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Mitochondrial Complex I Inhibition Produces Selective Damage to Hippocampal Subfield CA1 in Organotypic Slice Cultures

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The effects of mitochondrial respiratory chain inhibitors and the excitotoxin *N*-methyl-D-aspartate (NMDA) on cell death in hippocampal subfields CA1 and CA3 were examined in hippocampal organotypic slice cultures. Slice cultures, 2-3 week old, were exposed for 1 h to either the Complex I inhibitors, rotenone or 1-methyl-4-phenylpyridium (MPP^+), the Complex II inhibitor 3-nitropropionic acid (3-NP), or the excitotoxin NMDA. Cell death was examined 24 and 48 h following treatment, by measuring propidium iodide (PI) fluorescence. Treatment with 1 μM rotenone caused greater cell death in hippocampal subfield CA1 than CA3. Exposure of hippocampal slice cultures to 10 μM rotenone, to MPP^+ or to NMDA resulted in damage to both CA1 and CA3 subfields. 3-NP produced little damage in either subfield. The data suggest that mitochondrial complex I inhibition can produce selective cell damage in hippocampus and in this regard is similar to that observed following hypoxia /ischemia.

Keywords: Selective neuronal vulnerability; Mitochondrial toxins; Excitotoxicity

INTRODUCTION

Many neurological disorders are characterized by degeneration of specific neuronal populations. Global ischemia results in selective damage to hippocampal pyramidal cells in the CA1 subfield, dorsolateral striatum, and cortex (Kirino, 1982; 2000; Pulsinelli *et al.*, 1982). Parkinson's disease is characterized by selective damage to dopaminergic neurons in the substantia nigra (Betarbet *et al.*, 2002). Similarly, selective neuronal degeneration of neurons in nucleus basalis of Maynert and cortical motor neurons are common features of Alzheimer's disease (Whitehouse *et al.*, 1981) and amyotropic lateral sclerosis (Eisen and Weber, 2001), respectively.

Mitochondria have been implicated as mediators of neuronal death in a variety of neurological conditions (Fiskum *et al.*, 1999; Beal, 2000; Greenamyre *et al.*, 2001). Patients with Parkinson's disease showed deficiencies of respiratory chain complex I (Schapira *et al.*, 1989), and systemic administration of complex I inhibitors or their precursors produced degeneration of dopaminergic neurons in the substantia nigra in animals (Betarbet *et al.*, 2002) and humans (Langston *et al.*, 1983; 1999). Selective damage occurs to dopaminergic neurons in the substantia nigra following systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) because MPTP is oxidized to the toxic metabolite MPP^+ (Langston *et al.*, 1984) which is transported into dopaminergic neurons (Javitch and Snyder, 1984). Toxicity of MPP^+ appears to be, at least in part, due to its ability to inhibit complex I (Nicklas *et al.*, 1987). Another complex I inhibitor, rotenone, selectively damages dopaminergic neurons in the substantia nigra although it apparently inhibits complex I uniformly throughout the brain (Betarbet *et al.*, 2000).

Mitochondrial impairment, and specifically impairment of complex I of the respiratory chain have been reported in brain following ischemia [5,24,28,29] (Sims and Pulsinelli, 1987; Sciamanna *et al.*, 1992; Rosenthal *et al.*, 1995; Dave *et al.*, 2001). However, it is unclear whether inhibition of complex I is sufficient to damage neurons known to be selectively vulnerable

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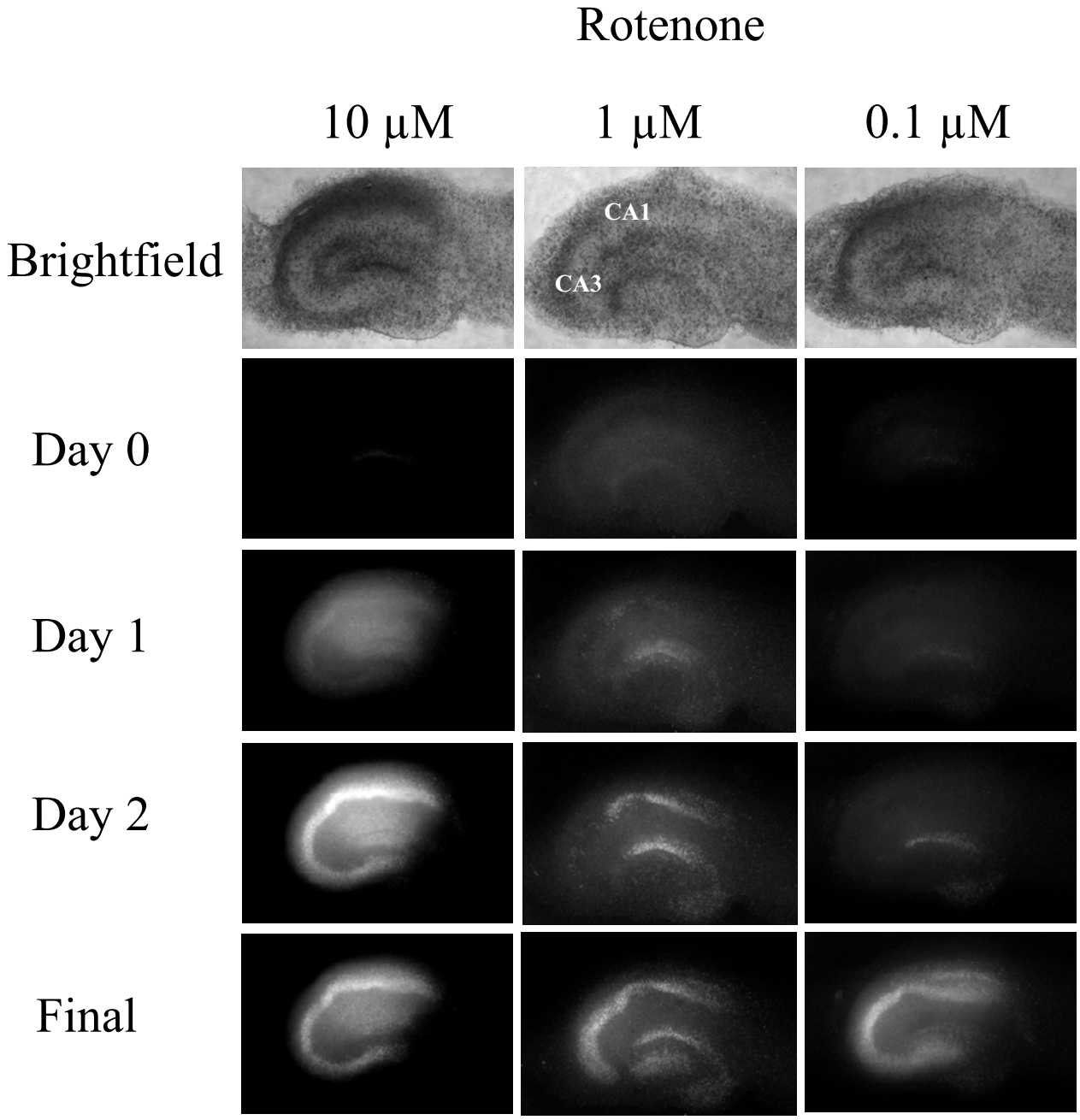

FIGURE 1 Examples of brightfield and propidium iodide (PI) fluorescence images of hippocampal organotypic slice cultures. Rotenone (10 μM, 1 μM, or 0.1 μM) was applied for 1 h after acquiring background images on Day 0. Final PI fluorescence was obtained 24 h after applying 100 μM NMDA for 1 h on Day 3.

to hypoxia/ischemia. We demonstrate here that exposure of hippocampal organotypic slice cultures to the complex I inhibitor rotenone results in selective damage to CA1 pyramidal cells similar to that reported following hypoxia/ischemia.

MATERIALS AND METHODS

Preparation of Cultures

Organotypic slice cultures of the hippocampus were prepared according to the methods described by Bergold and Casaccia-Bonnefil (1997) and the protocol was approved by the University of Miami, Animal Care and Use Committee. Neonatal Sprague Dawley rats (9-11 days old) were anesthetized by single intraperitoneal injections of ketamine (1.0 mg/pup). The pups were decapitated and the hippocampi dissected free from the cerebral hemispheres and transversely sliced (400 μm) on a McIlwain tissue chopper. Slices

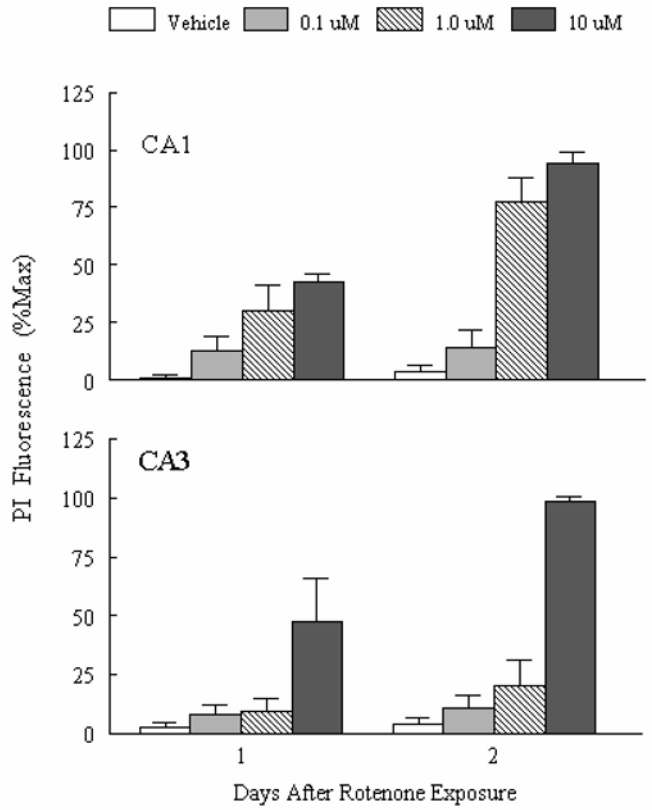

FIGURE 2 Average values (Mean ± SEM) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure, either to vehicle (0.1% ethanol), 0.1 μM, 1.0 μM, or 10 μM rotenone. Statistical comparisons of groups are provided in the text.

were incubated in Gey's Balanced Salt Solution (Gibco/Life Technologies, USA) supplemented with 6.5 mg/ml glucose (Sigma Chemical Co., St. Louis, MO, USA) for 1 h at 4°C. Slices then were transferred onto 30-mm diameter membrane inserts (Millicell-CM, Millipore, USA), and were transferred to 6 well culture trays with 1 ml of slice culture medium per well. The slice culture medium consisted of 50% Minimum Essential Medium (Gibco/Life Technologies), 25% Hank's balanced salt solution (Gibco/Life Technologies), 25% heat inactivated horse serum (Gibco/Life Technologies) supplemented with 6.5 mg/ml glucose and glutamine. The cultures were maintained at 37°C in an incubator (Nuaire, CF autoflow) with an atmosphere of humidified 21% O_2-5% CO_2. The slice culture medium was changed twice a week and slices were cultured for 14-15 days before experiments.

Assessment of Cell Death

The number of dead or dying cells in hippocampal subfields CA1 and CA3 was estimated by fluorescent staining with propidium iodide (PI) (Sigma). Prior to experimental treatment (exposure to mitochondrial inhibitors), slices were incubated in culture medium supplemented with 2 μg/ml PI for 1 h. Images of PI fluorescence in organotypic slices were acquired using an inverted fluorescence microscope (Olympus IX 50) and Spot CCD camera (Diagnostic Instruments Inc., Sterling Heights MI) and SPOT advanced software. Images of cultured slices were taken prior to toxin exposure, to estimate background fluorescence, and again 24 and 48 h later. Finally, the slices were superfused with NMDA (100 μM)(Sigma) for 1 h and a terminal image was acquired 24 h later to estimate maximum cell death. For quantification, regions of interest (ROIs) corresponding to hippocampal subfields CA1 and CA3 were selected from bright field images of each slice using Scion Image software (Windows Version). ROIs were transferred to individual images in an image stack after optical alignment of brightfield images. Relative cell death was calculated from each ROI as follows: Relative % cell death = (Fexp - Fmin)/(Fmax - Fmin)*100, where Fexp is the fluorescence of the test condition, Fmax is maximum fluorescence observed during an experiment (following toxin exposure or terminal treatment with 100 μM NMDA for 1 h), and Fmin is background fluorescence (prior to treatment).

Mitochondrial Inhibitors

Hippocampal organotypic slice cultures were treated for 1 h with either the complex I inhibitors rotenone (0.1 μM, 1 μM ,10 μM) or MPP^+ (10 μM, 100 μM, 1 mM), the complex II inhibitor 3-NP (10 μM, 100 μM, 1 mM), or the excitotoxin NMDA (1 μM ,10 μM, 100 μM). All compounds were dissolved in culture medium (see above) except rotenone, which was dissolved in culture medium containing 0.1% ethanol. Sham treated slices were incubated for 1 h in exchanged culture medium or culture medium containing 0.1% ethanol (vehicle for rotenone).

Statistics

Experimental groups were compared statistically using Analysis of Variance (ANOVA).

RESULTS

Hippocampal organotypic slice cultures were a reliable model for assessing cell death following exposure to mitochondrial toxins. Little background PI fluorescence was observed in hippocampal subfields CA1

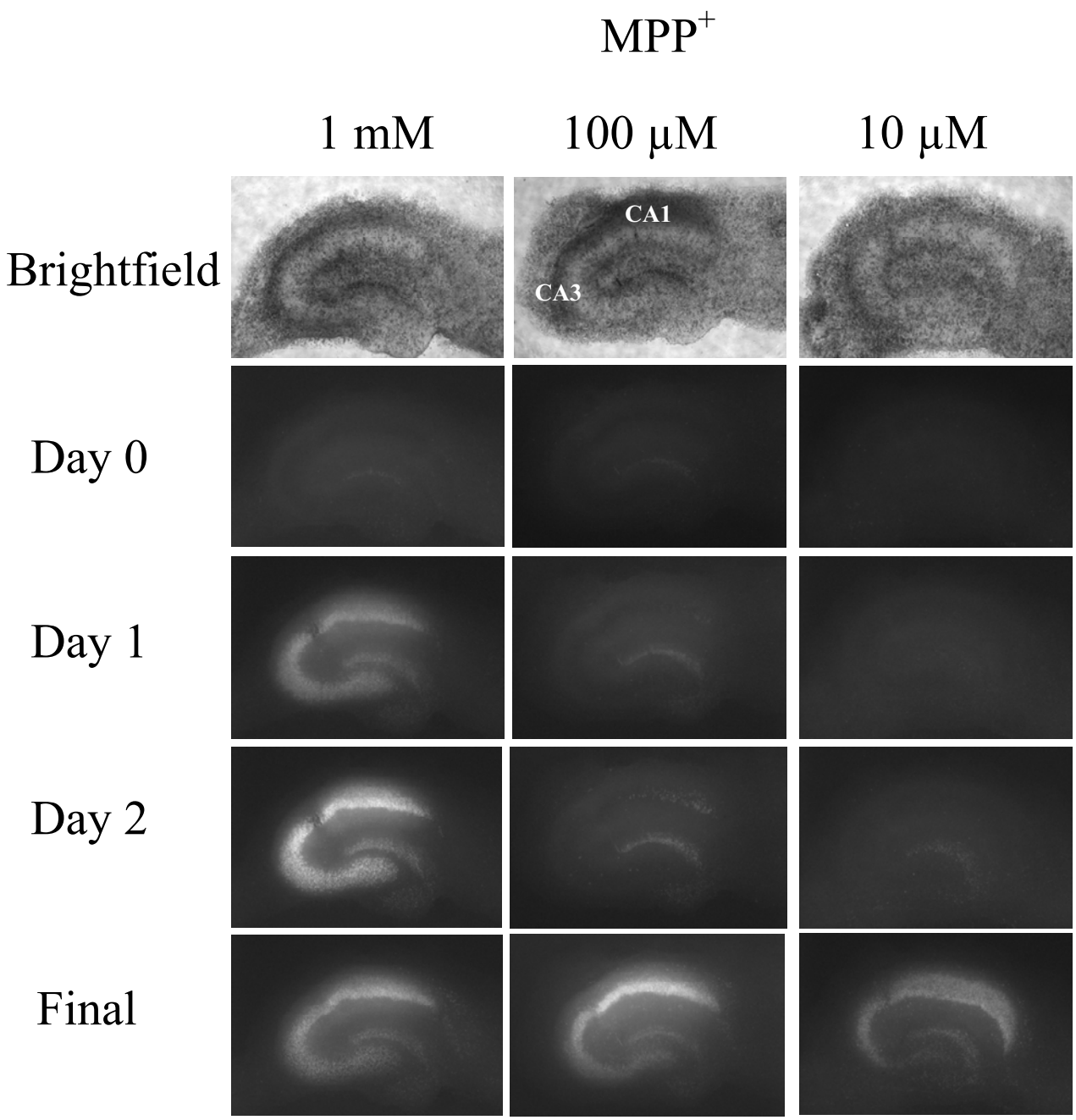

FIGURE 3 Examples of brightfield and propidium iodide (PI) fluorescence images of hippocampal organotypic slice cultures. 1-Methy-4-phenylpyridium (MPP^+, 1 mM, 100 µM, or 10 µM) was applied for 1 h after acquiring background images on Day 0. Final PI fluorescence was obtained 24 h after applying 100 µM NMDA for 1 h on Day 3.

And CA3 prior to toxin exposure or in sham-treated slices, indicating limited cell death in untreated slices after 2-3 weeks of culture. However, many slices showed PI staining in the upper blade of the dentate gyrus prior to toxin exposure. The variability in the dentate gyrus precluded any meaningful assessment of the effects of toxin exposure in this hippocampal subfield.

Rotenone

Hippocampal slices were exposed for 1 h either to vehicle, 0.1 µM, 1.0 µM, or 10 µM concentrations of

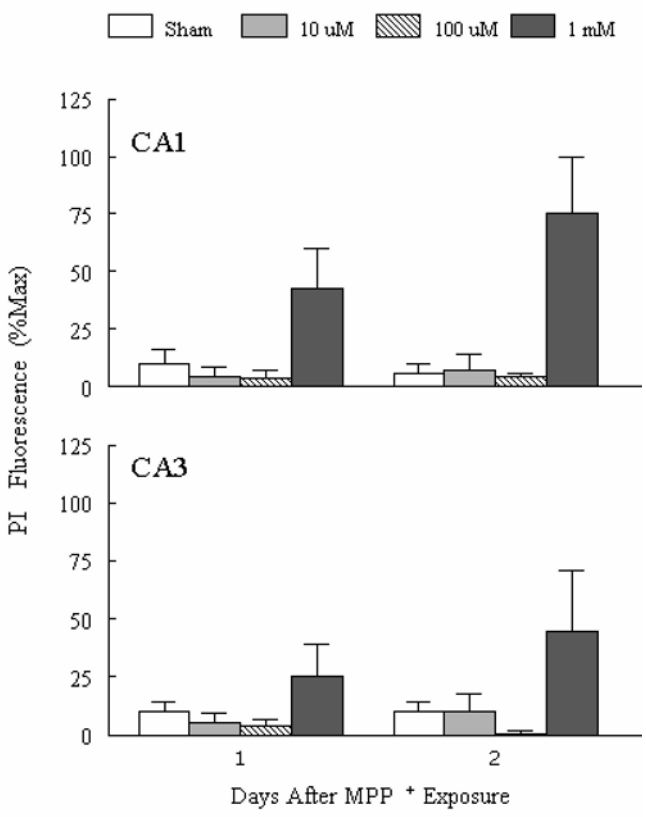

FIGURE 4 Average values (Mean ± SEM, n = 4) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to 1 mM, 100 μM, or 10 μM MPP^+. Statistical comparisons of groups are provided in the text.

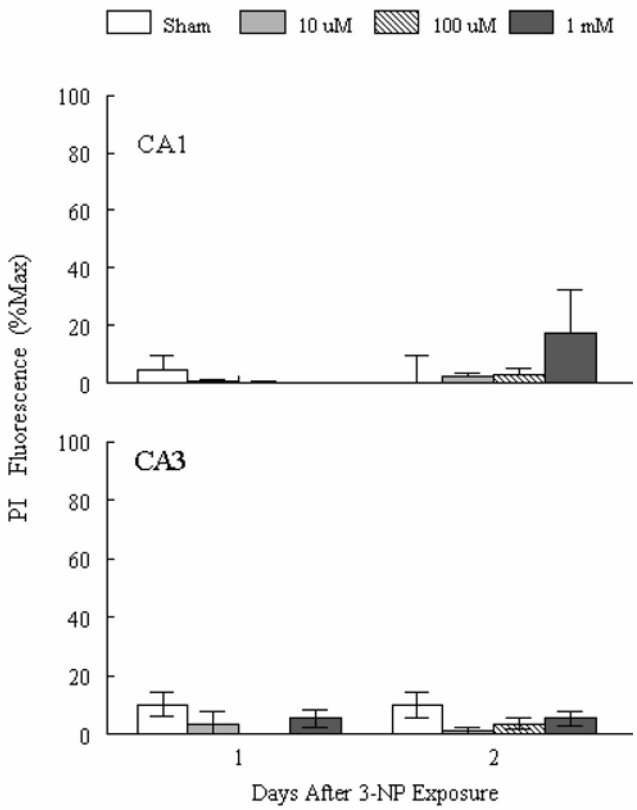

FIGURE 5 Examples of brightfield and propidium iodide (PI) fluorescence images of hippocampal organotypic slice cultures. *N*-methyl-D-aspartate (NMDA, 100 μM, 10 μM or 1 μM) was applied for 1 h after acquiring background images on Day 0. Final PI fluorescence was obtained 24 h after applying 100 μM NMDA for 1 h on Day 3.

the mitochondrial complex I inhibitor, rotenone. Examples of images showing the changes in PI fluorescence taken prior to exposure, and 24 h or 48 h after exposure, are shown in figure 1. Quantitative assessment of cell death in hippocampal subfields CA1 and CA3 following rotenone exposure is shown in figure 2. Rotenone treatment resulted in a highly significant dose-dependent increase in cell death in hippocampal pyramidal cells both in CA1 and CA3 (Main Effect of Dose, $F(3,24) = 90.0$, $p < 0.001$, $n = 4$). Significantly, cell death following rotenone exposure was more pronounced at lower doses in subfield CA1 than in subfield CA3 [Main Effect of Region, $F(1,24) = 17.2$, $p < 0.001$, Region X Dose Interaction, $F(3,24) = 6.5$, $p < 0.01$, $n = 4$]. For example, PI fluorescence following exposure to 1 μM rotenone was 30.1 ± 11.1% and 77.1 ± 10.9% on days 1 and 2 respectively in subfield CA1, and 9.4 ± 5.5% and 20.3 ± 10.7 on days 1 and 2 in subfield CA3. Higher doses of rotenone (10 μM) resulted in significant cell death in both hippocampal subfields.

1-Methyl-4-Phenylpyridium (MPP^+)

Examples of PI fluorescence images of slice cultures exposed to 10 μM, 100 μM, or 1 mM of the respiratory chain complex I inhibitor MPP^+, are shown in figure 3. Quantitative analysis of PI fluorescence following MPP^+ exposure is shown in figure 4. Similar to rotenone, MPP^+ caused dose-dependent death of pyramidal cells in both CA1 and CA3 [Main Effect of Dose, $F(3,24) = 9.8$, $p < 0.001$, $n = 4$]. After exposure to 1 mM MPP^+, cell death as indicated by PI fluorescence was 42.8 ± 17.1% and 75.5 ± 23.8% on days 1 and 2 respectively in subfield CA1. In subfield CA3, PI fluorescence was 25.0 ± 14.5% and 44.7 ± 26.4% on days 1 and 2 after exposure to 1 mM MPP^+. However, there was no significant difference in the damage to CA1 and CA3 following MPP^+ exposure [Main Effect of Region, $F(1,24) = 0.7$, $p = 0.5$, Dose X Region Interaction, $F(3,24) = 1.3$, $p = 0.3$, $n = 4$].

3-Nitropropionic Acid (3-NP)

In comparison to the complex I inhibitors rotenone and MPP^+, the complex II inhibitor 3-NP had a much

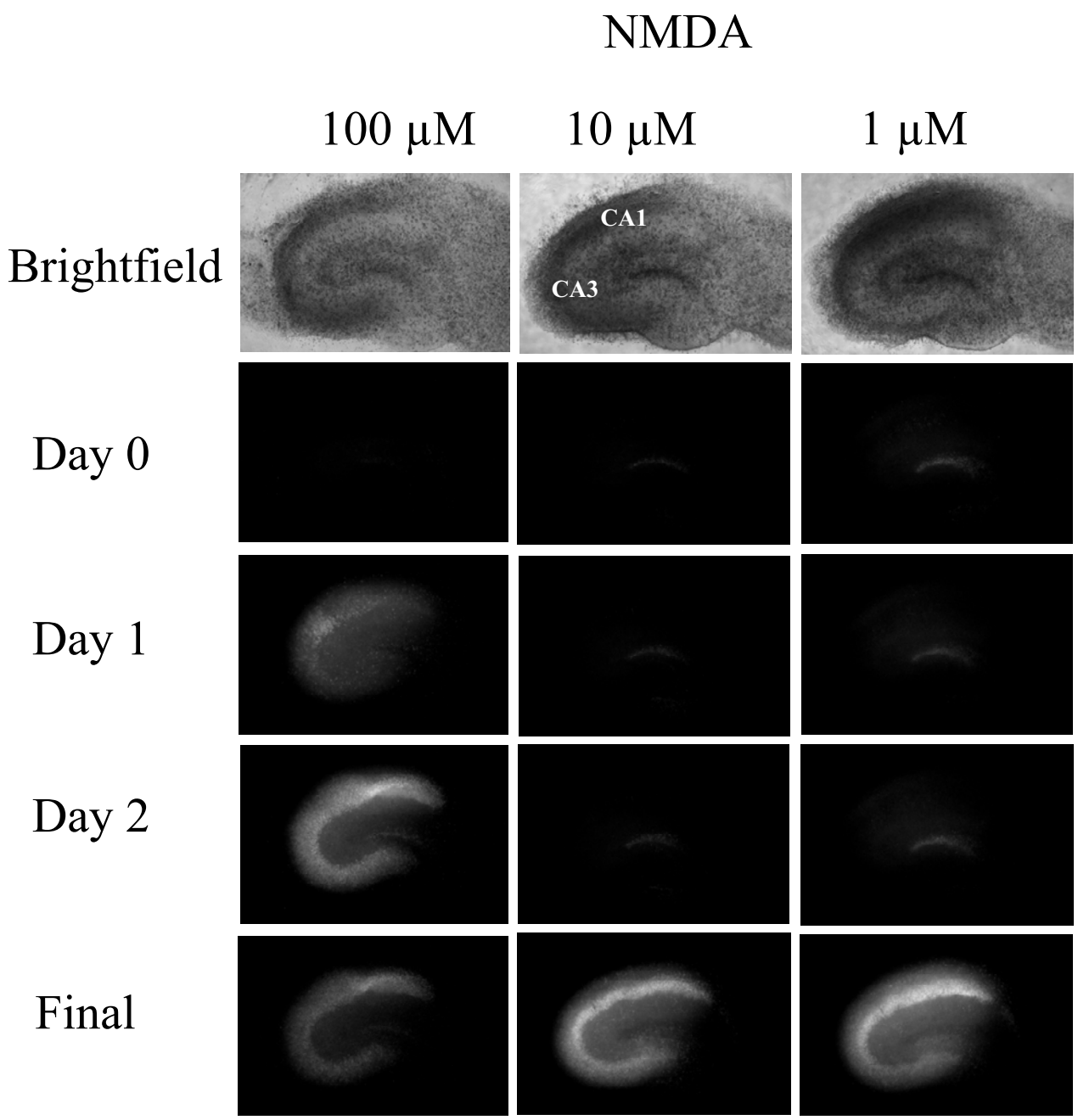

FIGURE 6 Average values (Mean ± SEM, $n = 4$) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to 100 μM, 10 μM, or 1 μM NMDA. Statistical comparisons of groups are provided in the text.

smaller effect on pyramidal cell death [Main Effect of Dose, $F(3,24) = 3.1$, $p < 0.05$, $n = 4$]. Also, there was no tendency for 3-NP to selectively damage either subfield [Main Effect of Region, $F(1,24) = 3.06$, $p > 0.05$, Dose X Region Interaction, $F(3,24) = 1.4$, $p > .05$, $n = 4$]. A summary of the data is presented in figure 5 (Images not shown).

N-Methyl-D-Aspartate (NMDA)

The excitotoxin NMDA caused pyramidal cell death only at the highest dose (100 μM) tested [Main Effect of Dose, $F(3,24) = 189.9$, $p < .0001$, $n = 4$]. Examples of PI fluorescence images acquired before and after exposure of organotypic slice cultures to 1 μM , 20 μM or 100 μM NMDA are shown in figure 6. A summary of the data is shown in figure 7. In contrast to rotenone,

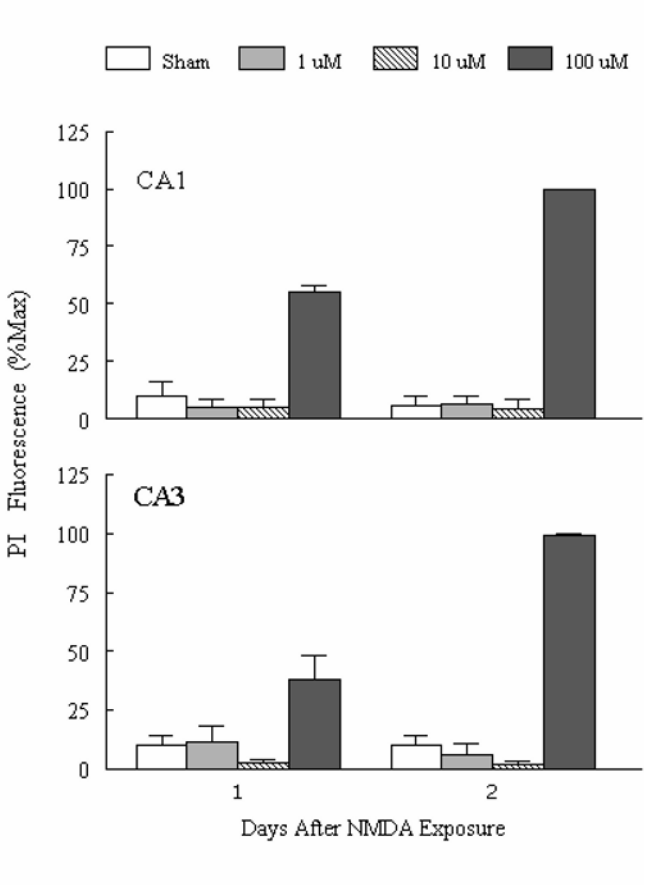

FIGURE 7 Average values (Mean ± SEM, n = 4) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to 1 mM, 100 µM, or 10 µM, 3-nitropropionic acid (3-NP). Statistical comparisons of groups are provided in the text.

and tentatively MPP^+, NMDA did not produce selective damage in subfield CA1 compared to CA3 [Main Effect of Region, $F(1,24) = 0.4$, $p > .05$, Dose X Region interaction, $F(3,24) = 1.4$, $p > 0.05$, $n = 4$].

DISCUSSION

In this report we demonstrate that inhibition of mitochondrial respiratory chain complex I with rotenone results in selective damage to pyramidal cells in subfield CA1. Selective neuronal damage was not observed following inhibition of complex I with MPP^+, complex II with 3-NP or following exposure to the excitotoxin NMDA. We believe this is the first report that complex I inhibition causes selective damage to CA1 pyramidal cells in the hippocampus.

Alterations in complex I activity of the mitochondrial respiratory chain have typically been associated with Parkinson's disease and damage to dopaminergic neurons in the substantia nigra and striatum. MPTP caused selective damage to neurons in the substantia nigra and striatum in man (Langston *et al.*, 1999), animals (Betarbet *et al.*, 2002), and produced neurological symptoms similar to those found in Parkinson's disease (Langston *et al.*, 1983). MPTP appears to damage dopaminergic neurons through its conversion to the complex I inhibitor MPP^+ (Langston *et al.*, 1983; 1984), and transport of MPP^+ into synaptic terminals of dopaminergic neurons by the dopamine transporter (Javitch and Snyder, 1984). Thus, enhanced toxicity of MPTP to dopaminergic neurons may result from a selective increase in the concentration of the complex I inhibitor MPP^+ in these cells. It has also been reported that patients with Parkinson's disease may show a deficiency in mitochondrial complex I (Parker *et al.*, 1989; Schapira *et al.*, 1989). More recently, it was reported that chronic systemic administration of rotenone, another complex I inhibitor, caused selective degeneration of dopaminergic neurons in the striatum and substantia nigra (Betarbet *et al.*, 2000).

In contrast, selective damage to pyramidal cells in hippocampal subfield CA1 has been commonly reported following global ischemia *in vivo* (Kirino, 1982; 2000). Oxygen-glucose deprivation (*in vitro* ischemia) also has been shown to selectively damage CA1 pyramidal cells in hippocampal organotypic slice cultures (Laake *et al.*, 1999). We report here that selective damage to CA1 pyramidal cells also occurred after treatment of hippocampal organotypic slice cultures with the complex I inhibitor rotenone. Betarbet at al (2000) did not report damage to CA1 pyramidal cells after systemic rotenone infusion *in vivo*. However it was unclear whether damage did not occur or whether a detailed analysis of hippocampus pathology was not conducted. Also, in their study exposure to rotenone was chronic and the doses used were lower than reported here in organotypic slice cultures. The mechanism of rotenone-induced cell damage in hippocampal subfield CA1 is currently unknown. However, it is interesting that inhibition of complex I activity is a consequence of brain ischemia (Dave *et al.*, 2001). Taken together, the data suggest that inhibition of complex I might be a mechanism of selective neuronal damage in hippocampus. Also, because a similar pattern of damage occurs with oxygen-glucose deprivation and rotenone treatment, it is safe to speculate that the mechanism of damage might also be similar.

The fact that inhibition of complex I with MPP^+ did not result in selective CA1 neuronal damage in the present study indicates 1) that selective damage to CA1 may be highly dependent upon the degree of complex I

inhibition and thus the concentration of inhibitor used, or 2) that the consequences of complex I inhibition after treatment with MPP^+ and rotenone are fundamentally different. One such possibility is the generation of reactive oxygen species (ROS). Oxygen-glucose deprivation results in increase ROS generation in organotypic hippocampal slice cultures (Frantseva *et al.*, 2001). Complex I inhibition with rotenone also increases ROS production in hippocampal slices (Saybasili *et al.*, 2001). However, if ROS production is key to selective neuronal damage in CA1 pyramidal cells, the increase should be greater in CA1 than in CA3 following either oxygen-glucose deprivation or complex I inhibition. To date, selective ROS production in CA1 has not been examined.

However, a recent study has shown that rotenone increased ROS production in rat brain mitochondria while MPP^+ did not, although both agents inhibited ATP production (Fonck and Baudry, 2003).

Elevation of intracellular calcium could also mediate selective neuronal vulnerability of CA1 pyramidal cells following either complex I inhibition or oxygen-glucose deprivation. Intracellular calcium levels were clearly elevated following oxygen glucose deprivation (Frantseva *et al.*, 2001) and more importantly the increase occurred earliest and was most severe in subfield CA1 Mitani *et al.*, 1994). Complex I inhibition also results in elevation of intracellular calcium (Leist *et al.*, 1998) but experiments have not been conducted to compare changes in hippocampal subfields CA1 and CA3. A recent report (Sousa *et al.*, 2003) has shown that calcium stimulates ROS production in brain mitochondria to a greater extent following inhibition of complex I with rotenone than with MPP^+. Thus, the combination of increased ROS production after inhibition of complex I with rotenone compared to MPP^+, enhanced calcium-mediated ROS production after complex I inhibition with rotenone, and selective elevation of intracellular calcium in subfield CA1 may all contribute to the selective neuronal damage observed in the present study after treatment with rotenone.

We did not observe reliable selective hippocampal CA1 pyramidal cell death following treatment either with the excitotoxin, NMDA, or the mitochondrial complex II inhibitor 3-NP. Previous studies (Kristensen *et al.*, 2001; Prendergast *et al.*, 2001) also showed comparable cell death in subfields CA1 and CA3 following exposure to high (>100 μM) concentrations of NMDA. However, Kristensen *et al.* (2001) reported selective damage to CA1 pyramidal cells after 2 days of exposure to 10 μM NMDA. There have been no previous studies examining selective neuronal vulnerability in hippocampus following inhibition of mitochondrial complex II. In fact, treatment with 3-NP has been shown to "pre-condition" or protect hippocampal neurons from subsequent hypoxia/ischemia (Sugino *et al.*, 1999).

In conclusion, we have demonstrated selective damage to pyramidal cells in subfield CA1 of hippocampal organotypic slice cultures following inhibition of complex I of the mitochondrial respiratory chain with rotenone. Selective damage to neurons in subfield CA1 was similar to that observed by other investigators after hypoxia/ischemia. Thus, it appears that severe inhibition of respiratory chain function may be sufficient to trigger CA1 neuronal death, whether inhibition occurs as a result of ischemia or exposure to mitochondrial toxins.

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