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Melatonin improves 3-nitropropionic acid induced behavioral alterations and neurotrophic factors levels

Inmaculada Tasset [a], Eduardo Agüera [c], Rosa Olmo-Camacho [a,b], Begoña Escribano [b], Fernando Sánchez-López [c], María J. Delgado [a], Antonio H. Cruz [d], Felix Gascón [d], Evelio Luque [e], José Peña [e], Ignacio M. Jimena [e], Isaac Túnez [a,*]

[a] Dpto. Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC)/Universidad de Córdoba, Spain
[b] Dpto. Biología Celular, Fisiología e Inmunología, Facultad de Veterinaria, Edificio C-1, Campus de Rabanales, Universidad de Córdoba, Spain
[c] Servicio de Neurología, Hospital Universitario Reina Sofía/IMIBIC, Spain
[d] Dpto. Análisis Clínicos, Hospital Valle de los Pedroches/IMIBIC, Spain
[e] Dpto. de Ciencias Morfológicas, Sección de Histología, Facultad de Medicina, Instituto Maimónides de Investigaciones Biomédicas de Córdoba (IMIBIC)/Universidad de Córdoba, Spain

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ABSTRACT

Objective: This study sought to determine whether melatonin causes changes in neurotrophic factors and it protects against the mycotoxin 3-nitropropionic acid (3-NP) in brain tissue.
Methods: Rats were given 3-NP over four consecutive days (20 mg/kg BW), while melatonin was administered over 21 days (1 mg/kg/BW), starting after the last injection of 3-NP.
Results: Rats treated with 3-NP displayed significant changes in neurotrophic factor (BDNF and GDNF) levels, together with alterations in behavior; they also displayed extensive oxidative stress and a massive neuronal damage.
Conclusions: Melatonin improved behavioral alterations, reduced oxidative damage, lowered neurotrophic factor levels and neuronal loss in 3-NP-treated rats. These results suggest that melatonin exerts a neuroprotective action.

1. Introduction

3-Nitropropionic acid (3-NP) is used to develop in rodent a model with similar profiles of neurotoxicity to those seen in patients with Huntington's disease (HD) (Tunez et al., 2010). The neurotoxin 3-NP, produced by certain fungi (e.g. *Aspergillus flavus)* and plants (e.g. *Indigofera endecaphylla*), triggers an irreversible inhibition of the enzyme succinate dehydrogenase (SDH; E.C. 1.3.99.1), which is present in the Krebs cycle and the electron transport chain. Inhibition of SDH causes excitotoxicity, oxidative stress and cell death, especially in the striatal nucleus. In addition, this toxin causes an increase in glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels as the disease progresses (Wu et al. 2010a).

Additionally, melatonin has demonstrated a protective action of melatonin against 3-NP-induced neurotoxicity (Nam et al. 2005; Tunez et al. 2004). Other researchers have found that melatonin increases the total volume of the granular cell layer and dendritic maturation in adult neurogenesis (Manda and Reiter 2010; Reiter et al. 1999), as well as it prompts proliferation of cultured neural stem cells obtained from adult mouse subventricular zone (Sotthibundhu et al. 2010). Finally, some data suggest that melatonin may increase the production of (GDNF) and (BDNF) (Kong et al. 2008).

On the basis of these findings, the present study sought to evaluate the effects of melatonin on 3-NP-induced changes in neurotrophic factor levels and neuronal density.

2. Material and methods

2.1. Animals

Thirty-two male Wistar rats (Charles River, Barcelona, Spain), weighing between 200 and 250 g at the beginning of the study, were maintained with standard conditions of temperature (20–23 ºC), illumination (12-h light/12-h dark cycle, lights on at 08:00 h) and were provided with free access to food (Purina®,

Abbreviations: BDNF, Brain-derived neurotrophic factor; FST, Forced Swim Test; GDNF, Glial cell-derived neurotrophic factor; HD, Huntington's disease; NGF, Nerve growth factor; 3-NP, 3-Nitropropionic acid; OFT, Open field test; SDH, Succinate dehydrogenase.

* Corresponding author at: Dpto. Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Córdoba, Avda. Menéndez Pidal s/n, 14004-Córdoba, Spain. Tel.: +34 957 218268; fax: +34 957 218229.
E-mail address: fm2tufii@uco.es (I. Túnez).

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Barcelona, Spain) and water. The animal study was in agreement with the European Communities Council Directive of 24 November 1986 (86/609/ECC) and the R.D. 223/1988, and was approved by Bioethics Committee of the University of Cordoba, Spain.

2.2. Drug administration

3-Nitropropionic acid (Sigma-Aldrich, St. Louis, MO, USA) was administered intraperitoneally (i.p.) (in saline, pH 7.4) at a dose of 20 mg/kg BW for 4 consecutive days. Melatonin (Sigma-Aldrich, St. Louis, MO, USA) was injected daily (in 5% ethanol in 0.9% NaCl) at a dose of 1 mg/kg BW/i.p./day during 21 consecutive days, after last injection of 3-NP. The length of in vivo exposure was determined on the basis of previous studies from our group that showed the protective effect of melatonin on 3-NP induced oxidative stress (Cruz et al. 2005; Tunez et al. 2004).

2.3. Experimental design

The animals were divided into four groups (n = 8 animals per group) as follows: i) control, ii) intact animals plus melatonin (melatonin); iii) 3-nitropropionic acid (3-NP), and iv) 3-nitropropionic acid plus melatonin (3-NP + melatonin).

At the end of study four animals per group were killed under light anesthesia by ether by decapitation and their brain were rapidly removed, frozen on dry ice, and stored frozen (−80 ºC) until the measurement of biochemical parameters. While, other four animals are killed under ketamine anesthesia and brains were rapidly removed, immersed and fixed in paraformaldehyde.

2.4. Lipid peroxidation products

The levels of lipid peroxidation products were determined using reagents purchased from Oxis International (Portland, OR, USA), i.e., LPO-586 kit. The level of lipid peroxidation is expressed as malondialdehyde (MDA) + 4-hydroxyalkenals (4-HDA) nanomoles of MDA + 4-HDA per milligram of protein (nmol/mg protein), and absorbance was evaluated in a spectrophotometer (UV-1603 Shimadzu, Kyoto, Japan) at wavelength 586 nm.

2.5. 8-Hydroxy-2′-deoxyguanosine (8-OHdG)

The levels of 8-OHdG were carried out using reagents purchased from JaICA (Japan Institute for the Control of Aging, Shizuoka, Japan), i.e., Highly Sensitive 8-OHdG Check Elisa. The level of 8-OHdG is expressed as nanomoles per milligram of protein (nmol/mg protein), and absorbance was evaluated in a Multimode microplate reader, Infinite® M1000 (Tecan Trading AG, Switzerland), at wavelength 450 nm.

2.6. Reduced glutathione (GSH) content

The levels of GSH were carried out using reagents purchased from Oxis International (Portland, OR, USA), i.e., GSH-420 kit. The content of GSH is expressed as GSH nanomoles per milligram of protein (nmol/mg protein), and absorbance was evaluated in a spectrophotometer (UV-1603 Shimadzu, Kyoto, Japan) at wavelength 420 nm.

2.7. Nitric oxide (NO) levels

This gas is evaluated through total nitrites (NOx) levels and determined following the Griess method (Ricart-Jane et al. 2002). Nitrates were reduced to nitrites by incubating a sample aliquot (150 µl) for 15 min at 37 ºC with the presence of 0.1 U/ml nitrate reductase, 50 µM NADPH and 5 µm flavin-adenine dinucleotide in a final volume of 160 µl. When nitrate reduction is completed, total nitrite is then determined spectrophotometrically using the Griess reaction. Griess reagent is composed of mixture of sulphanilamide 2% (w/v) and N-(1-naphthyl) ethylenediamine 0.2% (w/v). The reaction was monitored at 540 nm. The absorbance was evaluated in a spectrophotometer (UV-1603; Shimadzu). The values are presents in micromoles per milligram of protein (µmol/mg protein).

2.8. Caspase-3 activity

The caspase-3 activity in the brain homogenates were measured using reagents purchased from BioVision Inc. (Mountain View, CA, USA), i.e., Caspase-3/CPP32 colorimetric assay kit. The activity is expressed as optical density arbitrary units per milligram of protein (O.D. arbitrary units/mg protein), and absorbance was evaluated in a spectrophotometer (UV-1603 Shimadzu, Kyoto, Japan) at wavelength 405 nm.

2.9. Lactate dehydrogenase (LDH) activity

The assay is based on measurement of activity of lactate dehydrogenase (LDH) which is a stable enzyme normally found in the cytosol of all cells. LDH in the brain homogenate were assayed using kit purchased from BioVision Inc. (Mountain View, CA, USA), i.e., LDH-Cytotoxicity assay kit. The activity of LDH is expressed as units per milligram of protein (U/mg protein), and absorbance was evaluated in a spectrophotometer (UV-1603 Shimadzu, Kyoto, Japan) with maximum absorbance at 500 nm.

2.10. Brain-derived neurotrophic factor (BDNF)

BDNF levels were evaluated using reagents purchased from Promega (Madison, WI, USA), i.e., BDNF E_{max}® ImmunoAssay System. The content of BDNF is expressed as picogram of BDNF per milligram of protein (pg/mg protein), and absorbance was evaluated in a Multimode microplate reader, Infinite® M1000 (Tecan Trading AG, Switzerland), at wavelength 450 nm.

2.11. Glial-derived neurotrophic factor (GDNF)

GDNF levels were evaluated using reagents from Promega (Madison, WI, USA), i.e., GDNF E_{max}® ImmunoAssay System. The content of GDNF is expressed as picogram of GDNF per milligram of protein (pg/mg protein), and absorbance was evaluated in Multimode microplate reader, Infinite® M1000 (Tecan Trading AG, Switzerland), at wavelength 450 nm.

2.12. Tissue processing for histological analysis

The brain were rapidly removed, immersed and fixed in 10% buffer paraformaldehyde. Subsequently they were embedded in paraffin wax, cut into 8-µm thick sections and stained with cresyl-fast violet (Nissl-stained). Sections were examined under bright-field illumination on a Leitz Orthoplan microscope (Herramientas Leitz S.L., Barcelona, Spain).

We performed a semi-automated cell counting using an image-analysis program (Imag-Pro Plus, Media Cybernetics, Version 6, 2006). The neurons were counted bilaterally in eight areas (82×10^3 µm^2 each); in each area was evaluate the total number of neurons, healthy and pyknotic. The total area checked in each brain was of 656×10^3 µm^2. The areas were captured digitally at a magnification of ×400 with a Sony Exwaved HAD camera mounted on a Nikon Eclipse E1000 microscope.