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Neuroscience Letters 329 (2002) 86–90

Neuroscience Letters

www.elsevier.com/locate/neulet

3-Nitropropionic acid induces cell death and mitochondrial dysfunction in rat corticostriatal slice cultures

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Received 14 December 2001; received in revised form 11 April 2002; accepted 22 April 2002

Abstract

Exposure of organotypic rat corticostriatal slice cultures to the mitochondrial toxin 3-nitropropionic acid (3-NP) resulted in concentration-dependent loss of cresylviolet-stained cells and increase of lactate dehydrogenase and lactate efflux into the culture medium, indicators for cell death and metabolic activity in the slices, respectively. The involvement of apoptosis in these slices was suggested by using the terminal transferase-mediated biotinylated-UTP nick end-labeling (TUNEL) technique, and immunohistochemistry for the apoptosis-related markers Bax and Bcl-2. In 3-NP-exposed slices, TUNEL-positive cells were observed in both the striatum and the cortex but in different forms: striatal neurons were either diffusely stained or showed nuclear fragmentation, cortical neurons only exhibiting nuclear fragmentation. In 3-NP-exposed slices, the pro-apoptotic protein Bax was abundantly expressed, whereas the anti-apoptotic protein Bcl-2 was not expressed in striatal neurons. We suggest that both apoptosis and necrosis are involved in the 3-NP-treated slices, apoptosis as well as necrosis in the striatum and apoptosis in the cortex.

Keywords: Organotypic slice cultures; Striatum; Apoptosis; Necrosis; 3-Nitropropionic acid; Neuronal degeneration; Terminal transferase-mediated biotinylated-UTP nick end-labeling

The mitochondrial complex II (succinate dehydrogenase) inhibiting neurotoxin 3-nitropropionic acid (3-NP) induces selective striatal degeneration in aged rats. This degeneration is characterized by both neuronal loss and gliosis [2,5,19]. As such it has been used as a model for Huntington's disease, an inherited neurodegenerative disorder characterized by severe atrophy of the neostriatum with marked neuronal loss and gliosis [21]. The precise mechanism of this selective neuronal death is unknown, but inappropriate activation of apoptosis may be involved [1,6,14,18]. 3-NP induced neurodegeneration has also been demonstrated in striatal and hippocampal cell cultures [3,13] and in organotypic striatal slice cultures [17], and may result from apoptosis [4].

Cells that undergo apoptosis show characteristic morphological features [9] such as nuclear fragmentation and condensed nuclei. DNA fragmentation can be demonstrated by the terminal transferase-mediated biotinylated-UTP nick end-labeling (TUNEL; [8]). Members of the Bcl-2 family of proteins, including Bcl-2 and Bax play an important role in regulating the apoptotic cascade [4,10], Bax promoting apoptosis, and Bcl-2 inhibiting apoptotic cell death. Previously, we showed that in a rat model in vivo chronic 3-NP intoxication induces differential expression patterns of these apoptosis-related markers in the striatum [20]. In the present study we studied the expression of Bax and Bcl-2 in relation to DNA fragmentation in organotypic slice cultures. This model allows continuous monitoring of morphological and biochemical alterations over the lifetime of the cultures, information that is not easily obtained in in vivo models. Corticostriatal slices of 1-day old rats [7,12,16], cultured for 2 weeks, were exposed to 3-NP for 24 h, and, after a recovery period of 24 h, we analyzed the neurotoxic effects. As biochemical indicators for cell death and metabolic activity, we measured lactate dehydrogenase and lactate efflux into the culture medium.

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0304-3940/02/$ - see front matter
PII: S0304-3940(02)00581-5

Corticostriatal slice cultures were prepared using the interface culture method [16]. One-day old Wistar rat pups were decapitated, and their forebrains were quickly removed under sterile conditions. The forebrain was mounted on the specimen holder of a vibratome, held on ice, and embedded in agarose. The forebrain was divided into the two hemispheres, and transverse, 400 μm thick sections through the entire forebrain were cut with the vibratome. Sections were easily released from the agarose and were collected in chilled Gey's balanced salt solution with glucose (final concentration 11 mM). Four adjacent frontal corticostriatal slices of each hemisphere were placed on one membrane insert (Millicell-CM, 0.4 μm; Millipore Corp. Bedford, MA, USA). The inserts were transferred to culture trays with six wells, each well containing 1 ml of medium, containing 25% inactivated horse serum, 25% Hanks' balanced salt solution and 50% Optimun Medium (OPTI-MEM) culture medium, supplemented with D-glucose (final concentration 11 mM) and penicillin/streptomycin (100 IU/ml) (all GIBCO BRL). The culture trays were placed in an incubator at 37°C with 5% CO_2. After 3 days, the culture medium was replaced by 1 ml of serum-free Neurobasal medium, supplemented with D-glucose (final concentration 28 mM), L-glutamine (1 mM), B-27-supplement and penicillin/streptomycin (100 IU/ml) (all GIBCO BRL). The medium was changed twice a week during the entire culturing period of 14 days. During the culturing period, the slices were regularly examined by light and phase contrast microscopy. Initial studies were conducted using P-culture medium for the whole culturing period and for the dose-effect experiments of 3-NP, the main difference in composition being 50% MEM-Eagle instead of 50% OPTI-MEM.

The dose-effect relation of 3-NP (Sigma, St. Louis, MO, USA) was studied in 1-week-old interface cultures exposed for 24 h to 3-NP dissolved in P-culture medium in concentrations varying from 25 μM to 1 mM, respectively ($n = 4$ membrane inserts for each concentration; $n = 4$ for controls). Based on these experiments a concentration of 100 μM 3-NP was thereafter used and similar and consistent results were obtained using Neurobasal medium. In subsequent studies, 2-week-old interface cultures were exposed to 100 μM 3-NP for 24 h in Neurobasal medium, followed by a recovery period of 24 h in normal Neurobasal medium. Parallel control experiments were performed without 3-NP in the culture medium. In two independent experiments, lactate dehydrogenase (LDH), lactate and pyruvate efflux into the medium were measured before 3-NP exposure, after 24 h 3-NP (25–500 μM) exposure and after a 24 h recovery period, and compared with control slices. LDH, lactate and pyruvate concentrations (in U/l) were analyzed using a Cobas Mira automatic turbidometric analyzer (ABX, Eindhoven, the Netherlands) according to the manufacturer's instructions. LDH is an indicator for cell damage, whereas the levels of lactate and pyruvate give insight into the amount of mitochondrial stress, resulting in a rise of anaerobic metabolism from glucose to lactate.

After the 3-NP exposure, standard histological staining techniques, immunohistochemical detection of the apoptotic markers Bax and Bcl-2 as well as in situ labeling of DNA fragmentation were performed on cryosectioned slices of 3-NP treated slices ($n = 32$) and control slices ($n = 18$). The cultures were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 h, kept in a 20% sucrose solution at 4°C for at least 24 h, frozen using liquid nitrogen, embedded in Tissue-Tek (O.C.T. compound, Sakura, Japan), and cut into 8 μm thick frontal sections. Cryosections were immunostained using rabbit polyclonal antibodies directed against Bax and Bcl-2 (P-19 and N-19; dilution 1:250 and 1:100, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 in PBS for 10 min, and non-specific binding was blocked by 30 min incubation with 100% normal goat serum (Vector Laboratories, Burlingame, CA, USA). The primary antibodies were incubated overnight at 4°C. Antibody binding was visualized with a secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, dilution 1:200 in PBS/1% bovine serum albumin), followed by avidin-biotin-peroxidase-complex (Vectastain Elite, Vector Laboratories), and processed with diaminobenzidine (DAB; Sigma) and H_2O_2. The signal of the reaction product was enhanced by incubating sections for 5 min in 0.5% copper sulphate in saline, and sections were counterstained with hematoxylin. The specificity of the Bax and Bcl-2 antibodies was tested by incubating these antisera at room temperature with P-19 Bax blocking peptide and N-19 Bcl-2 blocking peptide (both from Santa Cruz Biotechnology), respectively, for 2 h prior to incubation of the sections. Nuclear DNA fragmentation was visualized using the Apoptag™ kit (Intergen Company, Gaithersburg, MD, USA) for TUNEL according to the manufacturer's recommendations. TUNEL-positive cells were stained using DAB as a chromophore. Staining was enhanced in 0.5% copper sulphate in saline, after which sections were counterstained using 'Kernechtrot'.

In general, during culture and after immunohistochemical staining the corticostriatal slices displayed the same organotypic histological organization as described by others [17]. Cresylviolet-stained corticostriatal slices demonstrated a dose-dependent effect of 3-NP on neuronal degeneration; with increasing 3-NP concentration, the number of cresylviolet-stained cells appeared to decrease in the striatum (Figs. 1A–C). Cultures exposed to 500 μM or 1 mM 3-NP displayed extensive striatal and cortical damage which disrupted the slices.

The mechanism of 3-NP induced cell death may be different in the cortex and the striatum. The number of TUNEL-positive cells was strongly increased in both the striatum and the cerebral cortex of the 3-NP-exposed sections compared to control slices. But many TUNEL-positive cells in the striatum after 3-NP treatment did not show the typical apoptotic morphology, with the nuclei being diffusely stained. In some striatal neurons, however nuclear fragmentation was

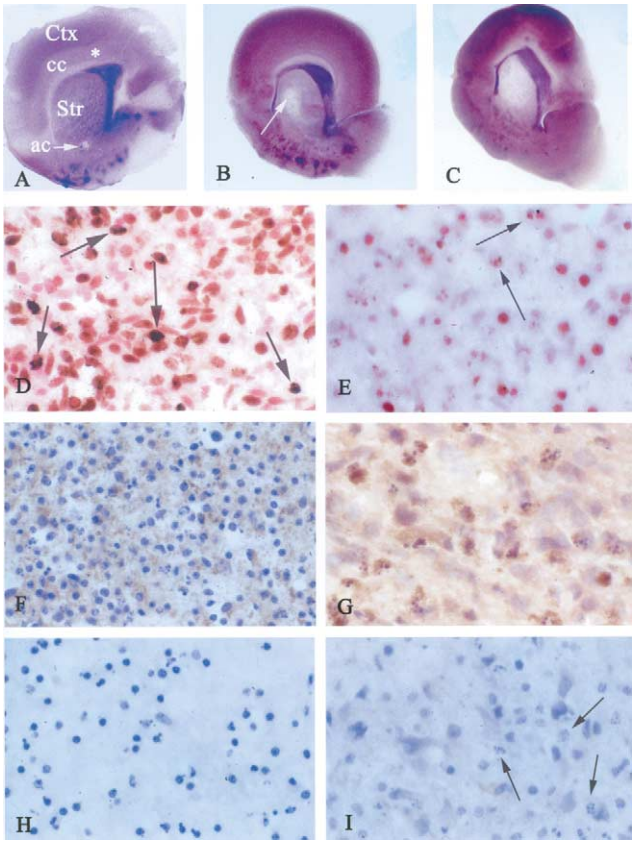

Fig. 1. (A–C) Low-power photomicrographs of cresylviolet-stained corticostriatal slices of control and 3-NP-exposed cultures (P-medium). (A) One-week-old control slice demonstrating major morphological features, including the cerebral cortex (Ctx), striatum (Str) consisting of the caudate-putamen, the anterior commissure (ac) and the corpus callosum (cc) (5×). The asterisk marks the deep part of the cerebral cortex, characterized by a decreased number of cresylviolet-stained cells. (B) Slice culture exposed to a concentration of 100 μM 3-NP for 24 h. Note the marked loss of Nissl-stained cells (arrow) in the dorsolateral part of the 3-NP-exposed striatum (10×). (C) Corticostriatal slice culture exposed to 250 μM 3-NP revealing a marked and widespread neuronal degeneration in the striatum (10×). The deeper layers of the cerebral cortex are also involved. (D–I) Expression of apoptosis-related markers in 3-NP-exposed corticostriatal slices (Neurobasal medium). (D) TUNEL-positive cells (dark-brown) in the 3-NP-treated striatum contained diffusely stained, condensed nuclei or showed nuclear fragmentation (arrows; 200×). Contrast was enhanced with a Photoshop program. (E) In the overlying, deep part of the cerebral cortex of unexposed slices the majority of the TUNEL-positive neurons exhibited characteristic apoptotic morphology (arrows) and extensive, brown TUNEL-labeling (400×). The sections shown in D and E are counterstained with 'Kernechtrot'. (F) Increased expression of the pro-apoptotic protein Bax in the striatum of a 3-NP-exposed cryosection (400×). (G) In the deep cortex of 3-NP-treated brain slices, Bax expression was increased and predominantly observed in the cytoplasm of neurons, demonstrating nuclear fragmentation (400×). (H) Bcl-2 immunostaining of an untreated cryosection demonstrating the striatum. The anti-apoptotic protein Bcl-2 was not detected in striatal neurons (400×). (I) Bcl-2 is not expressed by the dying cells in the deep parts of the cerebral cortex, exhibiting fragmentation (arrows; 400×).

observed in TUNEL-positive cells (Fig. 1D). In contrast, in the overlying deep parts of the cerebral cortex, both without and with 3-NP, the majority of the TUNEL-positive neurons exhibited characteristic apoptotic morphology and extensive labeling (Fig. 1E). Cortical cell loss and apoptotic features were also present in cresylviolet-stained cryosections of control brain slices, but only in the deeper layers nearest to the membrane insert. Here, DNA fragmentation was evident by TUNEL-staining, suggesting the occurrence of apoptotic cell death (see Fig. 1E). This cortical apoptosis may have been either an artifact of the sectioning or culturing procedures, or part of the physiologic brain modeling.

The expression of the pro-apoptotic protein Bax was dramatically increased in the 3-NP-exposed slices, both in the striatum and in the deeper layers of the cerebral cortex. In the striatum, Bax-expression was not restricted to the cytoplasm of cells, but was dispersed and detected in the neuropil (Fig. 1F). In the deeper layers of the cortex close to the membrane insert, however, Bax expression was predominantly observed in the cytoplasm of neurons that demonstrate nuclear fragmentation (Fig. 1G). The anti-apoptotic marker Bcl-2 was absent in striatal neurons of both 3-NP-treated and untreated brain slices (Fig. 1H). In the cortex of 3-NP-exposed slices Bcl-2 was weakly expressed, and was mainly observed in glial cells (Fig. 1I). Remarkably, it was generally not expressed in deep cortical cells that revealed nuclear fragmentation. Control experiments using Bax and Bcl-2 blocking peptides revealed no immunostaining of the cryoslices.

We found a 5–10-fold increase of LDH-efflux into the medium in response to increasing concentrations of 3-NP (Fig. 2A). This increase was only noted in the recovery period after the incubation with 3-NP, i.e. 48 h after the onset of the experiment, suggesting delayed cell damage after 3-NP. The lactate concentration in the medium also increased (2–3-fold) in response to 3-NP treatment (Fig. 2B), but this increase in lactate was an early event, already evident during 24 h of 3-NP incubation and not during the recovery period, i.e. after 48 h. The pyruvate concentration was decreased both during the 3-NP incubation period and the recovery phase (Fig. 2C), resulting in a strong increase in the lactate/pyruvate ratio (up to 10-fold) after incubation with 50 μM 3-NP or more (Fig. 2D), consistent with mitochondrial inhibition and a switch to anaerobic metabolism.

This study demonstrates that in organotypic slice cultures, prepared from the cerebral hemispheres of neonatal rats, 3-NP induces a concentration-dependent increase in neuronal death in the striatum of corticostriatal slice cultures. This striatal degeneration is accompanied by a dramatic increase of the LDH and lactate efflux into the culture medium. Our data are consistent with in vivo studies on systemic 3-NP [2,4,5,19] and intrastriatal 3-NP [11] injections reporting extensive damage in rat striatum. Since we used low glucose concentrations, 3-NP neurotoxicity was observed after relatively low 3-NP concentrations in line with recent data [17].

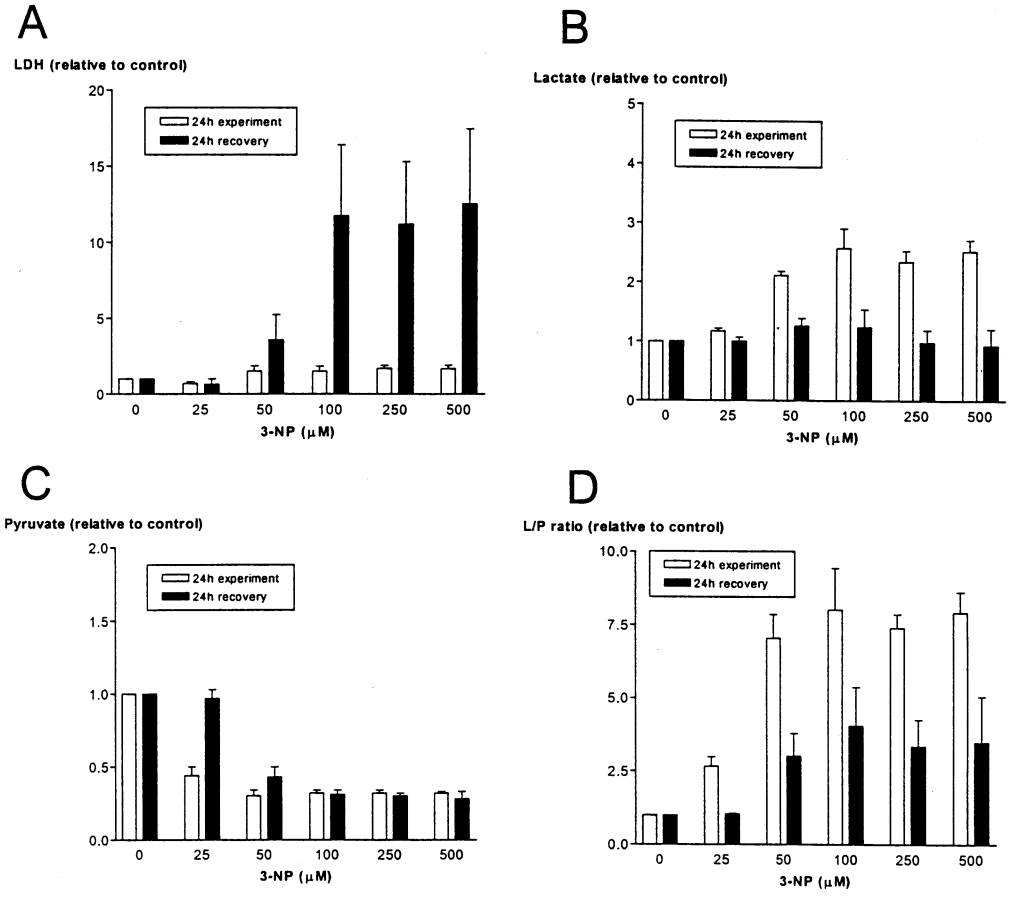

Fig. 2. 3-NP Dose-effect relations on LDH, lactate and pyruvate efflux into corticostriatal slice cultures. In two independent experiments, 1-week-old interface cultures were exposed to 3-NP dissolved in culture medium. Before 3-NP exposure, 24 h after 3-NP exposure and after a 24 h recovery period, the efflux of LDH, lactate and pyruvate was measured in the culture medium to determine cellular degeneration and metabolic activity, respectively. Additionally the lactate/pyruvate ratio is shown. Bars represent standard errors.

After 3-NP treatment both the striatum and the cortex contain numerous TUNEL labeled cells, while in control slices only the deeper layers of the cortex do. The upregulation of Bax as well as the presence of DNA fragmentation in 3-NP-exposed brain slices, suggests that 3-NP induces an apoptotic mode of cell death in organotypic slice cultures. This is in line with in vivo and other in vitro 3-NP studies [3,13,14,20]. Many TUNEL-labeled cells in the striatum of 3-NP-exposed slices do not show the morphology typical for apoptosis. Their nuclei are more diffusely stained. In some striatal neurons, however, nuclear fragmentation was observed. This is in contrast to the overlying cerebral cortex in which the majority of the TUNEL-positive neurons exhibits characteristic apoptotic morphology and extensive TUNEL-labeling. TUNEL-labeling may detect early stages of DNA fragmentation but careful assessment of the cellular morphology is necessary because not every TUNEL-positive cell is an apoptotic cell [20]. In 3-NP-exposed slices the striatal diffuse TUNEL-labeling raises the possibility of DNA damage as part of a necrotic type of cell death. However, the presence of nuclear fragmentation in TUNEL-stained cells and the diffuse Bax-immunostaining of striatal neurons, both cytoplasmic and dispersed into the neuropil, supports the notion of apoptosis as a mode of 3-NP induced neuronal death. If even more specific markers of apoptosis, like activated caspases, could be demonstrated in the 3-NP-treated striatum, apoptosis would become even more likely.

In primary cell cultures, both excitotoxic necrosis and apoptosis are implicated in cell death induced by metabolic impairment [22]. In dissociated hippocampal cultures exposed to 3-NP, Pang and Geddes [13] found both necrotic cell death characterized by cell swelling and nuclear shrinkage, and a delayed and more slowly evolving apoptotic cell death resulting in nuclear fragmentation. The mode of cell death is linked to mitochondrial dysfunction of the injured neuron [22], meaning that low doses of 3-NP induce less damage and an apoptotic-like cell death, while higher doses result in acute injury and necrotic neurons, although apoptosis could still occur in longer surviving neurons. Moreover, Portera-Cailliau et al. [15] showed that after excitotoxic lesions neuronal death in the striatum of newborn rats represent a morphological apoptosis-necrosis continuum, which depends on cell type, location and brain

maturity. Our data are in line with their observations. Striatal neurons in newborn slice cultures may have a higher potential vulnerability to 3-NP than cortical neurons, in being less mature and having less resistance to extensive mitochondrial dysfunction. Therefore, it is attractive to speculate that in our model the more severely affected striatal neurons underwent necrosis whereas less vulnerable striatal and cortical neurons were directed into the slower apoptotic pathway.

We thank M. Claessens-Linskens, J. Ibrahime-Stappers and E. Prudon-Rosmulder for biochemical analysis.

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