

3-Nitropropionic Acid Toxicity in Hippocampus: Protection Through *N*-Methyl-D-Aspartate Receptor Antagonism

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ABSTRACT: The over-activation of glutamate receptors can lead to excitotoxic cell death and is believed to be involved in the progression of neurodegenerative events in the vulnerable hippocampus. Here, we used an *in vitro* slice model to study toxicity produced in the hippocampus by the mitochondrial toxin 3-nitropropionic acid (3-NP). The organotypic slice cultures exhibit native cellular organization as well as dense arborization of neuronal processes and synaptic contacts. The hippocampal slices were exposed to 3-NP for 2–20 days, causing calpain-mediated breakdown of the spectrin cytoskeleton, a loss of pre- and postsynaptic markers, and neuronal atrophy. The *N*-methyl-D-aspartate (NMDA) receptor antagonist memantine reduced both the cytoskeletal damage and synaptic decline in a dose-dependent manner. 3-NP-induced cytotoxicity, as determined by the release of lactate dehydrogenase, was also reduced by memantine with EC₅₀ values from 1.7 to 2.3 μ M. Propidium iodide fluorescence and phase contrast microscopy confirmed memantine neuroprotection against the chronic toxin exposure. In addition, the protected tissue exhibited normal neuronal morphology in the major hippocampal subfields. These results indicate that antagonists of NMDA-type glutamate receptors are protective during the toxic outcome associated with mitochondrial dysfunction. They also provide further evidence of memantine's therapeutic potential against neurodegenerative diseases. © 2006 Wiley-Liss, Inc.

KEY WORDS: NMDA; memantine; neurodegeneration; neuroprotection; hippocampal slice cultures

INTRODUCTION

Glutamate, the fast-acting excitatory neurotransmitter in the brain, activates two ionotropic receptor subtypes, α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA)-type and *N*-methyl-D-aspartate (NMDA)-type. The glutamate receptors are well known for important signal transduction pathways that are involved in synaptic plasticity and memory (Riedel et al., 2003; Bast et al., 2005; Winters and Bussey, 2005). However, over-activation of glutamatergic responses has been found to disrupt neuronal maintenance and trigger excitotoxicity. The NMDA receptor is particularly well known for its role in a variety of excitotoxic processes including ischemia, traumatic brain injury, and oxidative stress (see Kemp and McKernan, 2002; Waxman and Lynch,

2005; Lipton, 2006), and perhaps contributes to Alzheimer's disease (AD) and other age-related disorders (Ulas et al., 1994; Hynd et al., 2004; Mishizen-Eberz et al., 2004). It may be the case that many disease states include an excitotoxic component.

Inhibiting the progression of excitotoxicity is a current therapeutic approach to treating a variety of neurodegenerative pathologies. Hence, targeting the glutamate system for development of neuroprotectants has been a focus in recent years. Glutamatergic antagonists have been shown to be protective in several models of neuropathology, including ischemia, stroke, and traumatic brain injury (e.g., Palmer, 2001; Danysz and Parsons, 2002). Much attention has been given to the specific NMDA receptor antagonist memantine, which reduces damage associated with excitotoxic lesions and other insults (Wenk et al., 1994, 1995; Miształ et al., 1996; Stieg et al., 1999; Willard et al., 2000) and acts against the neurodegeneration caused by β -amyloid (Miguel-Hidalgo et al., 2002). In addition, memantine has been reported to reduce AD-type dementia (Reisberg et al., 2003; Lipton, 2005). Thus, pharmacological inhibition of NMDA receptors is a potential strategy for preventing the progression of different types of disorders.

Here, we tested whether NMDA receptor antagonism protects against exposure to 3-nitropropionic acid (3-NP), a mitochondrial toxin. The toxin acts by inhibiting succinate dehydrogenase and disrupting cellular metabolism (Ludolph et al., 1992). 3-NP-induced toxicity has been used as an experimental model for certain age-related neurodegenerative diseases (Beal et al., 1993; Miller and Zaborszky, 1997; Bizat et al., 2003), and has been established *in vivo* to exacerbate β -amyloid neurotoxicity (Arias et al., 2002). In our study we made use of organotypic slice cultures prepared from the hippocampus, a brain region targeted by a variety of neurological disorders, including stroke and AD. Hippocampal slice cultures provide a sensitive model with similar cellular and genetic responses to pathogenic insults as compared to those found *in vivo* (see Vornov et al., 1994; Bahr, 1995; Bendiske et al., 2002; Caba and Bahr, 2004; Bonde et al., 2005). Their three-dimensional nature exhibits features characteristic of the adult hippocampus, such as the circuitry, morphological integrity, and organization of neuronal subfields. The present report

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describes the toxic effects of 3-NP in hippocampus using multiple measures of toxicity, and protection via blockage of NMDA receptors.

MATERIALS AND METHODS

Organotypic Hippocampal Slice Cultures

Hippocampal slice cultures were prepared as previously described (Bahr et al., 1995a,b). Sprague-Dawley rat litters (Janvier, France; Charles River Laboratories, Wilmington, MA) were housed in accordance with approved protocols. Animals 11–12 days postnatal were sacrificed and hippocampi rapidly dissected. Transverse slices of hippocampus (400 μm) were then quickly prepared and maintained on Millicell-CM inserts (Millipore Corporation, Bedford, MA) at 37°C in humidified air with 5% CO₂. Media was changed every 2–3 days, consisting of basal medium Eagle (50%), Earle's balanced salts (25%), horse serum (25%), and defined supplements (Bahr et al., 1995a). The slices were allowed to mature and stabilize for 2 weeks.

Induction of Toxicity and Lactate Dehydrogenase Assay

To induce toxicity, hippocampal slices were incubated with 35 μM 3-NP for 2–12 days. To study the neuroprotective potency of NMDA receptor antagonism, 3-NP-treated slices were treated with 1–10 μM memantine. Memantine is a moderate-affinity, uncompetitive NMDA receptor antagonist that has recently received approval for the treatment of AD in Europe and the United States. The release of lactate dehydrogenase (LDH) into the culture medium was determined colorimetrically using the CytoTox96R nonradioactive cytotoxicity assay kit (Fisher Scientific, Pittsburgh, PA). Resulting absorbances were recorded at a wavelength of 490 nm and corrected for background LDH activity present in the culture medium, as well as for spontaneous LDH release of nontreated control slices. Three to four independent assays per condition were conducted, consisting of ~50 slices per assay.

Immunoblot Analysis

Slice cultures were gently removed with a soft brush and homogenized in groups of 6–8 using ice-cold buffer consisting of 0.32 M sucrose, 5 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, and a protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin. Protein content was determined with a bovine serum albumin (BSA) standard and equal aliquots of the slice samples were denatured in SDS for 5 min at 100°C, then separated by 4–16% SDS-PAGE, and blotted to nitrocellulose. Immunodetection was accomplished by incubation overnight at 4°C with affinity-purified antibodies to calpain-mediated spectrin fragment BDP_N (Bahr et al., 1995b), anti-synapsin II (Boehringer Mannheim, Indianapolis, IN), and

affinity-purified antibodies against the AMPA receptor subunit GluR1 (Bahr et al., 1996). Secondary antibody incubation utilized anti-IgG-alkaline phosphatase conjugates, and color development used the 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system. Color development of immunoreactive bands was terminated before maximal intensity was reached in order to avoid saturation and to ensure a linear relationship with increasing amount of sample protein. Bands were scanned at high resolution to determine integrated density with BIOQUANT software (R&M Biometrics, Nashville, TN), and the means \pm standard error of mean (SEM) were compared using analysis of variance (ANOVA).

Propidium Iodide Staining

Organotypic cultures were stained with propidium iodide (10 $\mu\text{g}/\text{ml}$) for 1 h. Propidium iodide uptake is indicative of significant membrane injury, especially that induced by glutamate neurotoxicity in hippocampal slice cultures (Vornov et al., 1994). Propidium iodide fluorescence was elicited at 546 nm and recorded at 610 nm on an Axiovert 10 inverse fluorescence microscope. Phase contrast images were also captured to determine slice integrity.

Histology

Slices were treated with 30 μM 3-NP for 20 days in the absence or presence of 10 μM memantine. Along with untreated control cultures, the slices were rinsed with 0.1 M phosphate buffer, pH 7.4, and fixed for 2 h in cold buffer containing 4% paraformaldehyde. Slices were then cryoprotected and carefully removed from the insert, and serial sections were cut at 20 μm thickness using a sliding microtome and mounted on gelatin-coated slides. Sections were stained with cresyl violet, or were immunolabeled with antibodies to neurofilament-200 (Sigma, St. Louis, MO) and synaptophysin (Chemicon, Temecula, CA). Horseradish peroxidase (HRP) was applied to a small zone of the CA1 stratum pyramidale 2 h before the fixing procedure, to visualize transported HRP in apical dendrites and spine structures using diaminobenzidine.

RESULTS

Neuronal morphology and circuitry are stably preserved in organotypic slice cultures of hippocampus prepared at PND 11–12. An organized stratum pyramidale is seen in Figure 1A, a photomicrograph of a Nissl-stained section from a slice maintained in culture for 5 weeks. The characteristic intricate neuropil of the CA1 stratum radiatum was labeled with antineurofilament-200 (Fig. 1B). Similar dendritic and axonal arborizations were evident in the CA3 subfield, the molecular layer of the dentate gyrus (DG), and the hilus. Arrays of presynaptic terminals (Fig. 1C) and dendritic spines (Fig. 1D) were also evident by labeling the neuropil zone with antisynaptophysin and internalized HRP, respectively. Terminal density matched the organization of dense spine structures extending from the

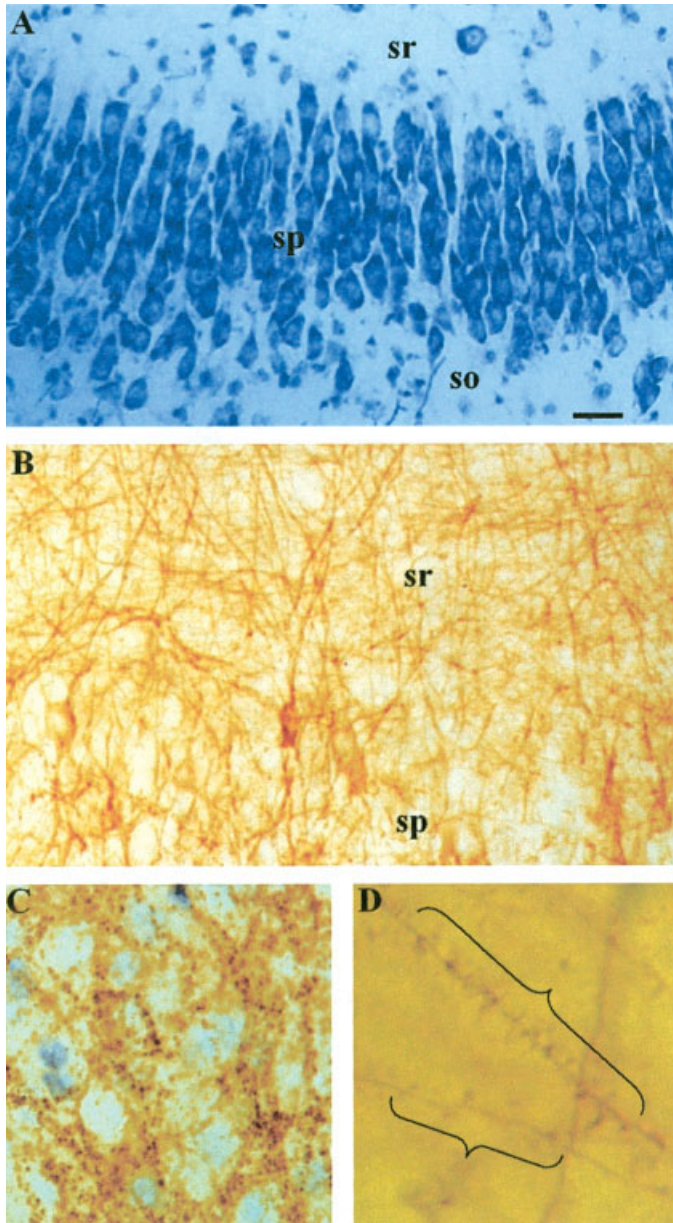


FIGURE 1. Hippocampal slice cultures exhibit native features of the adult hippocampus. Transverse slices prepared from postnatal day 11–12 hippocampi were maintained for 5 weeks in culture. (A) A section stained with cresyl violet exhibits normal organization of CA1 pyramidal neurons. (B) Immunocytochemistry using antibodies against neurofilament-200 revealed an intricate array of neuronal processes in the dendritic field of CA1. (C) Presynaptic terminals distributed along dendrites were stained for the synaptic vesicle marker synaptophysin. (D) Dendritic spines were also evident extending from dendritic processes loaded with HRP (see noted segments). so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Scale bar: (A, B), 35 μm ; (C, D), 5 μm .

surface of dendritic processes. The native features of the cultured slice render it an appropriate model to study mechanisms of neurotoxin action and synaptopathogenesis.

The hippocampal slice model was used to study the mitochondrial toxin 3-NP and its effects on synaptic integrity. As

shown in the immunoblots of Figure 2A, slice cultures subjected to 3-NP for 7 days exhibited marked declines in presynaptic (synapsin II) and postsynaptic proteins (GluR1). The synaptic determination was found to be reduced by the NMDA

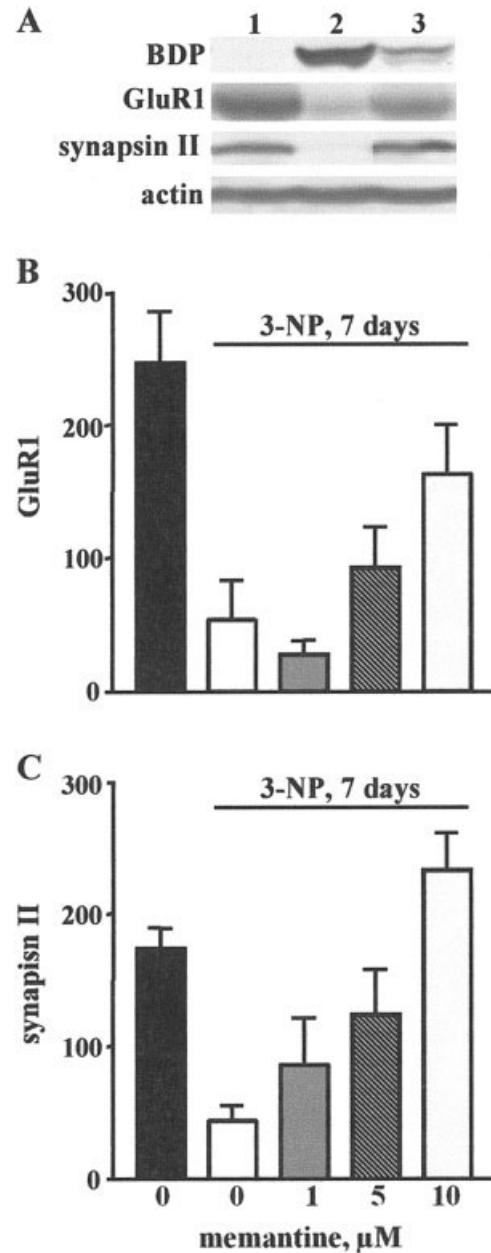


FIGURE 2. Blocking NMDA receptors protects against 3-NP-induced cytoskeletal damage and synaptic decline. Hippocampal slice cultures were exposed to 35 μM 3-NP for 7 days in the absence or presence of 1–10 μM of the NMDA receptor antagonist memantine. The slices were harvested in groups of 6–8 each, along with untreated sister cultures, and prepared for immunoblotting (A). Control slices (lane 1) and 3-NP-treated slices without (lane 2) or with 10 μM memantine (lane 3) were assessed for calpain-mediated spectrin breakdown product BDP_N, GluR1, and synapsin II. Graphs contain integrated optical densities (mean \pm SEM) as determined by image analysis for the postsynaptic marker GluR1 (B) and the presynaptic marker synapsin II (C). ANOVA: $P < 0.0001$.

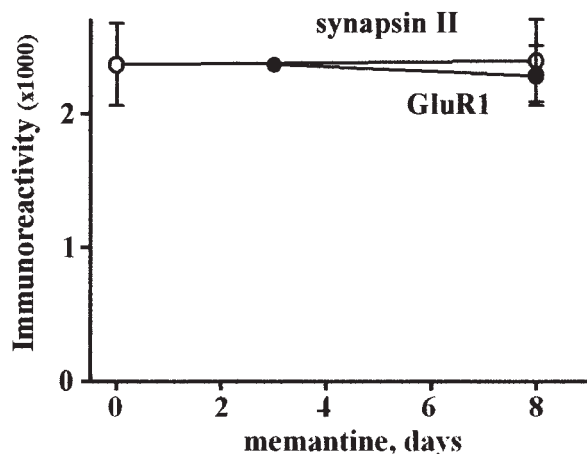


FIGURE 3. Memantine treatment alone does not cause synaptic decline. Hippocampal slice cultures were exposed to 10 μ M memantine for 8 days. Control slices and memantine-treated slices were assessed for the presynaptic marker synapsin II (open circles) and for the postsynaptic marker GluR1 (filled circles). Points represent integrated optical densities (mean \pm SEM) as determined by image analysis.

receptor antagonist memantine. In fact, treatment with memantine throughout the 7-day toxin exposure resulted in substantial synaptic protection in a dose-dependent manner. The NMDA receptor antagonist protected GluR1 levels by 82% (Fig. 2B; ANOVA $P = 0.0017$) and completely protected synapsin II levels (Fig. 2C; $P < 0.001$). Loss of synaptic markers is commonly linked to the pathogenic activation of the calcium-dependent protease calpain (see Bahr et al., 2002; Munirathinam et al., 2002; Karanian et al., 2005). Correspondingly, the synaptic decline in Figure 2A was associated with calpain-mediated spectrin breakdown product BDP_N (lane 2), an early and well-known marker of excitotoxic cytoskeletal damage (Vanderklish and Bahr, 2000). 3-NP-treated slice cultures that were co-incubated with 10 μ M memantine showed a marked reduction in BDP_N (Fig. 2A; lane 3). BDP_N immunoreactivity generated in response to 3-NP exposure was reduced by memantine from 444 ± 59 to 152 ± 9 (mean \pm SEM; $P < 0.0001$). Thus, NMDA-type glutamate receptors are involved in 3-NP-induced synaptic and cytoskeletal changes. As a control, memantine treatment alone was found to have no effect on synaptic markers (Fig. 3), nor was there any evidence of cytoskeletal breakdown (not shown).

Besides 3-NP-mediated synaptic and cytoskeletal damage in the slice model, histological analysis indicates obvious neuronal damage. Exposure to 35 μ M 3-NP for 12 days resulted in evident cytotoxicity in all hippocampal subfields (Fig. 4B), as compared to the neuronal strata in control tissue (Fig. 4A). Correspondingly, we used an LDH assay to quantitate the induced damage as LDH is released from damaged neurons into the media. The 3-NP-treated slice cultures exhibited increased levels of LDH release across exposure time (see Table 1). The LDH release was pronounced across 3-NP exposure times of 7 (Fig. 5A), 9 (Fig. 5B), and 12 days (Fig. 5C). Note that the

data (means \pm SEM) were normalized to the level of LDH released from slices treated with 3-NP for 12 days (Fig. 5C). Assessment of LDH release also indicates that memantine protects against signs of cellular damage in a dose-dependent fashion. Memantine administered during the 3-NP treatment periods significantly reduced LDH release ($P < 0.001$, ANOVA for each). The EC_{50} for memantine's protective effect ranged from 1.7 to 2.3 μ M. The lowest dose of memantine tested (1 μ M) produced a consistent reduction in LDH release of 37–39%, while the highest dose tested (10 μ M) reduced 3-NP toxicity by 65–82%. These results demonstrate the neuroprotective action of memantine following 3-NP exposure. Therefore, in addition to preserving cytoskeletal and synaptic integrity, memantine also reduces signs of cellular atrophy after 12 days of mitochondrial dysfunction.

To confirm the protective nature of memantine, the hippocampal tissue was monitored with propidium iodide, phase contrast microscopy, and Nissl staining. In Figure 6 (left

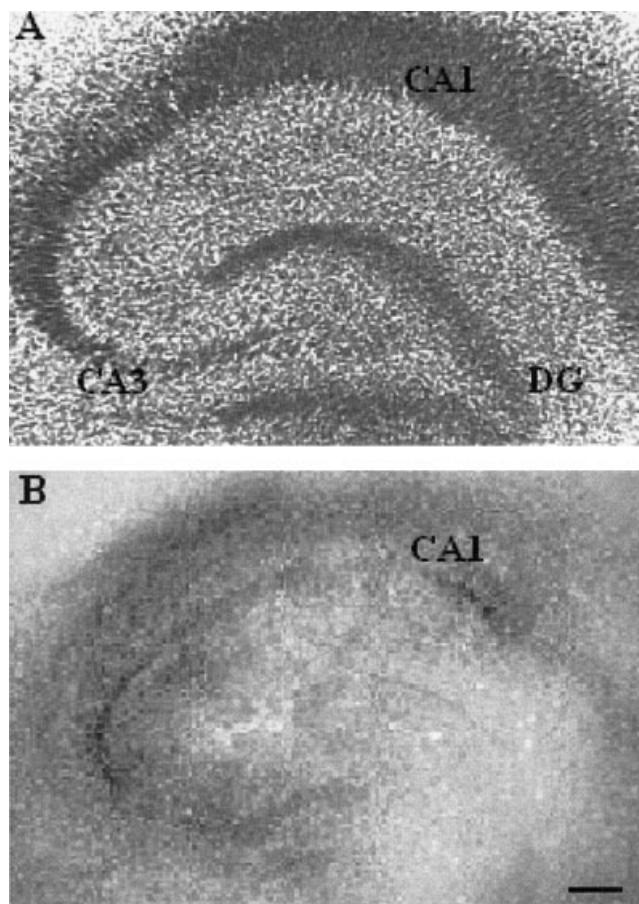


FIGURE 4. Chronic 3-NP exposure results in neuronal atrophy in subfields of hippocampus. Untreated hippocampal slice cultures (A) and cultures treated with 35 μ M 3-NP for 12 days (B) were fixed and stained with cresyl violet. Control tissue exhibits neuronal layers of the hippocampal subfields CA1, CA3, and DG, present with normal cellular morphology. Tissue treated with 3-NP was found to have clear neurodegeneration in all three hippocampal subfields. Scale bar: 250 μ m.

TABLE 1.

3-NP-Induced LDH Release in Hippocampal Slice Cultures

Treatment days	Control	Triton X-100	3-NP	3-NP + memantine
1–2	2 ± 0.4	188 ± 33	2 ± 0.3	2 ± 0.7
5	3 ± 0.4		11 ± 3	6 ± 3
7	3 ± 0.5		38 ± 9	9 ± 2
9	4 ± 2		49 ± 9	14 ± 2
12	4 ± 1		57 ± 11	21 ± 3

Slice cultures were treated with control media or with 35 μ M 3-NP for 2–12 days, and the tabulated measurements of LDH activity (absorbance units \times 100; mean \pm SEM) were determined from samples of culture medium using a colorimetric assay. Positive control for cell death used 0.9% Triton X-100 incubated with slices for 1 day. Slices treated with 3-NP in the presence of 10 μ M memantine exhibited reduced levels of LDH release ($P < 0.0001$, two-way analysis of variance).

images), uptake of propidium iodide revealed cellular damage in tissue exposed to 3-NP for 12 days. The degeneration extended to the major neuronal subfields in hippocampus. Note that the uptake correlates with LDH release as well as with the degree of histologic change in excitotoxic slice cultures (Vornov et al., 1994). Blocking NMDA receptors with memantine eliminated the 3-NP-induced propidium iodide uptake in a dose-dependent manner, and similar neuroprotection was seen in phase contrast images (Fig. 6, right column). Protection was also evident in Nissl-stained slices that were exposed to 3-NP for 20 days. The 3-NP exposure period was increased to examine memantine's effect on long-term survival. The toxin exposure caused substantial destruction of hippocampal subfields, as indicated by neuronal atrophy and pyknotic degeneration (Figs. 7B,E; control tissue in panels A and D). Concurrent application of memantine dramatically reduced the toxic effects, resulting in the preservation of neuronal morphology in pyramidal fields and in the stratum granulosum (Figs. 7C,F, respectively). Together, these findings suggest that memantine promotes long-term cell survival against the toxic actions of 3-NP.

DISCUSSION

The present report utilized an organotypic slice model to show that NMDA receptors are involved in the hippocampal degeneration produced by the mitochondrial toxin 3-NP. Protection by the NMDA receptor antagonist memantine was evident after 7–20 days of chronic exposure to 3-NP. The toxic response in the hippocampus included cytoskeletal breakdown, synaptic decline, pyknotic changes, and indications of cell death. Co-infusion of memantine with 3-NP resulted in cytoskeletal and synaptic protection as well as neuronal maintenance in hippocampal subfields.

The toxic action of 3-NP is of particular interest since mitochondrial dysfunction may contribute to both excitotoxic pro-

gression and age-related neurodegenerative disorders (see Beal et al., 1993; Miller and Zaborszky, 1997; Martin et al., 2006). In this study, stable hippocampal slice cultures exhibiting native

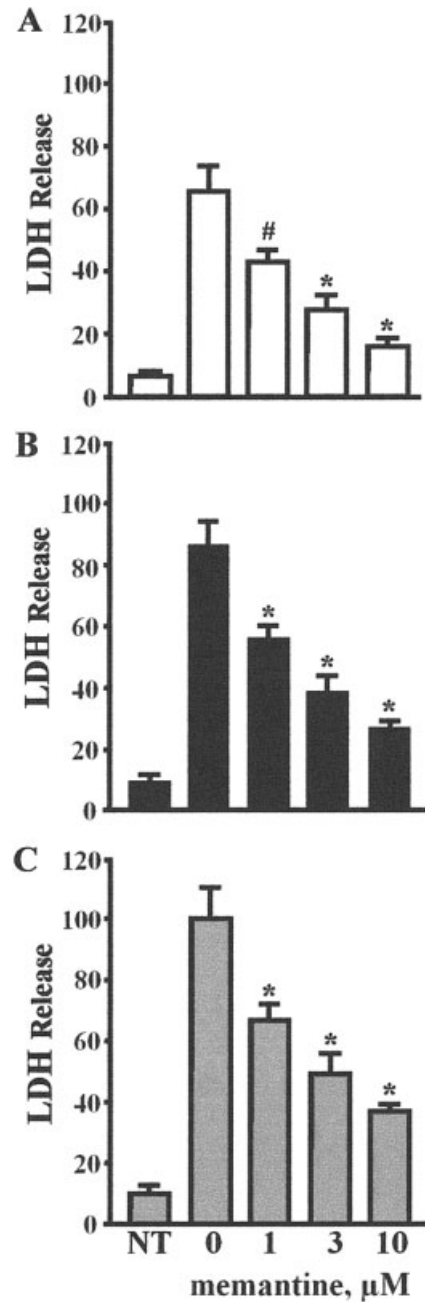


FIGURE 5. NMDA receptor blockade promotes cell survival in 3-NP-treated tissue. Hippocampal slice cultures were exposed to 35 μ M 3-NP for 7 (A), 9 (B), and 12 days (C) in the absence or presence of the NMDA receptor antagonist memantine (1–10 μ M). The treated slices and nontreated controls (NT) were assessed for LDH released into the culture medium. LDH assay absorbances were corrected for background activity, and the data (mean \pm SEM) were normalized to the level of LDH released from slices treated with 3-NP for 12 days (set at 100%). Dose-dependent reductions in LDH activity by memantine were assessed by ANOVA ($P < 0.001$ in each case; post-hoc tests compared to 3-NP alone: # $P < 0.01$, * $P < 0.001$).

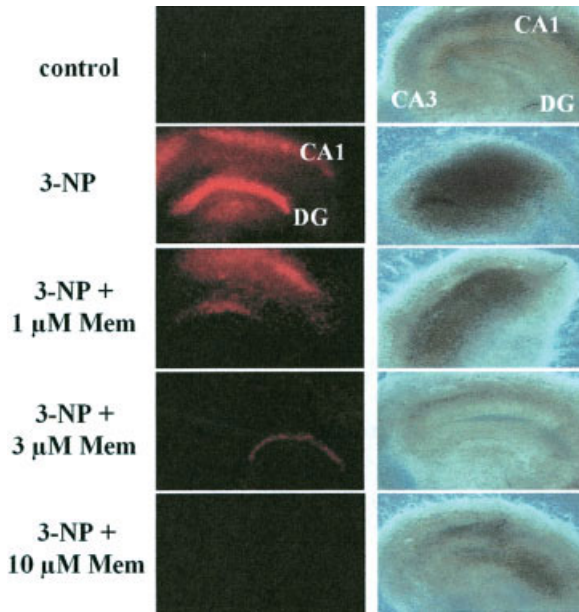


FIGURE 6. Memantine reduces cellular damage as revealed by propidium iodide staining (left images) and phase contrast microscopy (right images). Hippocampal slice cultures were treated with 35 μM 3-NP for 12 days in the absence or presence of 1–10 μM memantine (Mem). Indicative of membrane damage, the 3-NP exposure caused marked propidium iodide uptake into neuronal layers as compared to untreated control slices. Phase contrast microscopy also revealed evidence of swelling and cellular atrophy in slices treated with 3-NP. Memantine blocked the 3-NP-induced degeneration. The NMDA receptor antagonist reduced the damage in a dose-dependent manner, and tissue treated with the highest dose expressed little to no evident cytotoxicity. View-field width: ~ 4 mm.

neuronal organization and synaptic integrity were found to be vulnerable to the mitochondrial toxin. Gradual neurodegeneration was evident across the 3-NP exposure period, with damage

eventually being expressed in the three major hippocampal subfields. Others have reported CA1 selectivity for 3-NP-mediated damage in hippocampal slice cultures prepared from younger animals (Noer et al., 2002). Note that our protective results with memantine correspond with several studies using long-term slice cultures to describe the innate vulnerability of the hippocampus to over-activation of glutamate receptors including NMDA receptors (Bahr et al., 1995b; Bruce et al., 1995; Newell et al., 1997; Kristensen et al., 2001; Bonde et al., 2005). Blocking glutamatergic activity, especially through NMDA receptors, has also been shown to protect against ischemic insults in the cultured slices (Vornov et al., 1994; Newell et al., 1995; Strasser and Fischer, 1995; Pringle et al., 1997). Thus, the slice cultures have developed into a convenient system for studying not only mechanisms of toxicity but also effective avenues of neuroprotection (see Bahr, 1995; Breder et al., 2000; Ray et al., 2000; Bahr et al., 2002; Munirathinam et al., 2002; Karanian et al., 2005; Noraberg et al., 2005). The past and present findings indicate that the slice model allows an avenue for studying mitochondrial toxicity in complex brain tissue and in the absence of systemic issues.

By targeting mitochondria, 3-NP can induce several damaging processes. Cellular metabolism is disrupted, leading to a loss of cellular energy (Ludolph et al., 1991, 1992; Reynolds et al., 1998) and possibly making neurons more vulnerable to glutamate excitotoxicity. Slice cultures treated with 3-NP exhibited spectrin breakdown through the calcium-activated protease calpain, a common excitotoxic event linked to hippocampal cell death (see Vanderklish and Bahr, 2000; Bahr et al., 2002). The 3-NP exposure also associated declines in synaptic proteins with the cytoskeletal damage, and both signs of pathology were significantly reduced by memantine. In vivo, calpain has been implicated in the 3-NP-mediated cytoskeletal breakdown and concurrent striatal degeneration (Bizat et al., 2003, 2005). It

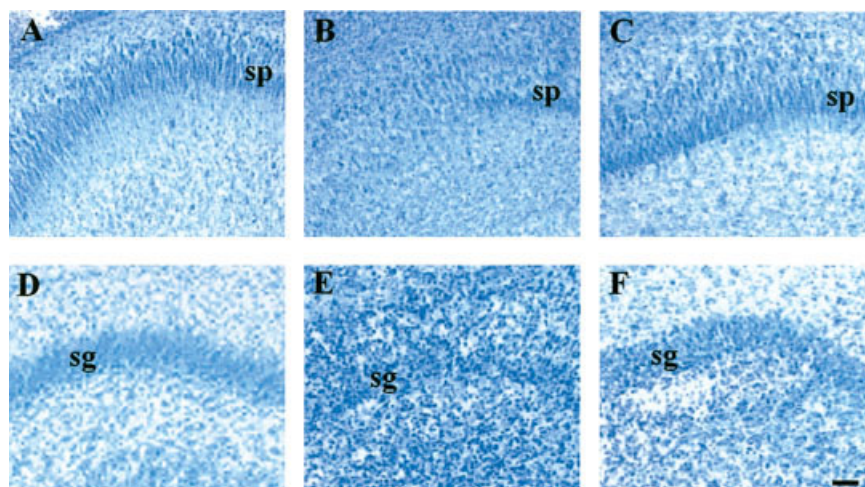


FIGURE 7. Memantine is neuroprotective in slices treated with 3-NP for 20 days. Untreated control slices (A, D) and slices treated with 35 μM 3-NP for 20 days in the absence (B, E) or presence of 10 μM memantine (C, F) were fixed and stained with cresyl violet. Photomicrographs of field CA1 (A–C) and the upper leaf of the

DG (D–F) are shown. 3-NP exposure was associated with the presence of pyknotic nuclei and loss of neurons. Blocking NMDA receptors with memantine greatly reduced 3-NP toxicity, preserving the typical neuronal morphology as found in control slices. sg, stratum granulosum; sp, stratum pyramidale. Scale bar: 35 μm .

should be pointed out that extended memantine treatment alone had no effect on pre- and postsynaptic markers in the slice model. The data suggest that calcium influx through NMDA receptors mediates 3-NP-induced calpain activation and subsequent neuronal compromise.

Memantine also reduced neuronal damage in 3-NP-treated slice cultures as indicated by marked decreases in LDH release and propidium iodide staining. The question remains as to the exact role the NMDA receptor plays in 3-NP toxicity. A likely pathogenic cascade stems from the metabolic compromise, causing glutamate release from the compromised neurons, and subsequent over-activation of NMDA receptors in those and neighboring neurons. Targeting distinct steps in this cascade indeed provides neuroprotective strategies, for example through improvement of energy metabolism or inhibiting glutamate release (Dessi et al., 1995; Schultz et al., 1996; Khodorov et al., 2002). As in the present study, over-activated NMDA receptors have been implicated by reports that antagonists such as memantine, AP-5, and MK-801 are effective protectants against rises in intracellular calcium, glutamate toxicity, and excitotoxic lesions (see Schultz et al., 1996; Wenk et al., 1996; Storozhevskiy et al., 1998). In the case of excitotoxic lesions produced by intrastriatal 3-NP, MK-801 was not protective, suggesting the severe localized insult activates NMDA and non-NMDA-type glutamate receptors (Beal et al., 1993). Alternative to receptor over-activation in response to excessive release of glutamate, constitutively activated NMDA receptors may be involved in the pathogenic cascade. Mitochondria act as calcium buffers to regulate intracellular calcium levels, and mitochondrial dysfunction would exacerbate any stress on calcium homeostasis (see Kristian and Siesjö, 1998; Danbolt, 2001). During chronic 3-NP exposure in the slice model, constitutive activation of NMDA receptors may result in a gradual rise in cytosolic calcium, eventually leading to damaging proteolytic events and atrophy. Note that Beal et al. (1993) described excitotoxic damage in the absence of any increase in extracellular glutamate after systemic 3-NP administration. The protective results with memantine reported here indicate a key role for NMDA receptors in the toxic cascades.

Mitochondrial dysfunction also has a propensity to produce reactive oxygen species and such oxidative stress can cause hyperexcitability of NMDA receptors (Avshalumov and Rice, 2002), resulting in a pathological cycle with concomitant generation of free radicals. The damaging free radicals can also lead to pathogenic activation of proteases, including calpain and caspases (see Butler and Bahr, 2005). NMDA receptor antagonists may have the potential to reduce the mitochondrial abnormalities and secondary events of cytochrome *c* release, protease activation, and associated apoptosis. Other factors to consider are potential mitochondrial release factors that promote secondary events that directly modulate NMDA receptors (Manzoni et al., 1992; Kristian and Siesjö, 1998; Choi et al., 2000). The ability of NMDA antagonists to treat AD may be related to the prevention of the noted apoptotic episodes and vicious cycles (Danysz and Parsons, 2002; Butterfield and Pocernich, 2003; Molinuevo et al., 2005; Sonkusare et al.,

2005). Excitotoxicity and free radical generation can enhance the risk of neurodegenerative events, and oxidative damage is thought to be a major factor in age-related diseases, involving mitochondrial membrane permeability, lysosomal dysfunction, and protein accumulation (see Bahr and Bendiske, 2002; Boya et al., 2003; Zhu et al., 2004; Butler and Bahr, 2005). Negative modulation of NMDA receptors may protect against distinctly different disease states that have in common the production of reactive oxygen species, and a combination with free radical scavengers may improve neuroprotection.

The neuroprotective action of memantine indicates that a certain degree of NMDA receptor antagonism is effective at attenuating excitotoxicity associated with acute and chronic brain injury as well as with dementia. Extended treatment with a potent NMDA receptor antagonist may be problematic since stimulation of NMDA receptors enhances the expression of neurotrophins that protect neurons against ischemic damage and traumatic injury (Sharma et al., 1998; Wu and Pardridge, 1999). However, memantine is proposed to modulate NMDA receptor activity rather than completely shut it down, due to its moderate affinity and associated fast kinetics and strong voltage-dependency (Parsons et al., 1993). Here, extended memantine treatment promoted neuronal maintenance. Moreover, trophic responses are not only mediated by NMDA-type glutamate receptors. The non-NMDA AMPA receptors are also active in repair signaling and cell maintenance (Bambrick et al., 1995; McKinney et al., 1999; Bahr et al., 2002). Furthermore, while NMDA receptors are involved in learning and memory, it is important to note that memantine does not disrupt learning, but rather prevents learning impairment and functional deficits caused by hippocampal excitotoxicity and other neurodegenerative insults (Wenk et al., 1994, 1995; Misztal et al., 1996; Stieg et al., 1999; Miguel-Hidalgo et al., 2002). In the slice model, memantine treatment alone had no effect on synaptic markers that are important for brain function, and memantine protected the markers against toxicity. The hippocampal slice model will be valuable for further elucidation of toxic mechanisms and for testing pharmaceutical actions against different disease states.

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