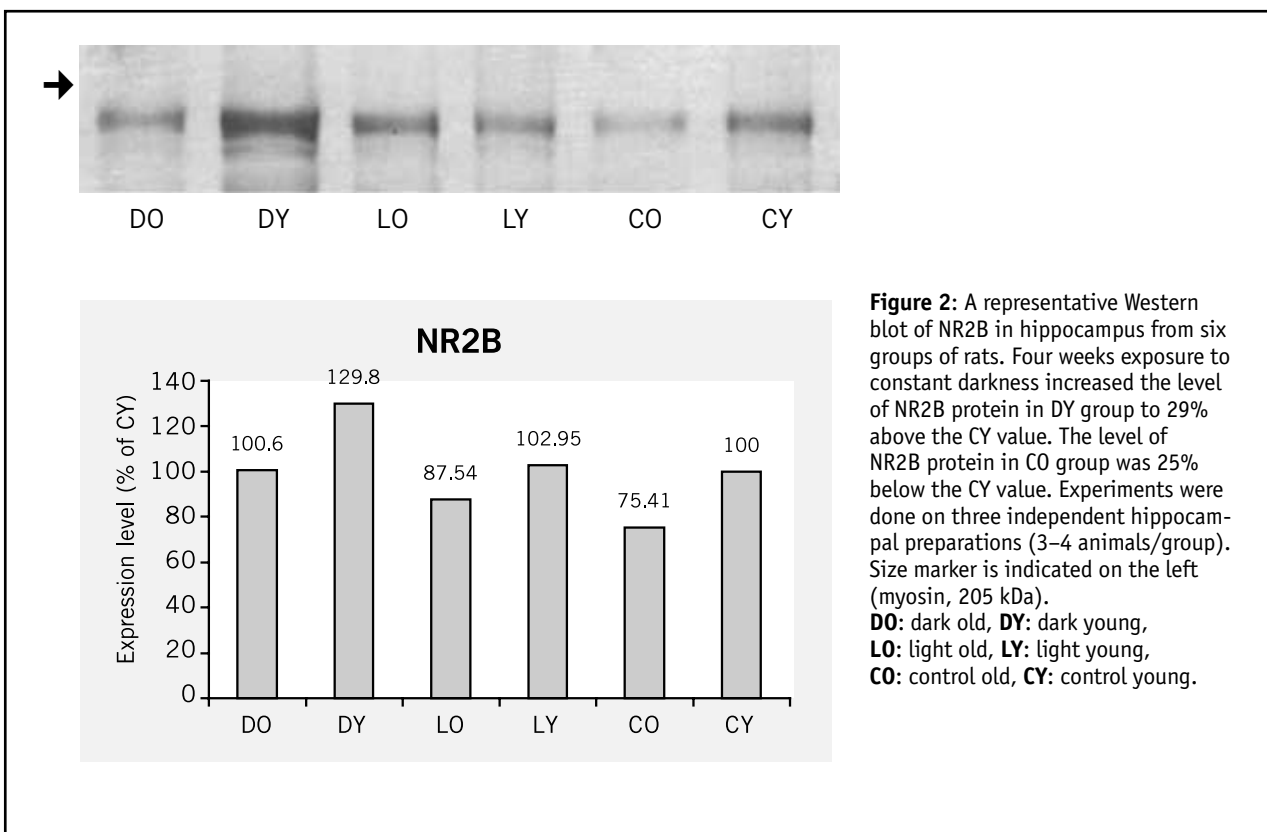
**Table II:** Significant p values based on the Bonferroni adjusted Mann Whitney U test.

Groups	GSH-Px	CuZn SOD	Mn SOD	MDA
CY-CO		0.002	0.001	0.001
DY-DO	0.001	0.001	0.001	
CY-DY	0.001	0.001	0.001	
CY-DO		0.001	0.003	
CO-LY		0.001	0.001	
CO-LO		0.038	0.001	
CO-DY	0.024	0.001	0.001	
CO-DO		0.001		
LY-DY	0.001	0.001	0.001	
LY-DO		0.001	0.001	
LO-DY	0.019	0.001	0.001	
LO-DO		0.001		

Statistically non-significant results were not given. CY: control young, CO: control old, LY: light young, LO: light old, DY: dark young, DO: dark old.



**Figure 1:** A representative Western blot of NR2A in hippocampus from six groups of rats. Four weeks exposure to constant darkness increased the level of NR2A protein in DY group to 40% above the CY value. Experiments were done on three independent hippocampal preparations (3–4 animals/group). Size marker is indicated on the left (myosin, 205 kDa).  
**DO:** dark old, **DY:** dark young, **LO:** light old, **LY:** light young, **CO:** control old, **CY:** control young.



**Figure 2:** A representative Western blot of NR2B in hippocampus from six groups of rats. Four weeks exposure to constant darkness increased the level of NR2B protein in DY group to 29% above the CY value. The level of NR2B protein in CO group was 25% below the CY value. Experiments were done on three independent hippocampal preparations (3–4 animals/group). Size marker is indicated on the left (myosin, 205 kDa).  
**DO:** dark old, **DY:** dark young, **LO:** light old, **LY:** light young, **CO:** control old, **CY:** control young.

MDA levels were significantly higher in the CO than in the CY group.

To evaluate protein concentrations of NR2A and NR2B, western blot analyses were done on the hippocampal homogenates of the rats. The density of protein band in CY group was accepted as 100% and data from other groups were calculated as percentages of the CY value. *Figure 1* and *Figure 2* show a significant increase in NR2A and NR2B concentration in DY group compared to all other treatments.

## Discussion

Long-term potentiation (LTP), the most intensively studied cellular and molecular model for learning and memory, in the hippocampus is generally dependent on NMDA receptor activation [19,20]. Induction of LTP in the hippocampus is generally dependent on postsynaptic  $Ca^{2+}$  influx after the activation of NMDA receptors. Furthermore, NMDA receptor stimulation has been shown to produce ROS, including the superoxide ( $O_2^-$ ), in hippocampal slices [21,22]. It has been suggested that  $O_2^-$  should be added to the list of signaling molecules necessary for the induction of LTP [19]. It was reported that ROS might modulate the activity of protein kinases and phosphatases during LTP [23]. Taken together, these data suggest the possibility that ROS may be required for the NMDA receptor-dependent pathways in hippocampus. However, prolonged activation of NMDA receptors under pathological conditions such as cerebral ischemia and traumatic injury causes neuronal cell death [24]. It has been hypothesized that NMDA receptor-mediated excitotoxicity may contribute to the etiology or progression of numerous neurodegenerative diseases [25]. Excitotoxicity refers to the excessive activation of glutamate receptors that results in neuronal death. Growing data implicate oxidative stress as a mediator of excitotoxic cell death [26]. In addition, free radicals themselves can increase the release and decrease the re-uptake of glutamate, thus leading to increased glutamate in the synaptic cleft [27]. It has been reported that there is a dramatic decrease in the expression of the NMDA receptor subunit NR1 in aged rats [11]. The level of NR2B protein in CO group was 25% below the CY value in the present study. But, no significant change was found in NR2A protein level between CY and CO groups.

In our study, NR2A and B concentrations were significantly increased in DY animals compared to the others. This suggests that the cause of this increase may be the increased level of melatonin in rats kept in dark room for 4 weeks. Presumably more melatonin would be produced in the pineal gland of dark exposed rats since it was free from the inhibitory effect of light. The bulk of the melatonin measured in the blood is derived from the pineal gland and blood melatonin concentrations were 5–15 times higher at night than during the day [1]. No significant increase in NMDA receptor concentration was found in DO group. This might be due to the low melatonin level in advanced ages [6].

MDA levels were not statistically different in DY group compared to the other groups. NR2A and NR2B concentrations increased while MDA concentrations remain unchanged in DY group. This result suggests that the principal factor that caused NMDA receptor increase but that not induced lipid peroxidation may be melatonin. The antioxidant properties of melatonin are well known [1,3,4]. It has been reported that oxidative apoptotic cell death induced by glutamate in the hippocampus is reduced by melatonin [28]. Furthermore, melatonin reduced kainic acid-induced lipid peroxidation in different areas of the brain including the hippocampus [29]. Based on these reports, we presume that melatonin increases NR2A and NR2B concentration in hippocampus without induction of lipid peroxidation.

CuZn and Mn SOD activity was significantly higher in dark groups than in the control and light exposed groups. This is in accordance with the stimulatory effect of melatonin on SOD. In agreement with this, the group that has the lowest level of SOD activity was the functional pinealectomy (24h light) group. GSH-Px activity was also higher in the DY animals than in the CY and LY groups. This also indicated the stimulatory effect of melatonin on antioxidant enzymes since the secretion of the indole is enhanced under constant dark conditions. Antioxidative enzymes respond much quicker than do changes in the quantity of oxidative damage. If this study had been made in longer period, we likely would have found a rise in MDA levels particularly in hippocampus.

Consequently this study showed that NR2A and B receptor concentrations in hippocampus of the rats maintained in dark showed significant increases compared to the control and functional pinealectomy groups. Furthermore, there was no significant increase in lipid peroxidation while the NMDA receptor concentration increased significantly.

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