NeuroToxicology 32 (2011) 734–741

Contents lists available at ScienceDirect

NeuroToxicology

Neuronal apoptosis in the striatum of rats treated with 3-nitropropionic acid is not triggered by cell-cycle re-entry

Joaquim Duran-Vilaregut [a,c], Gemma Manich [a], Jaume del Valle [a,c], Mercè Pallàs [b,c], Antoni Camins [b,c], Carme Pelegrí [a,c,1], Jordi Vilaplana [a,c,1,*]

[a] Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n., E-08028 Barcelona, Spain
[b] Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia, Institut de Biomedicina (IBUB), Universitat de Barcelona, Av. Joan XXIII s/n., E-08028 Barcelona, Spain
[c] CIBERNED Centros de Biomedicina en Red de Enfermedades Neurodegenerativas, Spain

ARTICLE INFO

Article history:
Received 3 May 2011
Accepted 22 July 2011
Available online 30 July 2011

Keywords:
3-Nitropropionic acid
Cell-cycle re-entry
Apoptosis
Neurodegeneration
Immunohistochemistry

ABSTRACT

Although terminally differentiated neurons lack the capacity to undergo cell division, they retain the capacity to reactivate the cell cycle. This reactivation, however, has been linked to the degeneration of neurons in many experimental models of neurodegenerative disease and in *post-mortem* brains of affected patients. Expression of markers of the G1 phase and apoptotic neurons has been detected in the striatal lesion of rats treated with 3-nitropropionic acid (3-NPA). Here we examined whether neuronal apoptosis induced by 3-NPA was mediated by the reactivation of the cell cycle. To this end, we studied whether TUNEL-positive neurons expressed the G1-phase markers cyclin-dependent kinase 4 (CDK4) and cyclin D (CyD). In addition, we also evaluated the neuronal expression of pRb and Ki67 antigens, both of which are involved in the regulation of cell-cycle progression. In 3-NPA-treated rats, CDK4 and CyD were not detected in TUNEL-positive neurons, but they were expressed in neurons in the core of the lesion, which were assumed to be in a more advanced stage of degeneration, since they had weaker NeuN staining and lacked Hoechst staining. In addition, injured neurons in the striatal lesion of 3-NPA-treated rats had lost the constitutive expression of pRb and Ki67 that we had detected in control animals. Taken together, these results indicate that neuronal apoptosis in the striatal lesion of 3-NPA-treated rats was not triggered by cell-cycle re-entry, and we conclude that expression of G1 markers may be considered an aberrant survival response, with no relation to the mechanisms of apoptosis.

1. Introduction

The cell cycle is a tightly regulated process that is essential to the control of development, differentiation and proliferation of eukaryotic cells. It proceeds through five phases, G0, G1, S, G2 and M, and requires the coordinated participation of several proteins, including cyclins, cyclin-dependent kinases (CDKs), the retinoblastoma family of "pocket" proteins, namely retinoblastoma protein (pRb), p107 and p130, and members of the E2F transcription factor family (Greene et al., 2007; Rashidian et al., 2007). Although it may seem contradictory, the fact that cell proliferation and cell death show similar morphological changes, including substrate detachment, cell rounding, cell shrinkage and chromatin condensation, and share some common cell-cycle regulators, suggests a link between cell cycle and apoptosis (Vermeulen et al., 2003; Wang et al., 2009). Moreover, it has been proposed that cell proliferation and cell death are linked in such a way that any initiation of mitotic machinery also primes the apoptotic cascades, which may serve as a safety measure to abort uncontrolled proliferation of cells (Stoica et al., 2009). Such a link is also found in the case of post-mitotic, terminally differentiated neurons, which, despite having no capacity to undergo cell division, retain the ability to reactivate the cell cycle in response to central nervous system (CNS) insults. However, this reactivation, far from being mitogenic, is lethal for the neurons (Yang and Herrup, 2007).

Furthermore, cell-cycle activation plays an active role in the acute CNS damage induced by cerebral ischemia, brain trauma or spinal cord injury, promoting apoptosis in neurons and cellular proliferation/activation in astroglia and microglia that may contribute to neurotoxicity (Byrnes and Faden, 2007; Stoica

Abbreviations: 3-NPA, 3-nitropropionic acid; AD, Alzheimer's disease; CDK, cyclin-dependent kinase; CNS, central nervous system; CyD, cyclin D; GFAP, glial fibrillary acidic protein; MCCII, mitochondrial respiratory chain complex II; NeuN, neuronal antigen nuclei; PBS, phosphate buffered saline; pRb, retinoblastoma protein; ROS, reactive oxygen species.

* Corresponding author at: Departament de Fisiologia, Facultat de Farmàcia, Av. Joan XXIII s/n., E-08028 Barcelona, Spain. Tel.: +34 93 4024505; fax: +34 93 4035901.
E-mail address: vilaplana@ub.edu (J. Vilaplana).

[1] Contributed equally to this study.

0161-813X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.neuro.2011.07.009

et al., 2009). Many studies have also revealed abnormal expression of cell-cycle proteins in *post-mortem* brains from patients suffering from neurodegenerative diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis and Parkinson's disease (Becker and Bonni, 2004; Herrup et al., 2004; Wang et al., 2009). Moreover, neuronal apoptosis linked to cell-cycle re-entry has been described in several models of neurodegeneration induced by neurotrophic factor deprivation (Park et al., 1997) or the administration of diverse neurotoxins such as kainic acid (Ino and Chiba, 2001; Smith et al., 2003; Verdaguer et al., 2002), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Höglinger et al., 2007), β-amyloid (Majd et al., 2008), camptothecin (Park et al., 2000), thrombin (Rao et al., 2007) and lead (Li et al., 2008).

3-Nitropropionic acid (3-NPA) is a natural toxin that irreversibly inhibits the mitochondrial respiratory chain complex II (MCCII). This inhibition leads to a rapid fall in ATP, with a concomitant rise in the production of reactive oxygen species (ROS) and delayed activation of *N*-methyl-D-aspartate glutamate receptors. This in turn induces a second rise in ROS production that eventually triggers neuronal cell death (Liot et al., 2009). 3-NPA is widely used to induce experimental models of Huntington's disease in laboratory animals such as rodents and non-human primates, taking advantage of its capacity to reproduce the brain lesions observed in the disease, which mainly consist of specific striatal neurodegeneration (Brouillet et al., 2005).

Some authors claim that cell-cycle activation has a significant role in 3-NPA-induced neuronal cell death. Flavopiridol, an inhibitor of CDKs, blocked the delayed death of cultured cortical neurons evoked by exposure to the toxin (Park et al., 2000). Moreover, it was recently shown, both *in vitro* and *in vivo*, that 3-NPA reduced the level of the CDK inhibitor p27 and induced phosphorylation of pRb in striatal neurons, thus indicating the reactivation of the cell cycle (Akashiba et al., 2008). In a previous study we detected, in the injured striatal areas of brains of treated rats, some neurons with increased expression of cell-cycle markers of the G1 phase, namely CDK4 and cyclin D (CyD) among others (Pelegrí et al., 2008). Moreover, in another study we identified apoptotic neurons with a pyknotic appearance which were positive for active caspase-3 and TUNEL staining in the striatal lesions of intoxicated rats (Duran-Vilaregut et al., 2010). Thus, the present study examines whether apoptotic neurons in the striatal lesion of 3-NPA-treated rats, identified by TUNEL staining, express the G1 cell cycle markers CDK4 and CyD. Both early G1 elements are believed to have a prominent role in neuronal apoptosis (Becker and Bonni, 2004; Wang et al., 2009). Furthermore, we also studied the pattern of expression of pRb and Ki67 antigens in the striatum of control and 3-NPA treated rats, since both proteins are involved in cell-cycle regulation. pRb is a nuclear protein that represses transcription of genes required for cell cycle progression and is regulated by CDK-mediated phosphorylation (Inoue et al., 2007). The antigen detected by Ki67 antibody is a large protein that is used as a marker of cell proliferation, since it is expressed in all active stages of the cell cycle and is absent in resting cells (Schlüter et al., 1993).

2. Materials and methods

2.1. Animals and 3-NPA treatment

Male Sprague-Dawley rats (220–250 g, Harlan Interfauna Ibérica, Barcelona, Spain) with access to food and water *ad libitum* were kept in standard conditions of temperature (22 ± 2 °C) and 12:12-h light–dark cycles (300 lx/0 lx). Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona. One group of 10 rats (treated group) was injected with saline solution containing 3-NPA (Sigma–Aldrich, St Louis, MO, USA) adjusted to pH 7.4 with NaOH, at a dose of 20 mg/kg i.p. once a day for 3 days (days 0, 1 and 2). The control group, which included 5 animals, was injected only with saline solution. On day 6, both groups of animals were sacrificed and their brains were excised. All rats were evaluated daily from day 0 until day 6 of the experiment for both body weight loss and motor impairment, and the evolution observed was similar to that described in previous studies in our laboratory (Duran-Vilaregut et al., 2009).

2.2. Brain processing

Brains for immunohistochemistry and TUNEL staining were obtained as follows. Rats were anaesthetized with 80 mg/kg i.p. of sodium pentobarbital and intracardially perfused with 100 ml of physiological saline. Brains were then excised and snap frozen by immersion in isopentane chilled in dry ice and stored at −80 °C. Thereafter, brains were embedded in OCT cryostat-embedding compound (Tissue-Tek, Torrance, CA, USA) and 20-μm-thick cryostatic sections containing striatum were obtained. Slices were picked up on common slides, fixed with acetone for 10 min at 4 °C, allowed to dry at room temperature and finally frozen at −20 °C until staining.

2.3. Staining of brain sections

For the standard immunostaining method, slides with brain sections were allowed to thaw at room temperature and rehydrated with phosphate buffered saline (PBS) for 5 min before being blocked and permeabilized with PBS containing 1% bovine serum albumin (Sigma–Aldrich) and 0.1% Triton-X-100 (Sigma–Aldrich) for 20 min. They were then washed twice for 5 min in PBS and incubated with freshly prepared (following the manufacturer's instructions) TUNEL reaction mixture containing TdT enzyme and fluorescein-dUTP (*in situ* cell death detection kit from Roche Diagnostics GmbH, Mannheim, Germany) for 60 min at 37 °C. For negative controls, some brain sections were incubated with fluorescein-dUTP alone. Slides were then washed again and incubated with primary antibodies (see below) for 90 min at room temperature. After another wash, incubation with secondary antibodies (see below) was performed for 1 h at room temperature. Five minutes before the end of the second incubation, nuclear staining was performed by adding Hoechst (H-33258, Sigma–Aldrich) reagent at 10 mg/ml in PBS to the incubation medium to a final concentration of 2 mg/ml. Finally, the slides were washed, mounted using Prolong Gold (Invitrogen, Carlsbad, CA, USA) anti-fade medium, allowed to dry for 3 h at room temperature and stored at 4 °C in the dark. Immunostaining controls were performed by incubating with PBS instead of the primary antibody or instead of both antibodies. All incubations were carried out in the dark.

The following primary antibodies were used: rabbit polyclonal anti-CyD1 (Abcam, Cambridge, UK; dilution 1:100), rabbit polyclonal anti-CDK4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:100), rabbit monoclonal anti-pRb (Cell Signaling, Danvers, MA, USA; dilution 1:50), rabbit polyclonal anti-Ki67 antigen (Neomarkers, Fremont, CA, USA; dilution 1:100), mouse monoclonal anti-neuronal antigen nuclei (NeuN) (Millipore, Billerica, MA, USA; dilution 1:100) and mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (Abcam; dilution 1:100). AlexaFluor 488 donkey anti-mouse IgG, AlexaFluor 555 donkey anti-rabbit IgG, and AlexaFluor 660 goat anti-mouse IgG (Invitrogen; dilution 1:250) were used as secondary antibodies.

2.4. Fluorescence microscopy analysis

Brain sections from 3-NPA-treated and control rats were examined under a laser confocal microscope (TCS/SP2, Leica