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Research paper

Direct exposure to *N*-methyl-D-aspartate alters mitochondrial function

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HIGHLIGHTS

- A previous study reported the detection of an NMDA-sensitive binding site on nervous system mitochondria.
- NMDA augmented mitochondrial ROS formation without altering membrane potential.
- NMDA attenuated cytochrome c release and delayed Ca^{2+}-induced mitochondrial swelling.
- Activation of the NMDA-sensitive binding site may stabilize mitochondrial function.

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ABSTRACT

N-methyl-D-aspartate (NMDA) receptors have long been known to be associated with the plasma membrane, providing a channel for the passage of extracellular Ca^{2+} into the cytosol during synaptic transmission. Recent results from our laboratory indicate that in addition to this classic location, an NMDA-sensitive site ($NMDA_m$) may also exist within the inner mitochondrial membrane. We report direct exposure of mitochondrial to NMDA enhances the production of reactive oxygen species and attenuate ROS-induced cytochrome c release, all the while slowing the rate of Ca^{2+}-induced mitochondrial swelling. Treatment with NMDA did not alter the mitochondrial membrane potential. The findings of this study lend further support for the existence of $NMDA_m$ and suggest that this site may serve to stabilize mitochondrial function.

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1. Introduction

Calcium is an intracellular messenger that plays a central role in neuronal signaling and homeostasis. The NMDA glutamate receptor subtype lies in the plasma membrane and gates the influx of extracellular Ca^{2+} into the cytoplasm. While physiological increases in neuronal Ca^{2+} contribute to normal synaptic communication, the influx of pathological levels of Ca^{2+} into the cell (For review see Ref. [1]) can initiate a variety of events which may underlie disorders such as cerebral ischemia, Alzheimer's diseases and HIV-associated dementia. In order to maintain the normal low levels of Ca^{2+} (<1 μM) and minimize its toxic effects, mitochondria (and endoplasmic reticulum) buffer cytosolic calcium following glutamate receptor stimulation [2–4]. While mitochondria can safely sequester moderate loads of Ca^{2+}, large increases can cause a number of potentially deleterious events including: (1) diminished ATP production, (2) activation of the permeability transition [5,6] and (3) release of pro-apoptotic factors such as cytochrome c [7–9]. In contrast to these detrimental effects, increases in mitochondrial Ca^{2+} have also been linked to enhanced cellular bioenergetics. This was initially shown in isolated mitochondria in which the addition of calcium stimulated several tricarboxylic acid cycle enzymes and increased NAD(P)H production and subsequently confirmed in cultured hepatocytes and in cardiac myocytes, where mechanical contraction and energy production must be closely coordinated [10,11].

Abbreviations: $A_{1/2}$, half maximum absorbance; A_{min}, minimum absorbance; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; CsA, cyclosporin A; DCF, dihydrodichlorofluorescein diacetate; $\Delta\Psi_m$, mitochondrial membrane potential; MK-801, dizocilpine; NMDA, *N*-methyl-D-aspartate; $NMDA_m$, *N*-methyl-D-aspartate-sensitive site; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester.

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Based on the above findings, it is clear that mitochondrial calcium is involved in a delicate balance between neuronal death and survival. How this single, seemingly ubiquitous cation can accomplish such diverse functions is a critical question and has, in part, been explained by the demonstration of cellular "microdomains" [12], which are transient oscillations of Ca^{2+} of varying frequency and amplitude that are sensed by mitochondria and readily distinguished from large, sustained increases and basal levels of cytoplasmic Ca^{2+} [13] In this "model" Ca^{2+} uptake is facilitated by the calcium uniporter and the rapid access mode and redistribution back into the cytoplasm subserved by Na^{+}-dependent and -independent exchangers [14].

While the above model may account for mitochondrial Ca^{2+} sensing under a number of conditions, recent data from our laboratory suggests that in the nervous system, there is another site that participates in Ca^{2+} homeostasis [15]. The uptake of Ca^{2+} by this site was shown to be activated by several known NMDA receptor agonists and inhibited by the non-competitive NMDA receptor antagonists MK-801 and 7-chlorokynurenic acid. Based on the similarity that this agonist/antagonist profile shares with plasma NMDA receptors, we termed this uptake pathway the "mitochondrial NMDA-sensitive site" ($NMDA_m$). Although its precise role has yet to be elucidated, a clue to the function of $NMDA_m$ was revealed when select overexpression of human NMDA receptor subunits GluN1 and GluN2a into mitochondria protected neurons from cell death following exposure to an excitotoxic insult [15]. Interestingly, following this same insult, mitochondrial Ca^{2+} levels in transfected cells were higher than in non-transfected cells, suggesting that $NMDA_m$ might serve to facilitate Ca^{2+} retention as a means by which to confer protection. In light of these observations and in an initial effort to better understand the function of $NMDA_m$, we have now begun to examine the effects that activation of this novel site has on several indices of mitochondrial function.

2. Materials and methods

2.1. Cell culture

GT1-7 cells are immortalized hypothalamic neurons that we have previously demonstrated express plasma NMDA receptors and functional $NMDA_m$ [15]. Cells were cultured in modified Dulbecco's Minimal Essential Medium (DMEM) containing 4500 mg/L glucose, 110 mg/L pyruvate and 548 mg/L L-glutamine (Mediatech, Herndon, VA), 10% fetal calf serum (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Gibco, Grand Island, NY). Cells were incubated at 37 °C with 5% CO_2. Cultures were passed weekly, by treating with 1× trypsin (Sigma, St Louis, MO) diluted in phosphate buffered saline for 1–2 min at room temperature. In all studies, cells were used between passages 5 and 10.

2.2. Mitochondrial preparation

Five to 10 million cells were trypsinized, washed with ice-cold PBS and centrifuged at 200*g* for 5 min. The pellet was re-suspended in 0.5 ml of ice-cold isolation buffer containing 0.3 M mannitol, 0.1% BSA, 0.2 mM EGTA, 10 mM HEPES pH 7.4 and 1× protease inhibitor cocktail (1/25 dilution Complete, Roche, Indianapolis, IN) and homogenized on ice with a 2 ml glass homogenizer, centrifuged at 1000*g* at 4 °C for 10 min and the supernatant collected. The supernatant was centrifuged at 14,000*g* for 15 min at 4 °C, the pellet collected, and re-suspended in 0.5 ml of isolation buffer without EGTA. The suspension was mixed with an equal volume of 10% Percoll (in isolation buffer without EGTA), layered on top of 2 ml of 15% Percoll in a new tube and centrifuged at 16,000*g* for 20 min. Purified mitochondria were collected at the Percoll gradient interface. The sample was washed twice with isolation buffer without EGTA followed by centrifugation at 13,000*g* for 10 min following the first wash and at 10,000*g* for 10 min after the second wash. The pellet was re-suspended and used for subsequent experiments. Protein measurements for this and all other assays were determined using the Bradford protein assay (Pearce, Rockford, IL).

2.3. Mitochondrial membrane potential

Changes in $\Delta\Psi_m$ were measured using the fluorescent probe TMRE (Life Technologies, Carlsbad, CA), at a high, quenching concentration, such that an increase in fluorescence intensity correlated to a decrease in $\Delta\Psi_m$. Mitochondria (25 μg) were incubated for 10 min at room temperature in assay buffer (125 mM KCl, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, 20 mM HEPES, 0.1% BSA) alone or assay buffer containing either 5 μM Ca^{2+} or 5 μM Ca^{2+} + 10 μM NMDA. Ten μM NMDA was selected for this and the remainder of studies herein based on results from our laboratory demonstrating maximum calcium uptake using this concentration of the agonist (Korde and Maragos, unpublished observations). Mitochondrial samples were briefly washed and centrifuged after which the pellet was suspended in assay buffer containing 150 μM TMRE. After 10 min, the following compounds were sequentially added at 5-min intervals: 5 mM pyruvate + 2.5 mM malate, 150 μM ADP, 1 μM oligomycin, and 1 μM carbonyl cyanide m-chlorophenyl hydrazine (CCCP). Samples were read (550 nm excitation, 580 nm emission) following each addition using a Synergy HT spectrofluorometer.

2.4. Mitochondrial reactive oxygen species formation

Twenty-five μg of mitochondria were added to assay buffer containing 5 mm pyruvate, 2.5 mm malate, 10 μm of DCF (Life Technologies, Calrsbad, CA) and 5 μm horseradish peroxidase (Sigma-Aldrich, St. Louis, MO). Samples were incubated for 10 min at 23 °C in buffer alone or with the addition of either (a) 10 μM NMDA, (b) 5 μM Ca^{2+} or (c) 5 μM Ca^{2+} + 10 μM NMDA, after which fluorescence was measured with an excitation frequency of 485 nm and emission frequency of 532 nM. ROS formation was also measured in replicate samples in which substrate-fed mitochondria were treated with 150 μm ADP, 1 μm oligomycin or 1 μm CCCP, the last of which was included to inhibit membrane potential-dependent ROS production. The experiment was replicated as described above, the only difference being the addition 16 μm of the mitochondrial uniporter blocker ruthenium red.

2.5. Mitochondrial cytochrome c release

Mitochondria were isolated from GT1-7 cells using the protocol described above. Cytochrome c was measured using a commercially available ELISA and the assay performed according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Briefly, 25 μg of mitochondria were incubated for 5 min in assay buffer or in assay buffer containing 5 μM Ca^{2+}, 10 μM H_2O_2, 10 μM H_2O_2 + 5 μM Ca^{2+} or 10 μM H_2O_2 + 5 μM Ca^{2+} + 10 μM NMDA. Samples were centrifuged at 10,000*g* for 5 min, and supernatant added to ferrocytochrome-c substrate solution at a ratio of 20:1. Absorbance was measured at A_{550} immediately following the addition of substrate. The concentration of cytochrome c was extrapolated from a standard curve that was generated using known quantities provided by the manufacturer.

2.6. Mitochondrial swelling

Mitochondria (25 mg) were incubated in 96-well plates in assay buffer containing 5 mM pyruvate + 2.5 mM malate and equilibrated for 5 min at room temperature. In control samples, 5 μM boluses of