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Dose-Dependent Modulation of NMDA Receptors: Neuroprotective Mechanisms against Oxidative Stress in Hippocampal Neurons

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ABSTRACT

N-Methyl-D-Aspartate (NMDA) receptors are involved in synaptic plasticity and neuronal communication. They have various responses to oxidative stress based on the dosage of agonists or antagonists that may be applied. This study focuses on modulation of NMDA receptors in primary hippocampal neurons in oxidative stress condition to understand the effects of NMDA receptor activation and inhibition. In our experiments, primary hippocampal neurons were treated with NMDA and MK-801 to assess their effect on cell viability and apoptosis. Oxidative stress was induced at different concentrations, to evaluate NMDA receptor activity and the neuroprotective effects of MK-801. Apoptosis rates were specified by applying flow cytometry, and assaying caspase-3 activity. Intracellular calcium levels were monitored using fluorescent dye Fura-2 AM. NMDA at 200 µM significantly prevented the cytotoxic effect induced by H_2O_2 ($P<0.001$). MK-801 with concentrations of 5 to 20 µM, could reverse the cytotoxic effect of H_2O_2. As a result, it significantly inhibited the toxicity of H_2O_2 on neuronal cells ($P<0.001$), while 40 µM could not reverse its effects. NMDA (200 µM) increased neuronal survival to 88.3% in the presence of H_2O_2 and prevented apoptosis. MK-801 (5 µM) also elevated cell survival to 87.2%. Treatment with NMDA (200 µM) + H_2O_2 also did not show any changes in the Fura-2AM fluorescence compared to the H_2O_2 group ($P>0.05$). However, MK-801+ H_2O_2 reduced the effects of H_2O_2 on the fluorescence ratio and calcium influx considerably in comparison with the H_2O_2 group ($P<0.01$). Treatment with MK-801 (5 µM) effectively mitigated the effects of H_2O_2 on caspase-3 activity compared to the H_2O_2 group ($P<0.001$). Importantly, the dose-dependent effects of NMDA receptors offer a new path into finding therapeutic strategies for neurodegenerative diseases.

Keywords: NMDA receptors, Oxidative stress, Hippocampal neurons, Neuroprotection, Apoptosis

Introduction

The disruption of the balance between reactive oxygen species (ROS) production and the antioxidant defense system leads to oxidative stress. This condition can be observed as an early factor for diagnosing various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (1). The hippocampus is implicated in synaptic plasticity, and as a result, it plays a role in learning and memory, and cognitive processing (2). Studies have shown the signaling pathways that are required for hippocampal function.

N-Methyl-D-Aspartate (NMDA) receptor, calcium/calmodulin-dependent protein kinase II (CaMKII), extracellular signal-regulated kinases (ERK) signaling, brain-derived neurotrophic factor, tropomyosin-related kinase receptor type B signaling, and cyclic adenosine monophosphate/protein kinase A-cAMP response element binding protein have been identified as signaling pathways, and they need to interact synergistically (3). Hippocampal neurons are sensitive to oxidative damage that causes neuronal injury and synaptic function disruption, leading to cognitive impairment in this region (4). Having special characteristics, such as elevated metabolic activity and high levels of polyunsaturated fatty acids exacerbate these negative effects (5). The NMDAR previously mentioned, is crucial for synaptic plasticity and memory formation (6).

NMDARs are involved in excitatory synapse maturation and the inhibitory circuits' regulation during the process of neural development (7). These receptors enhance dendritic growth and synapse stabilization by the influx of calcium through these receptors and the associated signaling cascades (8). This modification of synapses can be considered a guarantee for preserving the balance between excitatory to inhibitory inputs, essential for the functionality of neural circuit (9). Whenever excitation increases, NMDAR signaling can help decrease the firing rate of neurons or enhance the potential of inhibitory synapses to restore balance (10). Investigating how NMDAR respond to oxidative stress in this region could be effective in altering our understanding of synaptic plasticity, excitatory/inhibitory balance, and mechanisms underlying neuronal survival. These receptors are mainly located in the hippocampus, and they are highly permeable to calcium ions, which make them essential for excitatory neurotransmission (11). NMDA receptors mediate this process due to their involvement in excitotoxicity and calcium influx (12). Overactivation of the NMDA receptor and transient receptor potential melastatin2 channels (TRPM2) lead to intracellular calcium concentration ($[Ca^{2+}]i$) elevation. Consequently, the increase in calcium levels can activate caspases such as caspase-3, caspase-8, and caspase-9, factors that are implicated in apoptosis and cause the death of SH-SY5Y neuronal cells (13). NMDA receptor modulation can mitigate oxidative damage and enhance neuronal survival (14). However, dose-dependent modulation must be considered, as low doses can decrease oxidative stress without influencing the synaptic function disruption (15, 16), while high doses may impair neuronal connectivity (17). Therefore, it seems that targeting NMDAR might be associated with therapeutic potential for managing oxidative stress in the hippocampus (18). The NMDA receptor exhibits double-edged sword effect. (12). Excessive activation of these receptors can lead to excitotoxicity and excessive calcium influx, triggering detrimental effects, including mitochondrial dysfunction, production of ROS, and ultimately neuronal death (19). While NMDA receptors show neuroprotective effects through activation of synaptic NMDAR, not the extra-synaptic ones (20). Studies have shown that these receptors can exert their positive effects on neuronal survival and synaptic plasticity by facilitating calcium-dependent signaling pathways that activate antioxidant defenses and support cellular repair mechanisms (21). This dual function underscores the importance of understanding the dose-dependent effects of NMDA receptor modulation in context of oxidative stress. MK-801 is a selective non-competitive NMDA receptor antagonist that demonstrated neuroprotective capabilities in a dose-dependent manner (22). When administered at optimal doses, MK-801 effectively reverses the oxidative stress negative effects and limits the over entry of calcium, thus reducing oxidative stress and maintaining neuronal integrity (23, 24). Studies indicate that in ischemia-reperfusion conditions, administration of MK-801 can considerably improve the affected brain regions by decreasing neuronal death and improving functional recovery (25). Activation of NMDARs results in calcium influx and triggers downstream signaling cascades such as the CaMKII pathway (26). CaMKII is essential for enhancing synaptic connections by modulating the trafficking and

phosphorylation of AMPA receptors (27). Furthermore, cell function regulation by NMDARs, is mediated by the Calmodulin (CaM), CAMKII, and ERK pathways (28). MK-801 has been found to reverse all the pathways through inhibiting the downstream signaling pathways, including CaMKII, and ERK phosphorylation, as well as the CaM expression, mitochondrial calcium uniporter, and Toll-like receptor 4, the factors involved in cell death (29). As a result, MK-801 can be considered a therapeutic agent in ischemic stroke and related neurodegenerative diseases by attenuating ROS production, mitigating neuroexcitotoxicity, and maintaining mitochondrial function.

This study aimed to elucidate the dose-dependent effects of NMDA receptor modulation on oxidative stress in hippocampal neurons. The hippocampus takes into account as a location that has been designated for studying neuroplasticity and neurodegeneration. Findings from hippocampal studies can be generalized to other brain functions and pathologies in various regions. Thus, current research seeks to not only answer the specific questions about NMDA receptor modulation in the hippocampus but also to understand neuronal responses to oxidative stress, which could pave the way toward developing therapies for neurodegenerative diseases. By changing the NMDA receptor mode from activation to inhibition and vice versa, we attempt to identify the points at which NMDA receptor activity may become deleterious. This research will target the cellular and molecular mechanisms underlying NMDA receptor-mediated neuroprotection and excitotoxicity. Finally, the findings could lead to the development of targeted therapies that could minimize the risk of oxidative damage and simultaneously harness the protective potential of NMDA receptors, which can be effective for the treatment of neurological disorders.

Methods

Primary hippocampal neuronal culture

The experimental procedures in this study were conducted in accordance with the guidelines set forth by the National Institutes of Health (NIH) and received approval from the Ethics Committee of Iran University of Medical Sciences (IR.IUMS.AEC.1403.050). The procedure for culturing hippocampal neuronal cells was conducted as previously outlined (30). Hippocampal neurons were acquired from newborn Wistar rats aged between postnatal day 0 (P0) and postnatal day 1 (P1). Following decapitation, the brains were promptly transferred to ice-cold Hank's balanced salt solution (HBSS). The meninges were removed gently, and the hippocampi were separated quickly. These hippocampal tissues were then treated with 0.05% Trypsin-EDTA and incubated at 37 °C for 15 minutes. After trypsin was inactivated, the tissues were rinsed twice with HBSS and mechanically dissociated using a fire-polished glass Pasteur pipette. The resulting cell suspension was centrifuged at 1200 × g for 5 minutes. Afterwards, the cells were suspended again in neurobasal medium supplemented with 1% GlutaMAX, 2% B-27, 10% fetal bovine serum, and 1% antibiotic/antimycotic. The cells were cultured on plates coated with poly L-lysine- at a concentration of 0.1 mg/ml. Cultures were preserved in temperature of 37°C in 5% CO_2 , and half of the culture medium being replaced every two days. After 7 days in culture, immunofluorescence staining was performed to confirm the presence of neuronal cells and assess their purity.

Study design, treatments and oxidative stress induction

After culturing the cells for 7 days, drug treatments were initiated. To inducing oxidative stress, hippocampal neuronal cells were exposed to hydrogen peroxide (H_2O_2) at concentration of 400 μM for 24 hours (31). Additionally, the effects of different concentrations of NMDA (ranging from 50 to 800 μM) and MK-801 (ranging from 5 to 100 μM) were assessed over a 24-hour period. To examine the effects of combined treatment, NMDA or MK-801 was added to the cells 1 hour before H_2O_2 administration. Untreated cells served as the control group.

MTT Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was applied to evaluate the cell viability. Neuronal cells were seeded into 96-well culture plates at a density of 4×10^4 cells per well. After seeding, the cells were treated with the respective drugs according to the group specific group they belong to. Following drug exposure, each group was exposed to 0.5 mg/ml of MTT solution and incubated at 37°C for 4 hours. Following incubation, the MTT solution was discarded, and 100 μl of Dimethylsulfoxide was added

to dissolve the formazan crystals. Absorbance at 570 nm was then measured using a microplate reader (32).

Flow cytometry analysis for apoptosis

The percentage of apoptotic cells was specified by applying the Annexin V/PI (FITC) detection kit, according to the manufacturer's protocol. Neuronal cells were seeded in a 12-well tissue culture plate at a density of 4×10^5 cells per well. After drug treatment, the cells were collected using trypsin and centrifuged at 2,000 g × for 5 minutes. The supernatant was removed, and the cell pellet was washed twice with cold PBS. A solution containing 10 µl of Annexin V and 10 µl of PI was added to the cell suspension, which was then incubated in the dark at room temperature for 10 minutes. After incubation, cell apoptosis was analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA) (33).

Caspase-3 activity assessment

Caspase activity was evaluated using a reaction buffer containing 100 mM Tris-HCl (pH 8.0), 0.6 mM freshly prepared $MnCl_2$, and 20 mM D, L-trisodium isocitrate. The reaction was initiated by adding the caspase enzyme to the reaction mixture. Absorbance changes were recorded at 240 nm using a microplate reader (Biotek, Synergy HTX). The caspase activity was measured using an extinction coefficient of 3.6 $mM^{-1}.cm^{-1}$(34).

Calcium imaging

Following 24 hours of H_2O_2 treatment, cells were exposed to a fluorescent dye solution (DMEM:F-127AM = 500:1:1) for 20 minutes, followed by three washes with artificial extracellular solution. Fluorescence signals of Fura-2 AM were recorded at 340 nm (F340) and 380 nm (F380) using an Olympus Digital Calcium Imaging System (IX73, DG-4PLUS/OF30, Japan) with alternating excitation every second. Images were captured over 300 seconds to establish baseline Ca^{2+} levels in the presence of extracellular Ca^{2+} (1 mM). Subsequently, the extracellular solution was replaced with either polyamino carboxylic acid (BAPTA) (1 mM, Med Chem Express, USA) or $CaCl_2$ (2 mM) solutions, and the ratio (F340/F380) was observed. The change in ratio (Δ ratio, F340/F380) was calculated to assess the variation in intracellular Ca^{2+} ($[Ca^{2+}]i$) levels before and after treatment with BAPTA or high calcium solution. Each experiment was repeated independently at least three times (35).

Statistical analysis

All analyses were performed using GraphPad Prism 8 software. Experimental data were evaluated using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. Results are expressed as mean ± SEM. Statistical significance was defined as a p-value of less than 0.05.

Results

MK108 antagonize the NMDA stabilizing effect on Cell Viability in Hippocampal Neuronal Cells

To determine the impact of NMDA and MK-801 on H_2O_2-induced cytotoxicity, the appropriate concentrations of these components on hippocampal neurons were determined in the first step. Cells were exposed to varying concentrations of NMDA (50 to 800 µM) and MK-801 (5 to 100 µM) for 24 hours, and cell viability was assessed using the MTT assay. Our results showed that NMDA up to 800 µM had no cytotoxic effect ($P>0.05$), and MK-801 treatment did not exhibit toxicity at concentrations below 40 µM (Figure1 A, B). Consequently, we investigated NMDA at 50 to 800 µM and MK-801 at concentrations below 40 µM on H_2O_2- treated cells. To identify the optimal NMDA concentration that mitigates the cytotoxic impact of H_2O_2 on hippocampal cells, cells were pre-exposed to different NMDA concentrations for 1 hour, followed by cell viability assessment. As shown in Figure 1C, NMDA at 200 µM significantly reduced the cytotoxic effects of H_2O_2 ($P<0.001$), leading to increased neuronal survival ($P<0.01$). A similar experiment was conducted with MK-801, and the results showed that this antagonist could reverse the cytotoxic effect of H_2O_2 in a dose-dependent manner. Concentrations of 5 to 20 µM significantly inhibited the toxicity of H_2O_2 on neuronal cells ($P<0.001$), while 40 µM could not reverse its effects (Figure 1D). Based on these findings, we used NMDA at 200 µM and MK-801 at 5 µM for subsequent experiments.

Flow cytometry

The results from flow cytometry indicated that a majority of cells underwent apoptosis and necrosis, resulting in a decrease in neuronal survival rate from 93.1% to 27.8% in the presence of H_2O_2 (Figure 1E and

F). Conversely, NMDA (200 µM) increased neuronal survival to 88.3% in the presence of H_2O_2 and prevented apoptosis (Figure 1G). Similarly, MK-801 (5 µM) also elevated cell survival to 87.2% (Figure 1H). The agonist and antagonist effects on apoptosis appeared to be roughly equivalent to each other.

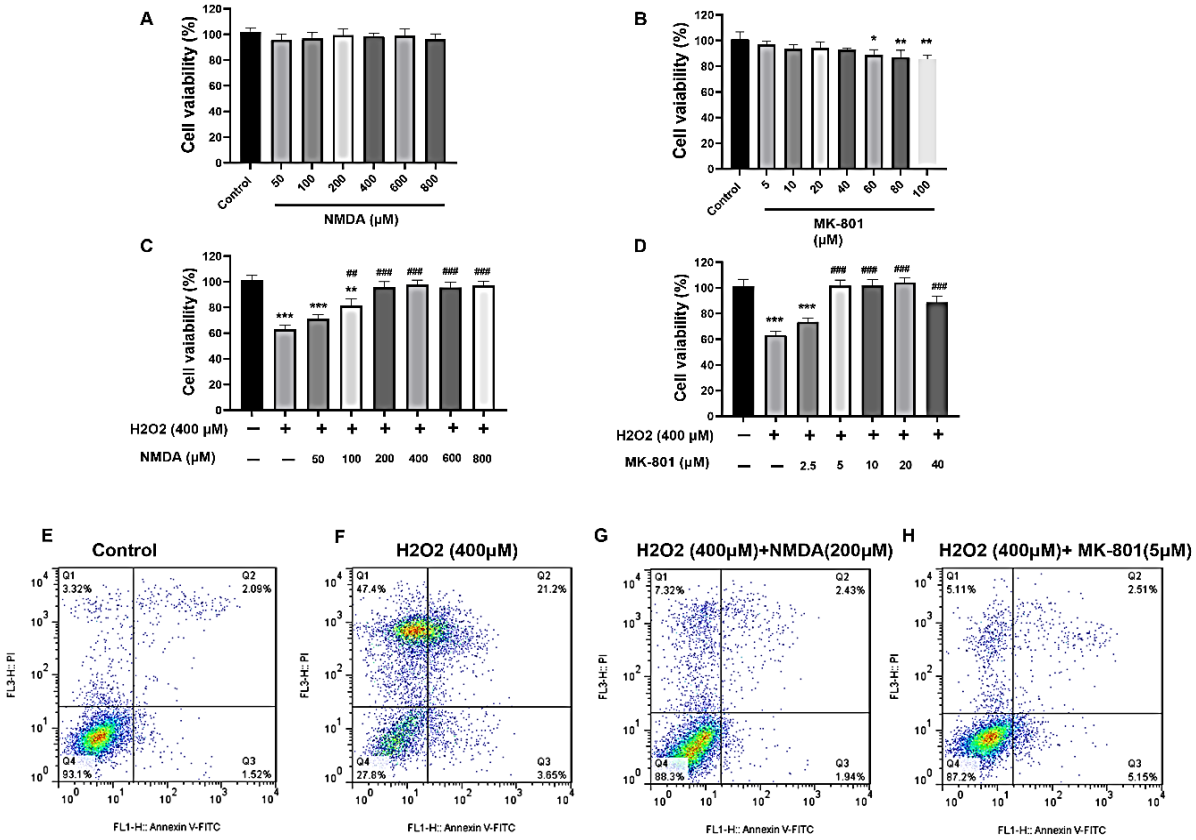

Figure 1. A and B) effect of different concentrations of NMDA and MK-801 on neuronal cells viability. C and D) Neuroprotective effects of NMDA and MK-801 in various concentrations on H_2O_2 induced neuronal toxicity which measured by using the MTT assay. E-H) Neuroprotective effects NMDA and MK-801 on H_2O_2 induced apoptosis of hippocampal neuronal cells which measured by flowcytometry test. Q1: Necrosis Q2: Late Apoptosis Q3: Early Apoptosis Q4: Live Cells. Data are presented as Mean ± S.E.M. *P< 0.05, **P< 0.01, ***P< 0.001 vs control group, ##P< 0.01, ###P< 0.001 vs H_2O_2 group.

Effects of NMDA and MK-801 on calcium influx in cells undergoing oxidative stress

As indicated in Figure 2A and B, Fura-2AM fluorescence for intracellular Ca^{2+} levels was estimated under NMDA and MK-801 treatment. In contrast to the control, the ratio of fluorescence intensities after excitation at 340/380 nm and emission at 510 nm increased (P<0.001) in the H_2O_2 group indicating an elevated intracellular Ca^{2+} in the presence of H_2O_2 (Figure 2B).

The fluorescence intensity peaked at a wavelength of 480 nm, reaching approximately 390 a.u. Treatment with NMDA (200 µM) + H_2O_2 also has no statistical change in the Fura-2AM fluorescence compared to the H_2O_2 group (P>0.05). However, MK-801+ H_2O_2 reversed the effects of H_2O_2 on the fluorescence ratio and calcium influx considerably when compared to the H_2O_2 group (P<0.01) (Figure 2A). As a result, the fluorescence intensity for MK-801+ H_2O_2 peaked at around 290 a.u., showing a wide disparity of 100 a.u. compared to the H_2O_2 treatment.

Caspase activity

The results demonstrated that H_2O_2 significantly increased caspase-3 activity compared to the control group (P<0.001).

However, treatment with MK-801 (5 µM) effectively reversed the effects of H_2O_2 on caspase-3 activity when compared to the H_2O_2 group (P<0.001, Figure 2C)

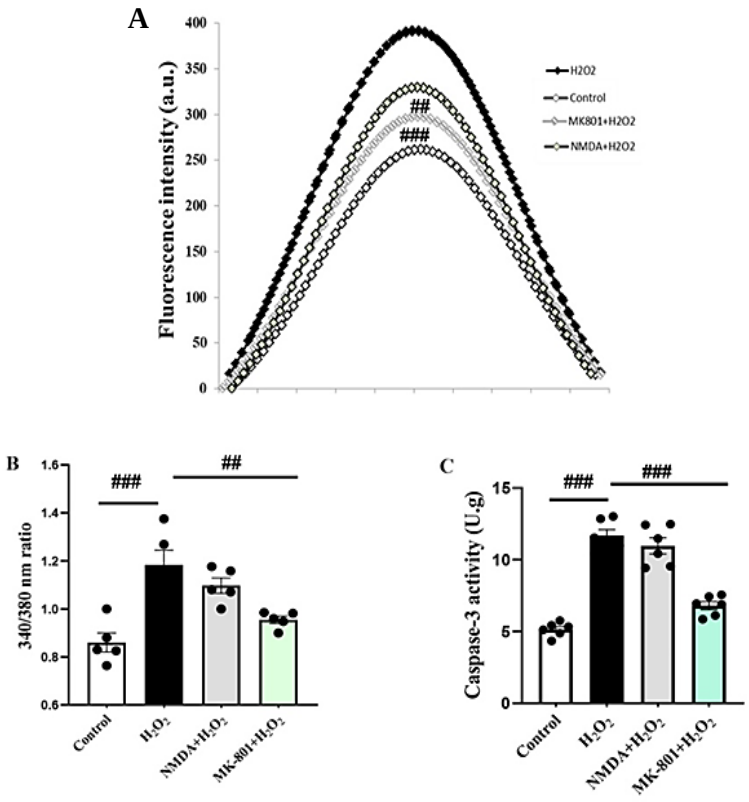

Figure 2. A) Emission spectra of Fura-2AM fluorescence intensity were analyzed to assess intracellular calcium levels in hippocampal cells following treatment with NMDA or MK-801 in the presence of H_2O_2. B) Ratio of fluorescence intensities after excitation at 340/380 nm and emission at 510 nm. C) Caspase-3 activity. Data are presented as Mean ± S.E.M. ##P< 0.01 and ###P< 0.001 vs H_2O_2 group.

Discussion

The modulation of NMDA receptors in response to oxidative stress opens a new window toward understanding mechanisms, particularly in the hippocampus, by being effective in neuronal protection, synaptic plasticity, and learning processes. This study investigated the dose-dependent effects of NMDA agonists and antagonists in the hippocampus under conditions of oxidative stress. We achieved the result that applying a low dose of NMDA agonist in mild oxidative stress conditions, attenuated apoptosis and enhanced cell viability. Our result demonstrates that under control conditions, NMDA receptor activation can show the neuroprotective effect. However, the agonist's effect faded in high oxidative stress conditions, and this could reveal a threshold at which excitotoxicity appears due to NMDA receptor overactivation. This finding is consistent with previous studies, in which calcium influx mediated by the NMDA receptor can be neuroprotective or detrimental depending on the activation status or cellular context (36). MK-801 significantly reduced neuronal apoptosis under oxidative stress. Additionally, this receptor prevented the excitotoxic cascades by reducing intracellular calcium levels and caspase-3 activity. These results confirm the hypothesis that NMDA receptor modulation can maintain the balance between excitation and inhibition that disrupted by oxidative stress. In brain ischemia and reperfusion, oxidative stress starts different pathways in ischemic tissue that will contribute to necrosis and apoptosis (37). Based on the previous studies, oxidative stress enters the negative side effects of neuronal damage of IR models into a vicious circle that increase apoptosis and necrosis (38). This brain region produces higher levels of superoxide anions and shows increased expression of genes involved in antioxidant defenses and the

production of ROS (39). In the mature neurons of the rat hippocampus and cortex, NR2A- and NR2B-containing NMDA receptors show distinct expression patterns, being localized at synaptic and extrasynaptic sites, respectively (40). In the CA1 and CA3 regions, the ratio of NMDA subunits NR2B to NR2A increased following ischemia, while the expression of both the NR2B subunit (associated with the activation of apoptotic pathways) and the NR2A subunit (associated with the activation of survival pathways) decreased in the CA1 region (41).

It seems that modulation of NMDA receptors by agonist and antagonist in the face of oxidative stress is not uniform across different concentrations of modulators. High concentrations of NMDA agonist may reduce oxidative stress by promoting cell viability, while lower concentrations did not exhibit protective effects, potentially by enhancing antioxidant defenses or modulating downstream signaling pathways. As regards its antagonist, MK-801 was effective at lower concentrations. Dose changes can be a therapeutic intervention to detect a dose-dependent response and target NMDA receptors under oxidative conditions. NMDA at 200 μM significantly reduced H_2O_2-induced cytotoxicity and prevented apoptosis and necrosis. This finding is consistent with the results of Bahrami et al. (2020), who reported neuroprotective effects of NMDA receptor agonists (42).

NMDA receptors have dual roles in both supporting neuronal survival and causing neuronal damage (43, 44). Some reasons for these paradoxical responses have been evaluated in previous studies. For instance, the promising therapeutic approach for stroke can be activation of NR2A-containing NMDA receptors selectively while applying an NR2B antagonist (45). Moreover, modification of thioredoxin-peroxiredoxin system enhances antioxidant defenses and this process is mediated by NMDAR signaling at the level of synapses (21). Synaptic activity increases thioredoxin activity, aids in reducing over-oxidized peroxiredoxins, and strengthens resistance to oxidative stress (46). The activation of NMDAR by ligands induces an inward flow of calcium. Research indicates that a temporary increase in Ca^{2+} levels lead to AMP-activated protein kinase (AMPK) activation, whereas prolonged high levels of Ca^{2+} inhibit AMPK activation (47, 48). The NMDAR/AMPK/ peroxisome-proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) signaling pathway supports mitochondrial balance and neuronal survival by increasing the expression of Sirtuin-3 (SIRT3) (49). Additionally, caspase-3 activity has slightly reduced in NMDA-treated rats as indicated in previous studies that NMDA's role in attenuating apoptotic pathways can be exerted by changing caspase-3 content (42). An example of oxidative reactions and NMDARs activation in neurodegenerative diseases can be revealed whenever ROS are typically generated in neurons and mitochondrial function intensified oxidative stress in the early phases of neurodegeneration, before occurring any neuronal cell death (50).

In the initial stage, Aβ interacts with NMDAR, triggering hyperactivation and disinhibition of multiple excitatory pathways that lead to the posterior cingulate and retrosplenial cortical regions, ultimately resulting in NMDAR hypoactivation (51). Aβ oligomers can engage and activate NMDAR, resulting in a swift rise in calcium levels and ROS production in cultured mature hippocampal neurons (52) Prolonged NMDAR hyperactivity and calcium dysregulation, persisting to months then years, can be detrimental, contributing to slowly progressing conditions like degenerative excitotoxicity in the development of Alzheimer's disease and related disorders (53). It is reasonable to suggest that memantine (MEM) and other antagonists of extrasynaptic NMDARs (eNMDAR) should be administered earlier, ideally during the presymptomatic stages of Alzheimer's disease and related disorders (54).

MEM treatment enhanced cell viability levels decreased by Aβ and high homocysteine while also reducing the expression levels of caspase 3, caspase 9, poly (ADP-ribose) polymerase 1 (PARP1), transient receptor potential cation channel subfamily A member 1 (TRPA1), TRPM2, and transient receptor potential cation channel subfamily V member 1 (TRPV1), which were elevated by Aβ and homocysteine (55). Research has demonstrated that the quantity of NMDARs, their subunit composition, and their postsynaptic linkers can be modified following the administration of NMDA antagonists such as MK-801, ethanol, and Phencyclidine (PCP) (56-58). MK-801, an irreversible blocker of open NMDA receptor channels, can only inhibit NMDA receptors activated by bicuculline (59, 60). The cell survival increments induced by NMDA and MK-801 were confirmed by applying flow cytometry method. A concentration of 5 μM MK-801 reduced neuronal necrosis effectively and enhanced cell

viability in cells exposed to oxidative stress. Excessive NMDA receptor activity and its downstream pathway, such as calcium influx and subsequent activation of apoptotic pathways, was blocked by MK-801. This reduction in apoptosis was accompanied by lower caspase-3 activity, a critical enzyme in the execution phase of apoptosis (61). Treatment with MK-801 results in the downregulation of genes associated with the intrinsic apoptotic pathway, including those encoding caspase-3 and similar proteins. Acute administration of MK-801 provides neuroprotection against trauma-induced hippocampal neuron loss and related cognitive deficits in rats (62). Other studies results indicated that MK-801 helps maintain membrane integrity and reduces the release of lactate dehydrogenase, a marker of cell damage in striatum (63). kynurenic acid (KYNA) is a naturally occurring antagonist that binds to the glycine site of NMDA receptors (64).

This neuroprotective property of KYNA has been suggested in various preclinical models of hypoxic–ischemic brain injury. Additionally, the inhibition of NMDA receptors by MK-801 has been shown to suppress Hypoxia-inducible factor 1-alpha (HIF-1α) expression, indicating that this mechanism may also play a role in KYNA's effects (65). In our study MK-801 significantly diminished calcium influx and decreased caspase-3 activation in hippocampal neurons subjected to oxidative stress, thereby reducing apoptosis. MK-801 administration in I/R rats diminished caspase-3 activity, thereby hindering excessive cell death (61). It seems that complementary NMDA receptor modulation has been investigated sporadically. To enhance overall therapeutic efficacy, the combination or alternative therapies could modulate the side effects associated with high-dose NMDA receptor modifications.

For instance, applying the antioxidant therapies may synergistically enhance the neuroprotective effects observed with NMDA receptor modulators. These therapies would target the ROS pathways or enhance endogenous antioxidant defenses. Genes involved in antioxidant defense mechanisms and cellular stress responses, such as nuclear factor erythroid 2–related factor 2 (Nrf2) and its downstream targets, were significantly upregulated in ischemic brain damage to mitigate the neurological dysfunction (66, 67). The combination of memantine, an NMDA antagonist, with clenbuterol significantly extended the therapeutic window of clenbuterol, lasting up to 2 hours after ischemia (68). Xanthine oxidase activation and superoxide ($O^{2\bullet-}$) production are completely inhibited by concomitant incubation of glutamate with MK-801(69). Practically, under oxidative stress conditions, the application of different doses of NMDA receptor can be applicable in mitigating neuronal damage associated with neurodegenerative diseases and oxidative stress-related disorders. The effects of NMDA antagonists, particularly MK-801, in reducing intracellular calcium content and apoptosis, may position them as treatments for oxidative stress-induced neuronal injury.

Theoretically, this study focuses on the NMDA receptor activity in hippocampal neurons under oxidative stress condition. Emphasizing balance between excitatory and inhibitory states under pathological conditions can stabilize neuronal function and survival. Furthermore, the findings suggest that intervention in receptor-mediated signaling pathways during oxidative stress could establish a foundation for future molecular research and therapeutic strategies. The findings highlight the complex relation among excitotoxicity, oxidative stress, and NMDA receptor function. Both NMDA agonists and antagonists can reduce hippocampal cell excitotoxicity by preventing calcium overload and inhibiting caspase-3 activation. It is noteworthy that blocking NMDA receptors can potentially lead to harmful effects; however, our study specifically utilized a low dose series of MK-801 (5-100 μM).

This method allowed us to minimize the risk of adverse effects typically associated with higher dosages. The results indicate that low-dose MK-801 could reduce oxidative stress and improve neuronal survival effectively. It seems that the upcoming studies need to focus on refining low-dose strategies for NMDA receptor modulation. Such an approach not only enhances our understanding of NMDA receptor dynamics in neurodegenerative conditions, but also it will help us be independent of any auxiliary compensatory responses.

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References

1. Li J, O W, Li W, et al. Oxidative stress and neurodegenerative disorders. Int J Mol Sci. 2013;14(12):24438-75.
2. Citri A, Malenka RC. Synaptic plasticity: multiple forms, functions, and mechanisms. Neuropsychopharmacology. 2008;33(1):18-41.
3. Wang H, Peng RY. Basic roles of key molecules connected with NMDAR signaling pathway on regulating learning and memory and synaptic plasticity. Mil Med Res. 2016;3(1):26.
4. Abbah J, Vacher CM, Goldstein EZ, et al. Oxidative Stress-Induced Damage to the Developing Hippocampus Is Mediated by GSK3β. J Neurosci. 2022;42(24):4812-27.
5. Lee KH, Cha M, Lee BH. Neuroprotective Effect of Antioxidants in the Brain. Int J Mol Sci. 2020;21(19).
6. Tian M, Stroebel D, Piot L, et al. GluN2A and GluN2B NMDA receptors use distinct allosteric routes. Nat Commun. 2021;12(1):4709.
7. Gu X, Zhou L, Lu W. An NMDA Receptor-Dependent Mechanism Underlies Inhibitory Synapse Development. Cell Rep. 2016;14(3):471-8.
8. Shankar GM, Bloodgood BL, Townsend M, et al. Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J Neurosci. 2007;27(11):2866-75.
9. Gupta SC, Ravikrishnan A, Liu J, et al. The NMDA receptor GluN2C subunit controls cortical excitatory-inhibitory balance, neuronal oscillations and cognitive function. Sci Rep. 2016;6:38321.
10. Marsden KC, Beattie JB, Friedenthal J, et al. NMDA receptor activation potentiates inhibitory transmission through GABA receptor-associated protein-dependent exocytosis of GABA(A) receptors. J Neurosci. 2007;27(52):14326-37.
11. Gao J, Duan B, Wang DG, et al. Coupling between NMDA receptor and acid-sensing ion channel contributes to ischemic neuronal death. Neuron. 2005;48(4):635-46.
12. Papadia S, Hardingham GE. The dichotomy of NMDA receptor signaling. Neuroscientist. 2007;13(6):572-9.
13. Akpınar H, Nazıroğlu M, Övey İ S, et al. The neuroprotective action of dexmedetomidine on apoptosis, calcium entry and oxidative stress in cerebral ischemia-induced rats: Contribution of TRPM2 and TRPV1 channels. Sci Rep. 2016;6:37196.
14. Doyle KP, Simon RP, Stenzel-Poore MP. Mechanisms of ischemic brain damage. Neuropharmacology. 2008;55(3):310-8.
15. Bambrick LL, Yarowsky PJ, Krueger BK. Glutamate as a hippocampal neuron survival factor: an inherited defect in the trisomy 16 mouse. Proc Natl Acad Sci U S A. 1995;92(21):9692-6.
16. Lipton SA. Pathologically activated therapeutics for neuroprotection. Nat Rev Neurosci. 2007;8(10):803-8.
17. Arundine M, Tymianski M. Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. Cell Mol Life Sci. 2004;61(6):657-68.
18. Jantzie LL, Talos DM, Jackson MC, et al. Developmental expression of N-methyl-D-aspartate (NMDA) receptor subunits in human white and gray matter: potential mechanism of increased vulnerability in the immature brain. Cereb Cortex. 2015;25(2):482-95.
19. Nicholls DG, Ward MW. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. Trends Neurosci. 2000;23(4):166-74.
20. Hardingham GE, Bading H. Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. Nat Rev Neurosci. 2010;11(10):682-96.
21. Papadia S, Soriano FX, Léveillé F, et al. Synaptic NMDA receptor activity boosts intrinsic antioxidant defenses. Nat Neurosci. 2008;11(4):476-87.
22. Janus A, Lustyk K, Pytka K. MK-801 and cognitive functions: Investigating the behavioral effects of a non-competitive NMDA receptor antagonist. Psychopharmacology (Berl). 2023;240(12):2435-57.
23. Selakovic V, Janac B, Radenovic L. MK-801 effect on regional cerebral oxidative stress rate induced

by different duration of global ischemia in gerbils. Mol Cell Biochem. 2010;342(1-2):35-50.
24. Yu LM, Zhang TY, Yin XH, et al. Denitrosylation of nNOS induced by cerebral ischemia-reperfusion contributes to nitrosylation of CaMKII and its inhibition of autophosphorylation in hippocampal CA1. Eur Rev Med Pharmacol Sci. 2019;23(17):7674-83.
25. Yi NX, Zhou LY, Wang XY, et al. MK-801 attenuates lesion expansion following acute brain injury in rats: a meta-analysis. Neural Regen Res. 2019;14(11):1919-31.
26. Lisman J, Yasuda R, Raghavachari S. Mechanisms of CaMKII action in long-term potentiation. Nat Rev Neurosci. 2012;13(3):169-82.
27. Herring BE, Nicoll RA. Long-Term Potentiation: From CaMKII to AMPA Receptor Trafficking. Annu Rev Physiol. 2016;78:351-65.
28. Zhou L, Duan J. The NMDAR GluN1-1a C-terminus binds to CaM and regulates synaptic function. Biochem Biophys Res Commun. 2021;534:323-9.
29. Han WM, Hao XB, Hong YX, et al. NMDARs antagonist MK801 suppresses LPS-induced apoptosis and mitochondrial dysfunction by regulating subunits of NMDARs via the CaM/CaMKII/ERK pathway. Cell Death Discov. 2023;9(1):59.
30. Matz A, Lee SJ, Schwedhelm-Domeyer N, et al. Regulation of neuronal survival and morphology by the E3 ubiquitin ligase RNF157. Cell Death Differ. 2015;22(4):626-42.
31. Chodari L, Sehati F, Hafazeh L, et al. Inhibition of histone methyltransferase promotes cognition and mitochondrial function in vascular dementia model. Behav Brain Res. 2024;473:115194.
32. Tang KS. Protective effect of arachidonic acid and linoleic acid on 1-methyl-4-phenylpyridinium-induced toxicity in PC12 cells. Lipids Health Dis. 2014;13:197.
33. Liu Y, Huang J, Zheng X, et al. Luteolin, a natural flavonoid, inhibits methylglyoxal induced apoptosis via the mTOR/4E-BP1 signaling pathway. Sci Rep. 2017;7(1):7877.
34. Islam MI, Nagakannan P, Ogungbola O, et al. Thioredoxin system as a gatekeeper in caspase-6 activation and nuclear lamina integrity: Implications for Alzheimer's disease. Free Radic Biol Med. 2019;134:567-80.
35. Han Y, Li X, Yang L, et al. Ginsenoside Rg1 attenuates cerebral ischemia-reperfusion injury due to inhibition of NOX2-mediated calcium homeostasis dysregulation in mice. J Ginseng Res. 2022;46(4):515-25.
36. Hardingham GE. Coupling of the NMDA receptor to neuroprotective and neurodestructive events. Biochem Soc Trans. 2009;37(Pt 6):1147-60.
37. Manzanero S, Santro T, Arumugam TV. Neuronal oxidative stress in acute ischemic stroke: sources and contribution to cell injury. Neurochem Int. 2013;62(5):712-8.
38. Wu L, Xiong X, Wu X, et al. Targeting Oxidative Stress and Inflammation to Prevent Ischemia-Reperfusion Injury. Front Mol Neurosci. 2020;13:28.
39. Esteves AR, Arduíno DM, Swerdlow RH, et al. Oxidative stress involvement in alpha-synuclein oligomerization in Parkinson's disease cybrids. Antioxid Redox Signal. 2009;11(3):439-48.
40. Tovar KR, Westbrook GL. The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. J Neurosci. 1999;19(10):4180-8.
41. Han XJ, Shi ZS, Xia LX, et al. Changes in synaptic plasticity and expression of glutamate receptor subunits in the CA1 and CA3 areas of the hippocampus after transient global ischemia. Neuroscience. 2016;327:64-78.
42. Bahrami F, Bahari Z, Abolghasemi R, et al. The neuroprotective effects of stimulation of NMDA receptors against POX-induced neurotoxicity in hippocampal cultured neurons; a morphometric study. Molecular & Cellular Toxicology. 2020;16:401-8.
43. Szydlowska K, Tymianski M. Calcium, ischemia and excitotoxicity. Cell Calcium. 2010;47(2):122-9.
44. Neves D, Salazar IL, Almeida RD, et al. Molecular mechanisms of ischemia and glutamate excitotoxicity. Life Sci. 2023;328:121814.
45. Engin A, Engin AB. N-Methyl-D-Aspartate Receptor Signaling-Protein Kinases Crosstalk in Cerebral Ischemia. Adv Exp Med Biol. 2021;1275:259-83.
46. 46. Yoshida T, Nakamura H, Masutani H, et al. The involvement of thioredoxin and thioredoxin binding protein-2 on cellular proliferation and aging process. Ann N Y Acad Sci. 2005;1055:1-12.

47. Tomar D, Jaña F, Dong Z, et al. Blockade of MCU-Mediated Ca(2+) Uptake Perturbs Lipid Metabolism via PP4-Dependent AMPK Dephosphorylation. Cell Rep. 2019;26(13):3709-25.e7.
48. Wang Y, Li X, Zhao F. MCU-Dependent mROS Generation Regulates Cell Metabolism and Cell Death Modulated by the AMPK/PGC-1α/SIRT3 Signaling Pathway. Front Med (Lausanne). 2021;8:674986.
49. Gu C, Kong F, Zeng J, et al. Remote ischemic preconditioning protects against spinal cord ischemia-reperfusion injury in mice by activating NMDAR/AMPK/PGC-1α/SIRT3 signaling. Cell Biosci. 2023;13(1):57.
50. Linciano P, Sorbi C, Rossino G, et al. Novel S1R agonists counteracting NMDA excitotoxicity and oxidative stress: A step forward in the discovery of neuroprotective agents. European Journal of Medicinal Chemistry. 2023;249:115163.
51. Chiang TI, Yu YH, Lin CH, Lane HY. Novel Biomarkers of Alzheimer's Disease: Based Upon N-methyl-D-aspartate Receptor Hypoactivation and Oxidative Stress. Clin Psychopharmacol Neurosci. 2021;19(3):423-33.
52. De Felice FG, Velasco PT, Lambert MP, et al. Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. J Biol Chem. 2007;282(15):11590-601.
53. Yu SP, Jiang MQ, Shim SS, et al. Extrasynaptic NMDA receptors in acute and chronic excitotoxicity: implications for preventive treatments of ischemic stroke and late-onset Alzheimer's disease. Mol Neurodegener. 2023;18(1):43.
54. Rogawski MA, Wenk GL. The neuropharmacological basis for the use of memantine in the treatment of Alzheimer's disease. CNS Drug Rev. 2003;9(3):275-308.
55. Övey İ S, Nazıroğlu M. Effects of homocysteine and memantine on oxidative stress related TRP cation channels in in-vitro model of Alzheimer's disease. J Recept Signal Transduct Res. 2021;41(3):273-83.
56. Anastasio NC, Johnson KM. Differential regulation of the NMDA receptor by acute and sub-chronic phencyclidine administration in the developing rat. J Neurochem. 2008;104(5):1210-8.
57. Suvarna N, Borgland SL, Wang J, et al. Ethanol alters trafficking and functional N-methyl-D-aspartate receptor NR2 subunit ratio via H-Ras. J Biol Chem. 2005;280(36):31450-9.
58. du Bois TM, Deng C, Han M, et al. Excitatory and inhibitory neurotransmission is chronically altered following perinatal NMDA receptor blockade. Eur Neuropsychopharmacol. 2009;19(4):256-65.
59. Tovar KR, Westbrook GL. Mobile NMDA receptors at hippocampal synapses. Neuron. 2002;34(2):255-64.
60. Huettner JE, Bean BP. Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. Proc Natl Acad Sci U S A. 1988;85(4):1307-11.
61. Yaghoobi Z, Ataei S, Riahi E, et al. Neuroprotective effects of MK-801 against cerebral ischemia reperfusion. Heliyon. 2024;10(13):e33821.
62. Cigel A, Sayin O, Gurgen SG, et al. Long term neuroprotective effects of acute single dose MK-801treatment against traumatic brain injury in immature rats. Neuropeptides. 2021;88:102161.
63. Kolar D, Kleteckova L, Skalova K, et al. Glycolytic and Krebs cycle enzymes activity in rat prefrontal cortex, hippocampus, and striatum after single and repeated NMDA inhibition by MK-801. Neurotoxicology. 2022;90:35-47.
64. Hilmas C, Pereira EF, Alkondon M, et al. The brain metabolite kynurenic acid inhibits alpha7 nicotinic receptor activity and increases non-alpha7 nicotinic receptor expression: physiopathological implications. J Neurosci. 2001;21(19):7463-73.
65. Chen X, Wu Q, You L, et al. Propofol attenuates pancreatic cancer malignant potential via inhibition of NMDA receptor. Eur J Pharmacol. 2017;795:150-9.
66. Yang T, Sun Y, Li Q, et al. Ischemic preconditioning provides long-lasting neuroprotection against ischemic stroke: The role of Nrf2. Exp Neurol. 2020;325:113142.
67. Chen-Roetling J, Regan RF. Targeting the Nrf2-Heme Oxygenase-1 Axis after Intracerebral Hemorrhage. Curr Pharm Des. 2017;23(15):2226-37.

68. Culmsee C, Junker V, Kremers W, et al. Combination therapy in ischemic stroke: synergistic neuroprotective effects of memantine and clenbuterol. Stroke. 2004;35(5):1197-202.

69. Atlante A, Gagliardi S, Minervini GM, et al. Glutamate neurotoxicity in rat cerebellar granule cells: a major role for xanthine oxidase in oxygen radical formation. J Neurochem. 1997;68(5):2038-45.