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Research article

Glutamate/NMDA excitotoxicity and HMGB1/TLR4 neuroimmune toxicity converge as components of neurodegeneration

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Abstract: Neurodegeneration in brain is linked to both excitotoxicity and neuroimmune gene induction, although the mechanisms are poorly understood. High-mobility group box 1 (HMGB1) is a cytokine like molecule released in brain by glutamate that has been found to enhance neuronal excitability through Toll-like receptor 4 (TLR4). To explore the role of HMGB1 in glutamate/NMDA excitotoxicity or neuroimmune-induced neurodegeneration we used an *ex vivo* model of organotypic hippocampal-entorhinal cortex (HEC) slice culture. Concentration response and time course studies find release of HMGB1 precedes neuronal death induced by glutamate, NMDA, TNFα and LPS. Blockade of glutamate receptors with antagonist MK-801 prevents glutamate/NMDA stimulation release of HMGB1 and neuronal death as well as blocking neuroimmune (LPS and TNFα) induced neuronal death. Similarly, HMGB1 neutralizing antibodies or inhibitor glycyrrhizin block glutamate/NMDA as well as neuroimmune (LPS and TNFα) induced neuronal death. Further, delayed neuronal cell death mediated by LPS and TNFα was rescued by NR2B inhibitor ifenprodil. Together, these findings suggest HMGB1 contributes a critical element of both glutamate/NMDA as well as neuroimmune induced neurodegeneration indicating HMGB1 may be a novel target crossing multiple neurodegeneration pathologies.

Keywords: excitotoxicity; neuroinflammation; cytokines; toll-like receptors; endogenous TLR4 ligand; brain slice culture

Abbreviations:

Toll-like receptor = TLR; High-mobility group box 1 = HMGB1; N-methyl-d-aspartate = NMDA; receptor for advanced glycation end products = RAGE; endotoxin lipopolysaccharide = LPS; hippocampal-entorhinal cortex = HEC

1. Introduction

Neuronal degeneration and cell loss in brain are features of neurological disorders such as Parkinson's Disease (PD), Amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and Alzheimer's disease (AD) [1,2,3]. Although a great deal of effort has been devoted to explore the molecular and cellular mechanism responsible for the development and progression of these pathologies, the neuropathogenesis of the initiation or progression of neurodegeneration remains unclear. Neuroinflammation and excitotoxicity are considered to be distinct but interconnected mechanisms contributing to the neuropathogenesis of neurodegenerative disorders [4]. Excitotoxicity is caused by excessive activation of glutamate/ N-methyl-d-aspartate (NMDA) receptors due to accumulation of glutamate in extracellular space and is considered to be one of the main causes of neurodegeneration. Evidence of excessive glutamatergic signaling has been found in several neurodegenerative disorders [5]. In recent years, it has been postulated that neuroinflammation and excitotoxicity frequently coexist in neurological disorders. Many studies indicate that proinflammatory cytokines released from dying neurons activate microglia and contribute to the process of excitotoxicity through blocking glial glutamate transporters, releasing glial glutamate [4,6,7] and/or potentiating glutamate receptor function [8]. In general, neuroimmune activation has been considered to involve responses to dying cells inducing a wide variety of proinflammatory molecules that endanger neighboring cells in autocrine/paracrine fashions by spreading the proinflammatory signaling. In this manuscript we present data indicating that glutamate toxicity and HMGB1/TLR signaling in parallel contributes to neurotoxicity.

High mobility group box-1 (HMGB1), a ubiquitous nuclear and cytosolic non-histone DNA-binding protein, has multiple signaling mechanisms including activation of TLR4 receptors. HMGB1 can be released into extracellular space in one of two settings: active secretion or passive release from necrotic or damaged cells [9,10,11,12]. Extracellular HMGB1 functions as a damage associated molecular pattern (DAMP) or an alarmin [13] and activates proinflammatory signaling pathways by activating pattern recognition receptors including Toll-like receptor (TLR) 2 and 4 and receptor for advanced glycation end products (RAGE) [14]. HMGB1 has multiple forms related to oxidation state that have different activities at these receptors, with a disulfide-HMGB1 form having full TLR4 agonist activity, that is not a property of the reduced all thiol-HMGB1, although it retains cytokine-like activation of RAGE and other receptors, whereas fully oxidized HMGB1 is inactive [15,16]. Actively released HMGB1 is acetylated and disulfide-HMGB1, whereas cell death induced release from necrotic cells involves disulfide-HMGB1 and other active forms, whereas release from apoptotic cells is inactive HMGB1 [17]. Despite extensive studies in immunocompetent cells showing that HMGB1 is actively released into the extracellular space during sterile inflammation, few studies have investigated release of HMGB1 from neuronal cells. We and others find HMGB1 is abundantly expressed in neurons [11,18,19,20] including human cortical neurons [21]. HMGB1-TLR4 signaling is linked to epilepsy kindling of hyperexcitability and seizures [22]. Other studies have also reported that targeting HMGB1 can offer neuroprotection in animal models of brain injuries [23,24,25] and reverse memory impairments [26].

We used an *ex vivo* model of hippocampal-entorhinal cortex (HEC) slice cultures that contain all brain cell types and maintain the morphological structure of the intact brain to test the hypothesis that delayed neuronal death is due to convergence of glutamate-excitotoxic and HMGB1/TLR signaling that ultimately lead to neuronal death. We report here that HMGB1 is released by

glutamate/NMDA or mGluR agonists and that neuroimmune signaling induced neuronal death involves glutamate/NMDA receptors. HMGB1 release precedes neuronal cell death induced by glutamate/NMDA as well as by neuroimmune signaling activated by proinflammatory cytokine TNFα and endotoxin lipopolysaccharide (LPS). Furthermore, antagonists to glutamate/NMDA receptors or HMGB1 block neuronal death independent of the initial triggering stimulus. Although glutamate antagonists protect against neurotoxicity, HMGB1 antagonists may allow protection without directly disrupting normal glutamate synaptic signaling, a critical component of normal brain function.

2. Materials and Methods

2.1. Reagents

The following reagents were purchased from Sigma-Aldrich (St Louis, USA): Glutamic acid, NMDA, LPS from *Escherichia coli* 0111:B4, glycyrrhizin, ifenprodil and HMGB1. TNFα was purchased from R&D System (Minneapolis, USA). Non oxidizable chemokine-HMGB1 and cytokine-HMGB1 came from HMG Biotech (Milano, Italy). Lipopolysaccharide from the photosynthetic bacterium *Rhodobacter sphaeroides* (Lps-Rs) came from InvivoGen (San Diego, USA). HMGB1 ELISA kit and neutralizing antibodies were purchased from IBL International (Hamburg, Germany).

2.2. Hippocampal-entorhinal cortex slice culture

All protocols followed in this study were approved by the Institutional Animal Care Use Committee at UNC and were in accordance with National Institute of Health regulation for the care and use of animal in research. The preparation of organotypic hippocampal-entorhinal cortex (HEC) slice cultures was described elsewhere [8]. Briefly, hippocampal-entorhinal cortex complexes were dissected from neonates on postnatal day 7 in Guey's buffer and transversely sectioned at thickness of 375 μm. Individual HEC slice was placed onto tissue insert membrane (10–12 slices/insert) and cultured with medium containing 75% MEM with 25 mM HEPES and Hank's salts + 25% horse serum (HS) + 5.5 g/L glucose + 2 mM L-glutamine in a humidified 5% CO_2 incubator at 36.5 °C for 7 days *in vitro* (DIV), followed by 4 DIV in medium containing 12.5% HS and then 3 DIV in serum-free medium supplemented with N2. The cultures after 14 DIV were used for experiments and drug treatments with serum-free N2-supplemented medium.

2.3. Drug treatments

All drug treatments were initiated on slices at 14DIV in serum-free N2-supplemented medium. For dose-response studies, HEC slices were treated with various concentrations of drugs (see below) for a fixed time (24 or 48 hrs), and then slices and media were removed for further analysis. In time-course studies, HEC slices were treated with a fixed concentration of drug depending on the experimental design (see below) and a total of 100 μL media was removed at each time point from culture for ELISA measurements of HMGB1. After removing media, the same amount of 100 μL fresh N2 medium was added into culture.

2.4. ELISA measurements of HMGB1

Media HMGB1 levels were determined with ELISA kit (IBL, Germany) according to the manufacturer's instruction. A total of 50 μL of culture medium from each sample was used for ELISA. All samples were run in triplicate.

2.5. Assessment of neuronal cell death

The uptake of the fluorescent exclusion dye propidium iodide (PI) was used for determination of neuronal cell death. PI is a polar compound that is impermeable to a cell with an intact cell membrane but penetrates damaged cell membranes. Inside the cells it binds nuclear DNA to generate the brightly red fluorescence. This method has been well characterized as accurately measuring neuronal degeneration in organotypic slice cultures [8,27,28] and the PI uptake was confirmed to significantly correlate with LDH efflux [28,29] , which is another well accepted marker for neuronal cell death [30]. For each experiment, PI was added into the culture medium at the beginning of treatment at a concentration of 5 μg/mL and PI fluorescence images were captured at indicated time points. PI fluorescent intensity was measured and analyzed with the AxioVision 3.1 software. Using an interactive drawing tool, hippocampal CA1 field was outlined as regions of interest (ROIs), and mean PI fluorescent intensity of ROI was then determined by the program [31].

2.6. Preparation of conditioned medium

HEC slices at 14 DIV were treated with NMDA (200 μM) for 40 min. After NMDA treatment, NMDA-containing medium was removed from cultures and replaced with fresh serum-free N2-supplemented medium. After 24 hrs, culture media were collected and pooled as conditioned medium (CM). Pooled CM was diluted (1:3) and then used for treatment of new slice cultures.

2.7. Immunofluorescent staining and confocal analysis

HEC slice cultures were removed at the end of the experiment and fixed with 4% paraformaldehyde + 5% sucrose in 0.01 M PBS for 24 hrs at 4 °C. Free-floating slices were used for immunofluorescent staining. For detection of HMGB1 in PI-labeled neuronal cells, PI-labeled (red) slices after images captured for measurements of neuronal cell death were directly fixed and removed for performing immunofluorescent staining of rabbit anti-HMGB1 (1:500, AbCam). For double immunofluorescent staining, HEC slices were stained with neuronal marker MAP2 (mouse, 1:1000, sigma) and HMGB1 (Rabbit, 1:500, AbCam). All primary antibodies were incubated for 48 hrs at 4 °C. Either Alexa Fluor 594 or Alexa Fluor 488 secondary antibodies (1:2000; Molecular Probes, Eugene, OR) were used for immunofluorescent staining and incubated for 1 hr at room temperature. The slices were coverslipped with anti-fade mounting medium (pro-long; Molecular Probes). Confocal analysis was performed using a LeicaSP2 AOBS Upright Laser Scanning Confocal in Michael Hooker Microscopy Facility (University of North Carolina, Chapel Hill, NC).

2.8. Western blotting

Cell lysates were prepared from the entire HEC slices. Briefly, HEC slices were incubated in RIPA lysis buffer plus phosphatase and/or protease inhibitor cocktail (Thermo Scientific, USA) for 15 min and then disrupted with sonication (3 time, 2 min apart in ice). After centrifugation of the slice homogenate, the supernatant were collected, and protein level determined using a BioRad Bradford reagent kit (BioRad). For Western blotting, an equal amount of protein (50 μg) was mixed with 10 μL 5× loading buffer, and separated using a 4–15% Tris mini-gel (Bio-Rad) and transferred onto PVDF membrane. After blocking with LI-COR blocking buffer overnight, the membrane was probed with mouse anti-phospho-NR2B (1:1000, Santa Cruz), rabbit anti-total NR2B antibodies (1:200, Santa Cruz) and mouse anti-β-actin (1:1000, Santa Cruz) at 4 °C overnight. After washing, membrane was incubated with second antibodies coupled with fluorescence from LI-COR Bioscience and then scanned with Odyssey machine (Lincoln, NE).

2.9. Statistical Analysis

Data are expressed as a mean values ± standard error of mean from the indicated number of slices or experiments. Statistical comparisons were made with *ANOVA* and the difference between the experimental groups was further compared by using *post hoc* Fisher PLSD test. Differences were considered to be statistically significant if p value of < 0.05.

3. Results

3.1. Glutamate/NMDA concentration response curves on HMGB1 release and neuronal cell death

Hippocampal-entorhinal cortex (HEC) brain slice cultures provide an *ex vivo* model validated by many to investigate mechanisms of neurotoxicity [8,27,28]. In the present study, we tested the hypothesis that HMGB1 contributes to glutamate/NMDA receptor excitotoxicity. The glutamate/NMDA concentration-dependence of HMGB1 release into the media is compared to neuronal death as indexed by measurements of PI intensity within hippocampal CA1 field of HEC slice. Glutamate at lower concentrations triggered robust release of HMGB1 while neuronal cell death shows no significant increase (Figure 1). Glutamate released HMGB1 by 50%, 350%, and 700% with 100 μM, 500 μM, and 1 mM, concentrations which show no increase in neurotoxicity (Figure 1). Treatment of HEC slices with glutamate at concentration of 5 mM increased media HMGB1 by 1700%, accompanying with a marked 300% increase in neuronal death measured as PI fluorescent intensity (Figure 1A-dash line, 1B-c). Similarly, treatment of HEC slices with various concentration of NMDA for 24 hrs drastically increased media HMGB1 level by 500–800% at concentrations up to 1 μM, all concentrations showing no significant increase in neuronal death (Figure 2). NMDA at 10 μM and 100 μM increased media HMGB1 by 1400% and 2000% and significantly increased neuronal cell death by 350% and 650% respectively (Figure 2A-dash line, 2B-c). Immunoprecipitation of media proteins using an antibody for acetyl-lysine followed by western blot using an antibody for HMGB1 found that glutamate and NMDA increased release of acetyl-HMGB1, a marker of actively released HMGB1 [11]. We also tested the effects of stimulating mGluR on secretion of HMGB1. We treated HEC slices with mGluRII agonist LY-354740 (100 nM) or non-selective mGluR agonist

(1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD, 100 μM). Both LY-354740 and ACPD stimulated significant increases in media HMGB1 levels (Control: 1.8 ± 0.2; LY-354740: 11.2 ± 0.4; ACPD: 14.2 ± 2.6, $p < 0.001$, $n = 3$) without causing significant neuronal death (PI Intensity: Control 24.4 ± 3.5; LY-354740 32.1 ± 6.9; ACPD 26.7 ± 5.9, $p < 0.09$, $n = 6–8$). LY-354740 and ACPD-stimulated HMGB1 release was blocked by mGluR1/II antagonists LY3578 and MCPG (data not shown). Together, these results indicate that glutamate receptor activation can release HMGB1 through mGluR receptors that are not neurotoxic and through NMDA receptors at concentrations below those that trigger neurotoxicity. In addition, neurotoxicity further increases HMGB1 release.

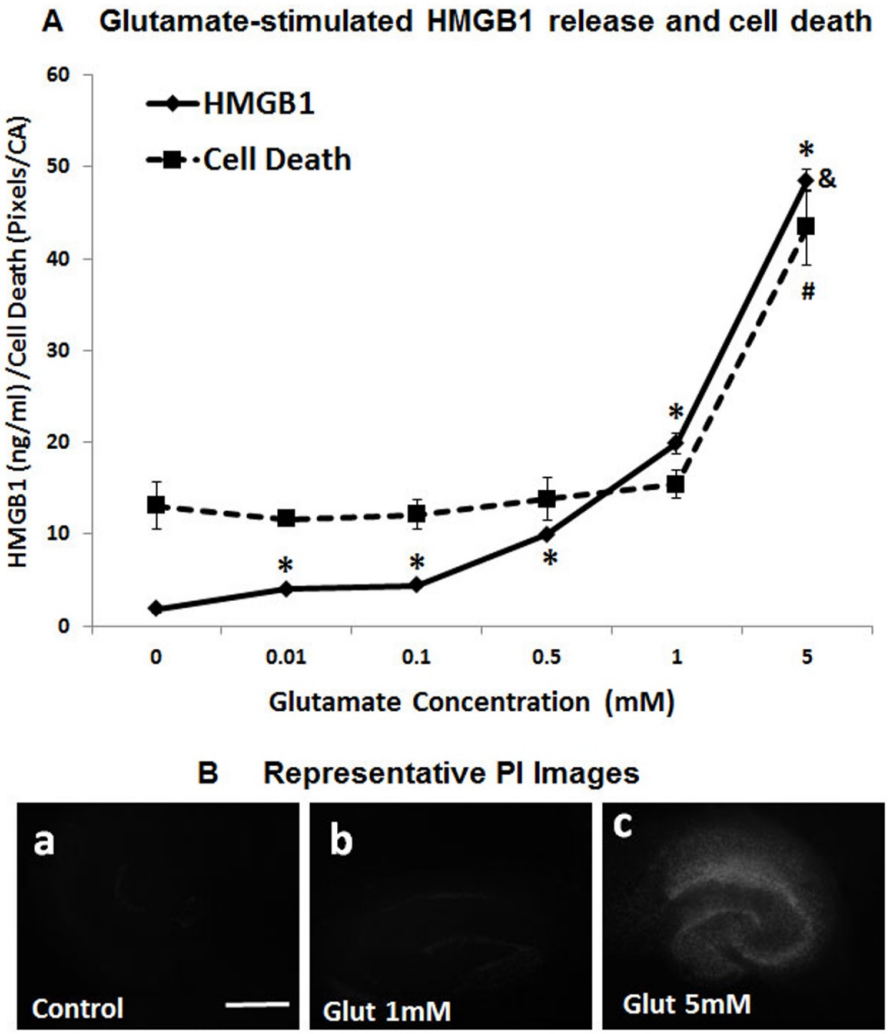

Figure 1. HMGB1 release in response to glutamate. A: HEC slices were treated with different concentrations of glutamate for 24 hrs. Media HMGB1 levels (solid line) were measured with ELISA kit and neuronal cell death (dash line) was monitored by using propidium iodide (PI) uptake. Shown are mean ± SEM of media HMGB1 level or PI intensity measured from CA1 field of hippocampus in a representative experiment. Glutamate treatment dose-dependently elevates media HMGB1 level (*$p < 0.001$ compared with Control, $n = 3$); Glutamate at concentration of 5 mM caused profound neuronal cell death (#$p < 0.001$ compared with Control, $n = 8–10$); &: HMGB1 levels at

5 mM glutamate may be underestimated due to over saturation of the ELISA standard curve. B: Representative PI images were shown from Control and slices treated with 1 and 5 mM glutamate. Bright fluorescence represents dying neurons (scale bar = 500 μM).

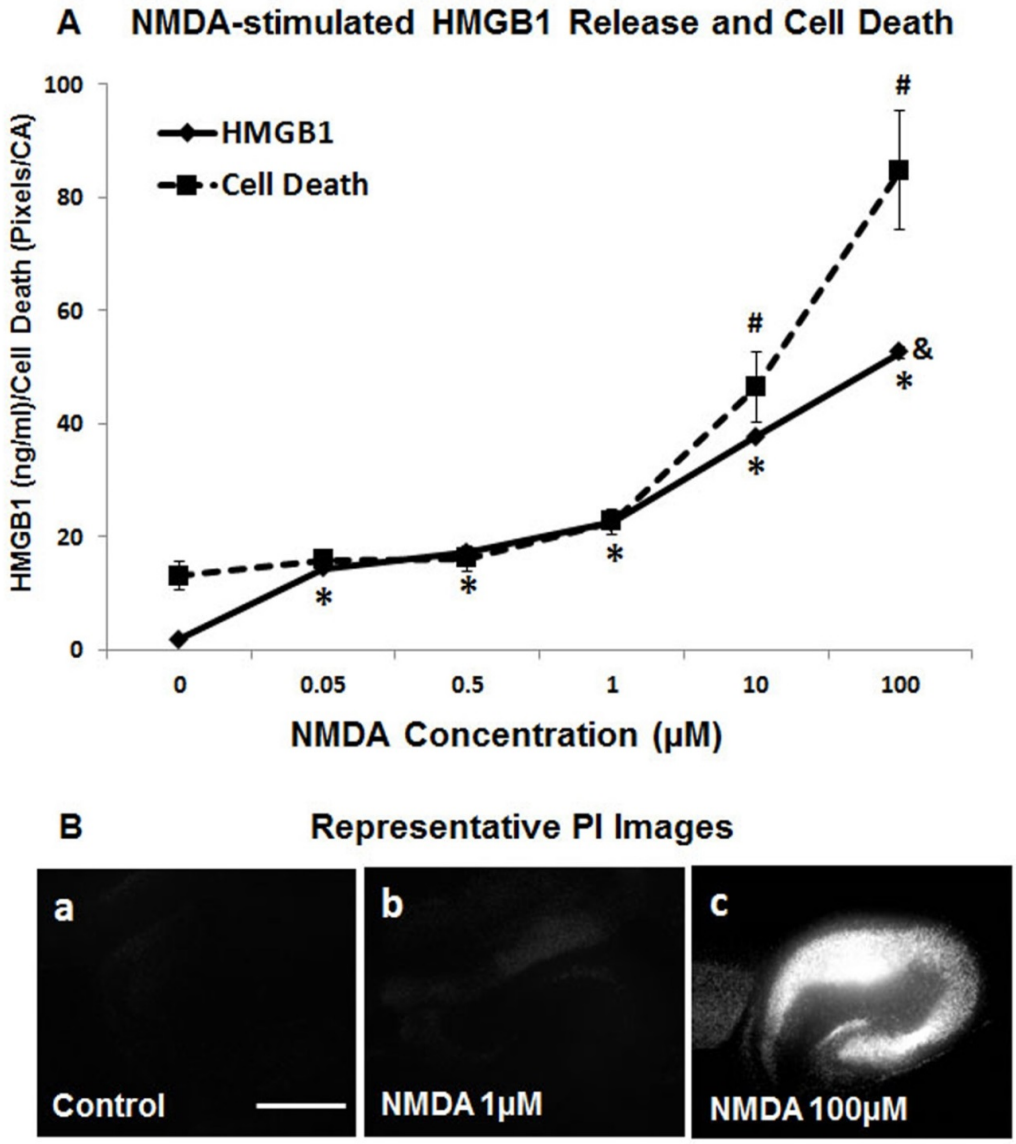

Figure 2. NMDA-stimulated HMGB1 release. A: Media HMGB1 levels (solid line) and neuronal cell death (dash line) were quantified from slices treated with different concentrations of NMDA for 24 hrs. Shown are mean ± SEM of media HMGB1 or PI fluorescent intensity measured from CA1 field of the hippocampus. While stimulating robust HMGB1 release into media, NMDA at concentrations of 10 and 100 μM caused drastic increase in neuronal cell death (*$p < 0.001$ compared with Control, $n = 3$; #$p < 0.0001$ compared with Control, $n = 8–10$); &: HMGB1 levels at 100 μM glutamate may be underestimated due to over saturation of the ELISA standard curve. B: Representative PI images were shown from Control and slices treated with 1 μM and 100 μM NMDA. Bright fluorescence represents dying neurons (scale bar = 500 μM).

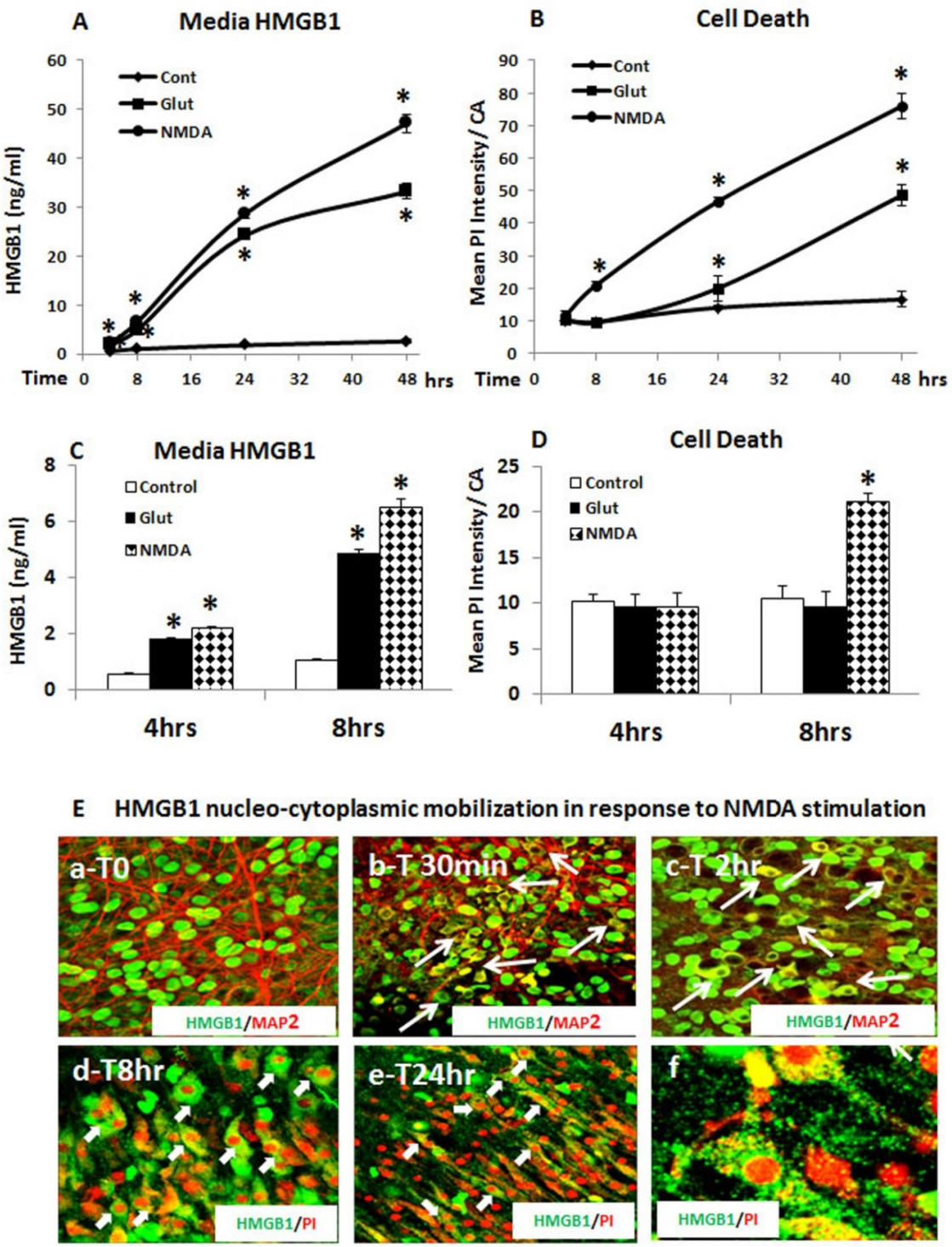

Figure 3. Time-course study of glutamate/NMDA treatment and HMGB1 release. A: Shown are mean ± SEM of media HMGB1 levels from slices treated with glutamate (1 mM) and NMDA (10 μM) at different time points up to 48 hrs. HMGB1 levels at 4 hrs were significantly elevated and progressively increased (*$p < 0.001$ compared to control); B: Neuronal cell death was monitored at the same time points. Significant increases in neuronal cell death were evident after 24 hrs for glutamate and 8 hrs time point for NMDA (*$p < 0.001$ compared to control, $n = 8–10$). Bar graphs in C and D represent the

data of 4 and 8 hrs time points showing significant HMGB1 release (C) precedes neuronal cell death in response to glutamate/NMDA. E: Shown are confocal images labeled with HMGB1 (green) and MAP2 (red a–c) or PI (red, d–e). Depletion of nuclear HMGB1 was evident in slice following NMDA treatment for 30 min (b, arrows) and 2 hrs (c, arrows) compared to control (a). After 24 hrs NMDA treatment, majority of neurons are dead and labeled by PI (red, d-e), however, HMGB1 cytoplasmic retention in dying neurons are evident (arrow heads). High magnification of images showing HMGB1 retention was depicted in e (magnification: 80× in a–d; 360× in e)

3.2. HMGB1 release precedes neuronal cell death

Time course studies were conducted to correlate glutamate/NMDA stimulated release of HMGB1 with neuronal cell death. In this set of experiments, HEC slices were treated with neurotoxic concentrations of glutamate (3 mM) or NMDA (10 μM). Glutamate stimulated a rapid increase in media HMGB1 with 3 and 4 fold increases at 4 and 8 hrs time points when there was no measurable neuronal cell death (Figure 3). Similarly, NMDA treatment increases media HMGB1 after 4 hrs of treatment, without causing measurable neuronal death. Treatment for longer time periods by glutamate (24 and 48 hrs) or NMDA (8, 24 and 48 hrs) increased neuronal cell death and induced greater increases in media HMGB1 (Figure 3). To visualize cellular HMGB1, we performed double immunofluorescent staining and confocal image analysis from slice culture treated with NMDA (100 μM). The representative confocal images are depicted showing nuclear HMGB1 in neuronal cells at zero time (T0), with increased depletion of nuclear HMGB1 and cytoplasmic accumulation in neuronal cells 30 min (Figure 3E, b), and 2 hrs after NMDA (Figure 3E, c), early time points when there is no neuronal death. Treatment with NMDA for 8 and 24 hrs finds PI-labeled dying neurons (dense red nuclei) with little nuclear HMGB1 (Figure 3E, d, e, f). Higher magnification of confocal images after NMDA treatment for 24 hrs results in granule-like HMGB1+ staining that is confined within cytoplasm and proximal neuronal processes (Figure 3E, f). These findings suggest that stimulated HMGB1 release precedes neuronal death by excitotoxic concentrations of glutamate and NMDA and that neuronal death further increases HMGB1 release.

3.3. Glutamate antagonists block HMGB1 release correlating with reduction of neuronal death

To further investigate the effects of glutamate on release of HMGB1 into the media, we determined the effect of glutamate receptor antagonists. MK-801 (25 μM) completely prevented glutamate and NMDA induced neuronal cell death. Glutamate and NMDA increased media HMGB1 from a control value of 2.6 ± 0.2 ng/mL (Figure 4A–B) to 18 ± 0.6 ng/mL and 53 ± 2.0 ng/mL for glutamate and NMDA respectively. MK801 reduced media HMGB1 to 3.7 ± 0.6 ng/mL and 4.3 ± 0.8 ng/mL in Glutamate-MK801 and NMDA-MK801 treated slices respectively. Confocal analysis of HMGB1-immunofluorescent staining found that nuclear HMGB1 was reduced in the nuclei of neurons treated with NMDA (Figure 4C-e). Controls and NMDA/MK-801 groups show prominent nuclear HMGB1 suggesting blockade of NMDA receptors blocks HMGB1 translocation to the cytoplasm and release of HMGB1 (Figure 4C-d, f). We also examined the effects of glutamate-AMPA receptor antagonist NBQX. Treatment with AMPA antagonist NBQX (30 μM)

also reduced glutamate (1 and 3 mM, data not shown) stimulated HMGB1 release. These findings indicate that MK-801 blocks glutamate and NMDA stimulated HMGB1 release and neurotoxicity.

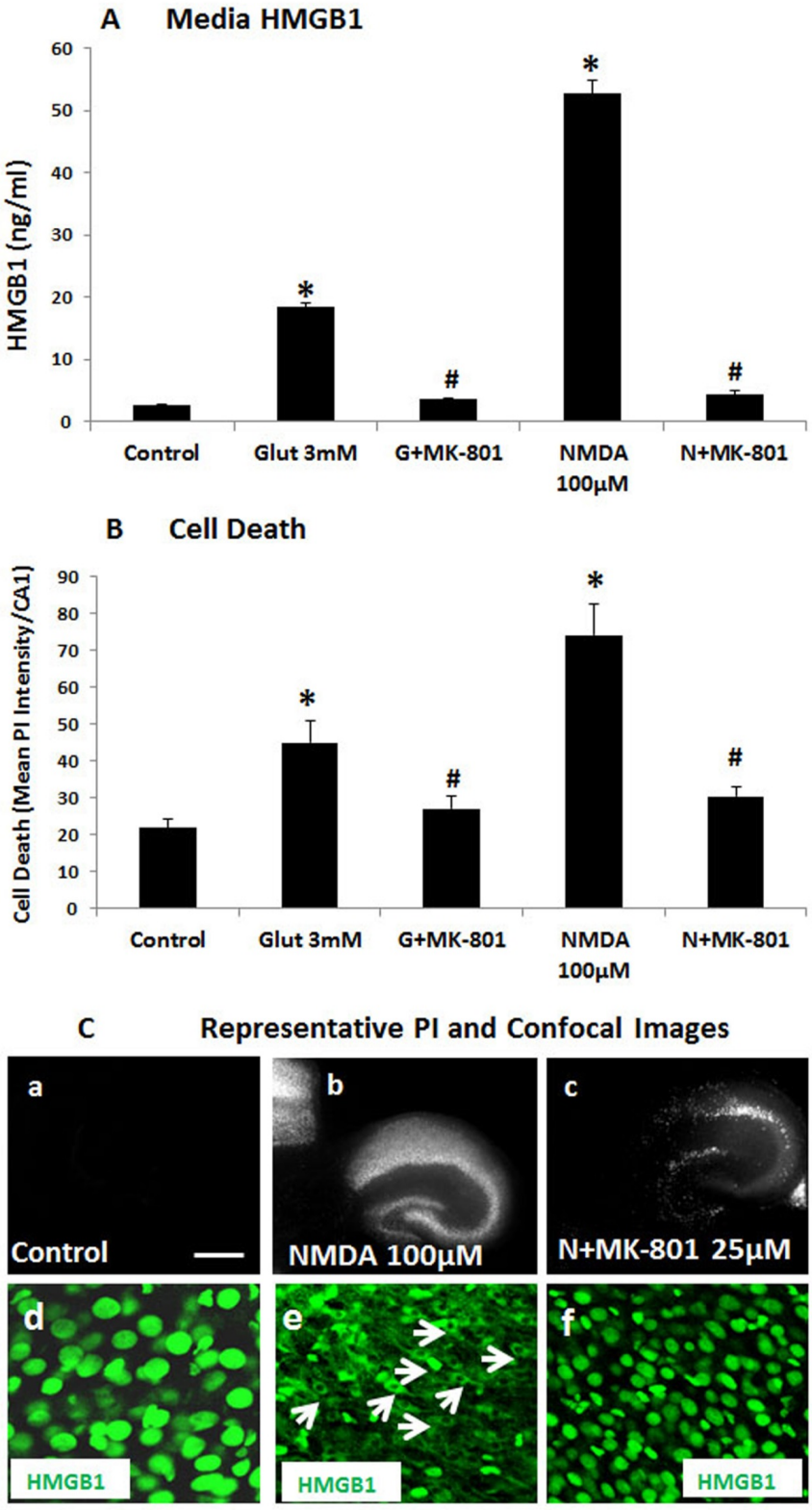

Figure 4. MK-801 blocks HMGB1 release and neuronal death. A: Media HMGB1 levels in response to glutamate/NMDA were reduced by MK-801 (30 µM). B: In

correlation with HMGB1 release, neuronal cell death was significantly prevented by MK-801. C: Representative PI images showing neuronal cell death were shown from Control (a), NMDA (b) and NMDA+MK-801 (c) (scale bar = 500 μM), and confocal images of HMGB1 fluorescent staining (green) were depicted from control (d), NMDA (e) and NMDA+MK-801 (f). NMDA-treated neurons show empty of nuclear HMGB1 (arrows) and MK-801 restores nuclear HMGB1 (magnification 80×).

3.4. HMGB1 contributes to glutamate-induced neuronal cell death

To investigate the direct impact of HMGB1 on glutamate/NMDA excitotoxicity, we conducted experiments by adding exogenous HMGB1 to HEC slice cultures during treatments of glutamate or NMDA. From a separate experiment testing the effects of exogenous HMGB1 on neuronal cell viability, HMGB1 alone for 48 hrs caused modest increases in PI intensity at concentrations less than 500 ng/mL, but clearly increased cell death at 500 ng/mL and 1000 ng/mL. The PI intensity values for HMGB1 alone were Control: 17.5 ± 1.8; HMGB1 100 ng/mL: 22.3 ± 2.6, $p > 0.5$ compared to control; HMGB1 500 ng/mL: 32.6 ± 3.2, $p < 0.01$ compared to Control; HMGB1 1000 ng/mL: 41.5 ± 6.2, $p < 0.0003$ compared to Control; $n = 6–8$). These are high concentrations, which may be related to the commercial preparation of HMGB1 which is known to vary across lots from different vendors (11) or due to some loss of HMGB1-TLR4 activation due to HMGB1 inactivated by oxidation. HMGB1 (100 ng/mL) significantly and synergistically increased neuronal cell death when combined with glutamate (1 mM) or NMDA (10 μM) for 24 hrs (Figure 5A). Furthermore, we prepared conditioned media (CM) collected from NMDA-stimulated slice cultures, in which HEC slice cultures were pretreated with NMDA (200 μM) for 40 min and then replaced with fresh N2-suplemented medium to remove NMDA and capture released HMGB1 and other factors for 24 hrs (see method), and treated HEC slice with CM during glutamate/NMDA treatments. Combined treatments of HEC slices with glutamate (1 mM) or NMDA (10 μM) for 46 hrs significantly potentiated neuronal cell death by 103% and 49% respectively (Figure 5B). Addition of anti-HMGB1 neutralizing antibodies or HMGB1 inhibitor glycyrrhizin blocked CM-potentiated glutamate/NMDA neurotoxicity (Figure 5B). Taken together, these results indicate that HMGB1 contributes to glutamate/NMDA neurotoxicity and that HMGB1 released from NMDA excited neurons can increase excitotoxic cell death.

To facilitate our understanding of the role of HMGB1 in excitotoxic delayed neuronal cell death induced by glutamate/NMDA, we performed experiments targeting HMGB1 by adding HMGB1 neutralizing antibodies or the HMGB1 inhibitor glycyrrhizin into HEC slice cultures during glutamate/NMDA treatment. Glutamate (1 mM) increases media HMGB1 by about 5, 8 and 9 fold at 8, 24 and 48 hrs, respectively (Figure 6A) consistent with glutamate releasing HMGB1 from cells that contributes to glutamate excitotoxicity. Glutamate stimulated HMGB1 release was significantly reduced by glycyrrhizin (G+Gly) likely due to blocking HMGB1 activation of TLR4 receptors that further stimulates release of HMGB1 [11]. Assessment of neuronal cell death indicated that glutamate treatment for 8 hrs does not show an increase in cell death, but at 24 and 48 hrs neuronal cell death is progressively increased (Figure 6A). Glutamate stimulated cell death was reduced by both HMGB1 neutralizing antibodies and by the HMGB1 antagonist glycyrrhizin (Figure 6B). Similarly, treatment with the receptor specific agonist, NMDA (10 μM), increased media HMGB1 level by about 7, 9 and 10 fold at 8, 24 and 48 hrs time points respectively, which corresponds to

220%, 400% and 310% increases of PI fluorescent intensity, e.g. neuronal cell death at each time point (Figure 6C–D). The addition of HMGB1 neutralizing antibodies and inhibitor glycyrrhizin during NMDA treatment significantly reduced neuronal cell death (Figure 6C–D). Together, these results provide evidence that HMGB1 released by glutamate receptor activation can contribute to glutamate/NMDA neurotoxicity.

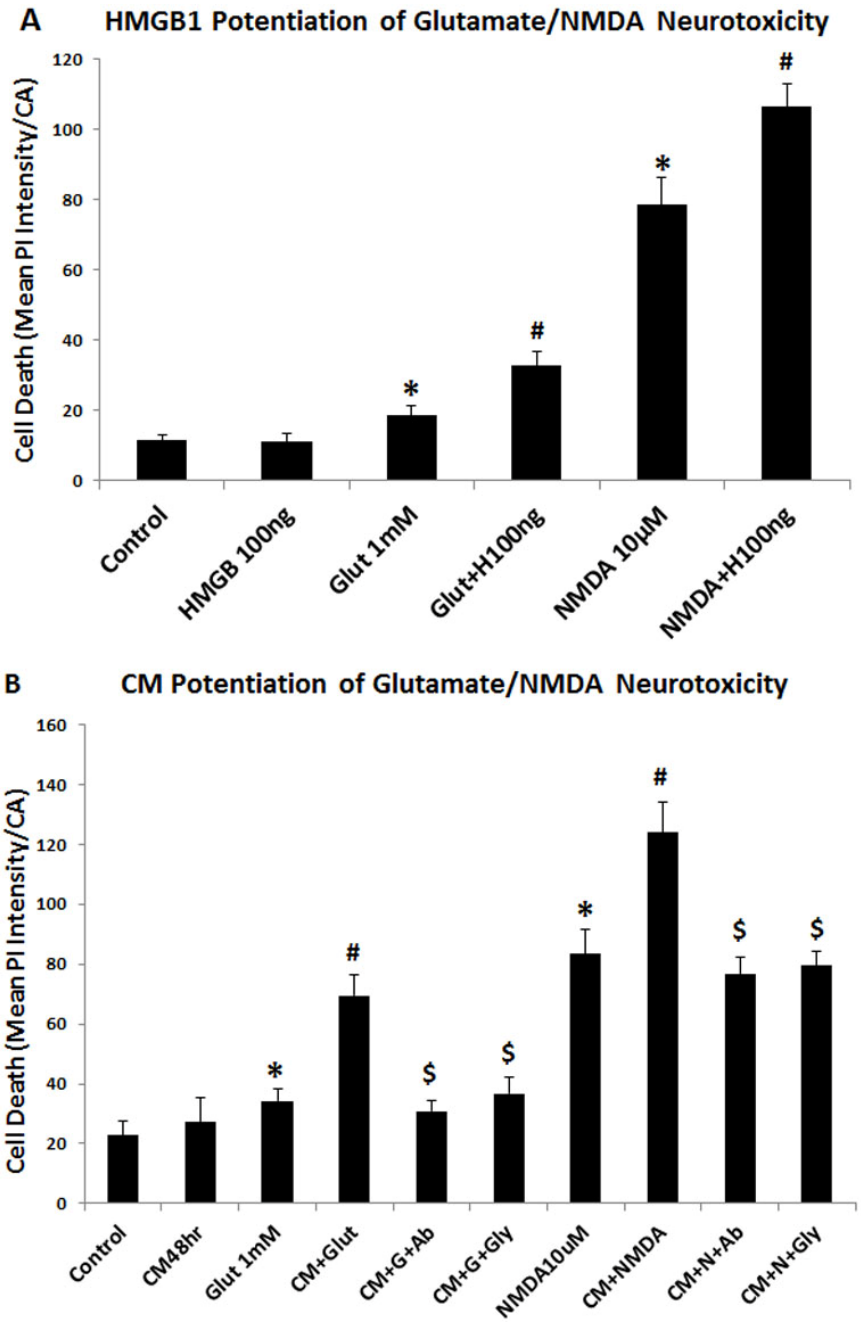

Figure 5. HMGB1 contributes to glutamate/NMDA-induced neuronal death. A: Exogenous HMGB1 potentiated glutamate/NMDA neurotoxicity (* $p < 0.001$ compared to control, n = 8–10). B: NMDA-conditioned medium potentiated glutamate/NMDA neurotoxicity and blockade of antagonizing HMGB1 (*$p < 0.001$ compared to control; #$p < 0.001$ compared to glutamate or NMDA; \$$p < 0.01$ compared to corresponding group, n = 8–10).

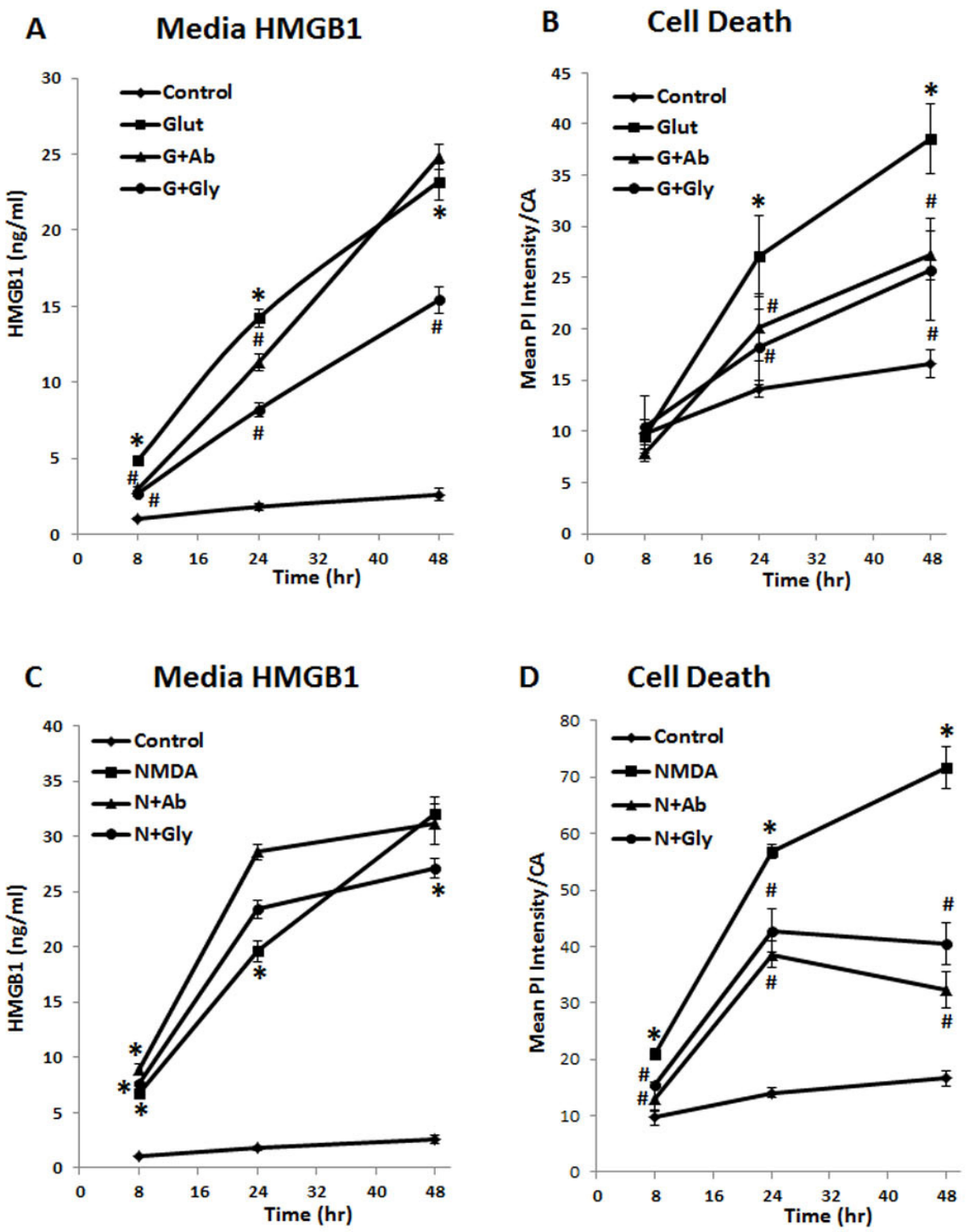

Figure 6. Effects of HMGB1 neutralizing antibody and inhibitors on glutamate/NMDA neurotoxicity. Time-course studies were performed to monitor the effects of antagonizing HMGB1 using neutralizing antibody (Ab) or inhibitor glycyrrhizin (Gly) on HMGB1 release and neuronal cell death in response to glutamate/NMDA stimulation. Glutamate-induced HMGB1 release and neuronal death were shown in A-B and NMDA in C-D. Glutamate triggered robust HMGB1 release proceeding to neuronal cell death as indicated by 4 fold increase of media HMGB1 without increase in neuronal death. Massive increase of media HMGB1 at 24 and 48 hrs

time points was correlated with significant neuronal death. The presence of HMGB1 neutralizing antibodies or inhibitor glycyrrhizin attenuated glutamate-induced neuronal death accompanying with reduction of media HMGB1 at 8 and 24 hrs time points. Similar results were found in response to NMDA treatment (C–D) except that both Ab and Gly had no effect on media HMGB1 (*$p < 0.001$ compared to control; #$p < 0.01$ compared to corresponding glutamate or NMDA, $n = 8–10$).

3.5. Neuroimmune agonists LPS and TNFα stimulate release of HMGB1 proceeding neuronal death

To facilitate our understanding whether HMGB1 contributes to neuronal cell death caused by neuroimmune action, we investigated HMGB1 release and neuronal cell death induced by endotoxin LPS, the prototype TLR4 agonist, and the proinflammatory cytokine TNFα that we have previously found to increase sensitivity to glutamate neurotoxicity [31]. We first performed dose-response curve experiments, in which HEC slices were treated with different concentrations of LPS (25, 50 and 100 ng/mL) or TNFα (25, 50 and 100 ng/mL) for 48 hrs and media HMGB1 determined. The results indicate that media HMGB1 was increased by around 13, 14 and 16 fold with LPS and by 7, 10 and 11 fold with TNFα at concentrations tested respectively (data not shown). Time course determinations with LPS (50 ng/mL) and TNFα (50 ng/mL) find rapid increases in media HMGB1, with a 6 and 4 fold increase by LPS and TNFα respectively after treatment for 2 hrs (not shown). At the 8 and 24 hrs time points, media HMGB1 levels were 8 and 12 fold increases with LPS and 6 and 9 fold increases with TNFα relative to corresponding control level at each time point (Figure 7). All of these increases in HMGB1 release occur without a significant change in neuronal cell death relative to controls (Figure 7). However, after 48 hrs a significant increase in neuronal cell death is associated with further increases in media HMGB1 levels (Figure 7). The presence of HMGB1 neutralizing antibodies or inhibitor glycyrrhizin did not prevent HMGB1 release in response to LPS and TNFα, but it did reduce neuronal cell death induced by LPS and TNFα at the 48 hrs time point (data not shown). Co-treatment of LPS and TNFα with minocycline, an inhibitor of microglial cell activation, reduced both the release of HMGB1 and neuronal cell death (Figure 8), suggesting that microglia contribute to HMGB1 signaling and delayed neuronal cell death. Taken together, these results suggest that active release of HMGB1 precedes neuronal cell death in response to LPS and TNFα that contributes to neurotoxicity.

It has been demonstrated that HMGB1-TLR4 signaling sensitizes glutamate-NMDA receptors through NR2B containing NMDA receptors [22,32]. We further confirmed that blockade of all NMDA receptors with MK801, blocked both LPS and TNFα induced neuronal death, but not HMGB1 release into the media (Figure 9). The subunits of NMDA receptors contribute to altered glutamate sensitivity as well as neuronal localization. The NR2B subunit is particularly associated with neurotoxicity perhaps due to an extra-synaptic localization on neurons. We tested the hypothesis that NR2B may be involved in LPS and TNFα induced neurotoxicity using ifenprodil, a selective blocker of NR2B-containing NMDA receptors [33]. Ifenprodil blocked neuronal cell death caused by LPS and TNFα, but appears not to effect media HMGB1 release (Figure 9). These findings indicate that neuroimmune agonists stimulate HMGB1 release independent of glutamate-NMDA NR2B receptor induced neurotoxicity. Further, they indicate neuroimmune agonists increase glutamate/NMDA receptor stimulation, likely through glutamate release, contributing to neuroimmune agonist neurotoxicity.

To further understand the role of NR2B, we performed Western blot analysis. Treatments with HMGB1 and LPS increased protein level of phosphorylated NR2B (Figure 9C). LPS- and TNFα-induced expression of NR2B phosphorylation was reduced by HMGB1 antagonist glycyrrhizin (Figure 9C). Taken together, these results indicate that HMGB1 modulation of NR2B is involved in delayed neuronal cell death induced by LPS and TNFα**.**

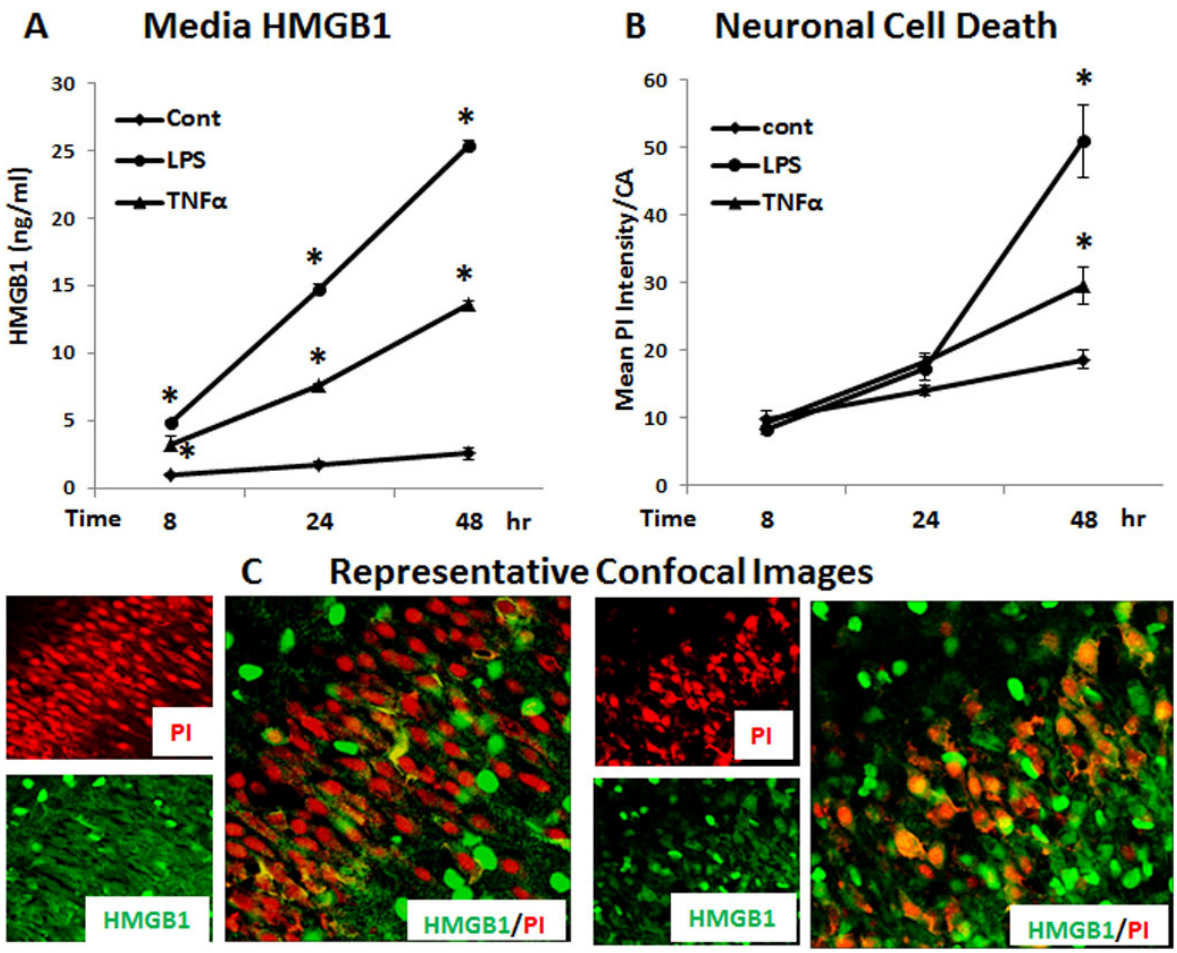

Figure 7: LPS and TNFα-induced release of neuronal HMGB1 and neuronal death. A: Shown are mean ± SEM of media HMGB1 levels measured at 8, 24 and 48 hrs time points in response to LPS (50 ng/mL) and TNFα (50 ng/mL). (*$p < 0.001$ compared with Control, $n = 3$). B: Neuronal cell death was monitored at the same time points and significant increase in PI uptake was only found at 48 hrs time point. (*$p < 0.0001$ compared to control; $n = 8–10$). C: Representative confocal images showed dying neurons labeled by PI (red) with depletion of nuclear HMGB1 (green) in slices treated with LPS or TNFα (magnification 80×).

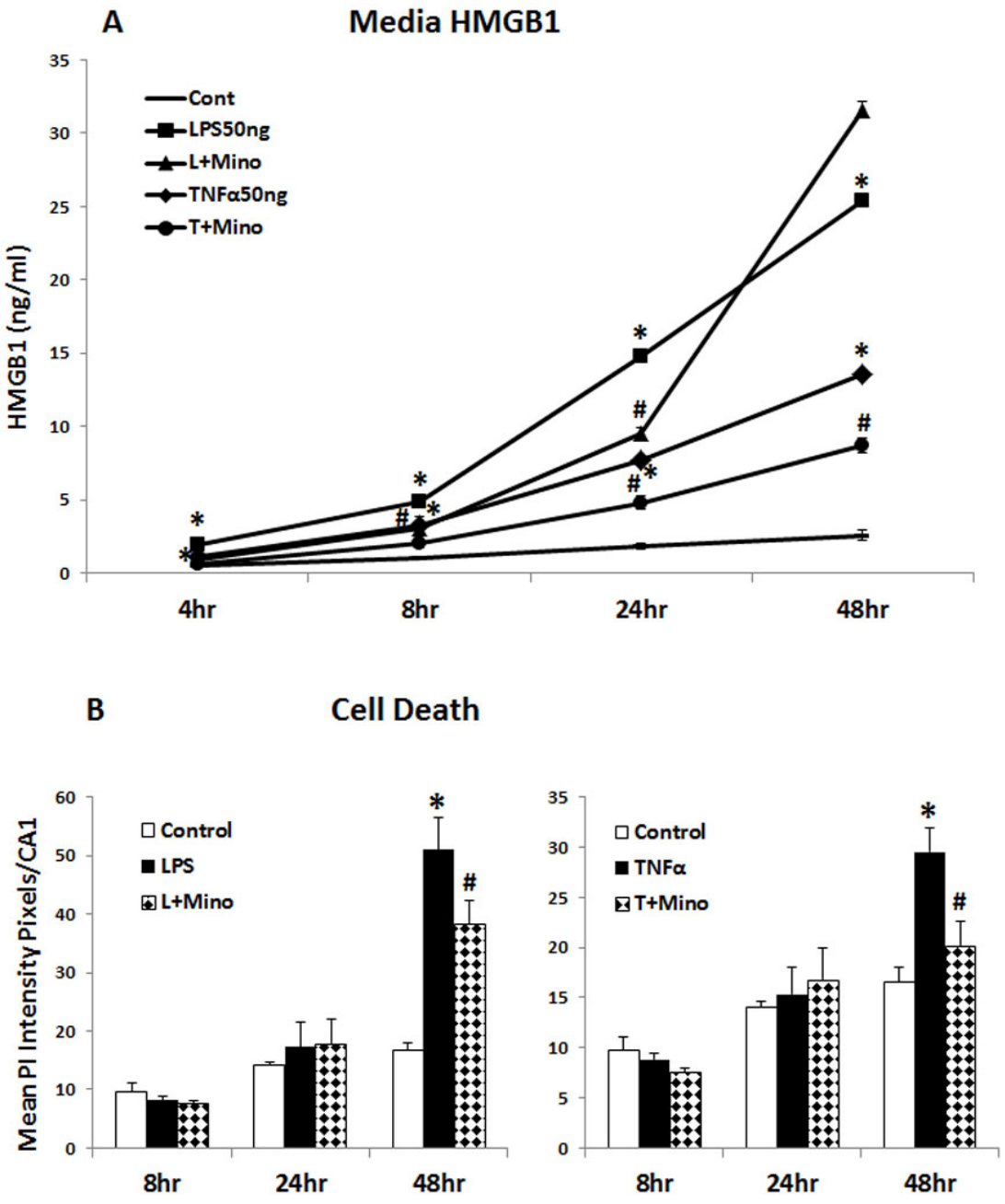

Figure 8. Inhibitory effects of minocycline on LPS and TNFα induced HMGB1 release and neuronal death. A: Shown are time course study of HMGB1 release in response to LPS (50 ng/mL) and TNFα (50 ng/mL). Increase of media HMGB1 level was reduced by minocycline at time points of 4, 8 and 24 hrs for LPS and 4, 8, 24 and 48 hrs TNFα (*$p < 0.01$ compared to control; #$p < 0.05$ compared to corresponding groups of LPS or TNFα; $n = 3$). B: Bar graphs represent neuronal cell death index of PI fluorescent intensity from treatments of LPS (left) and TNFα (right). Delayed neuronal cell death induced by LPS or TNFα was significant at 48 hrs time point and the presence of minocycline reduced neuronal cell death (*$p < 0.001$ compared to control; #$p < 0.01$ compared to LPS or TNFα alone; $n = 8–10$).

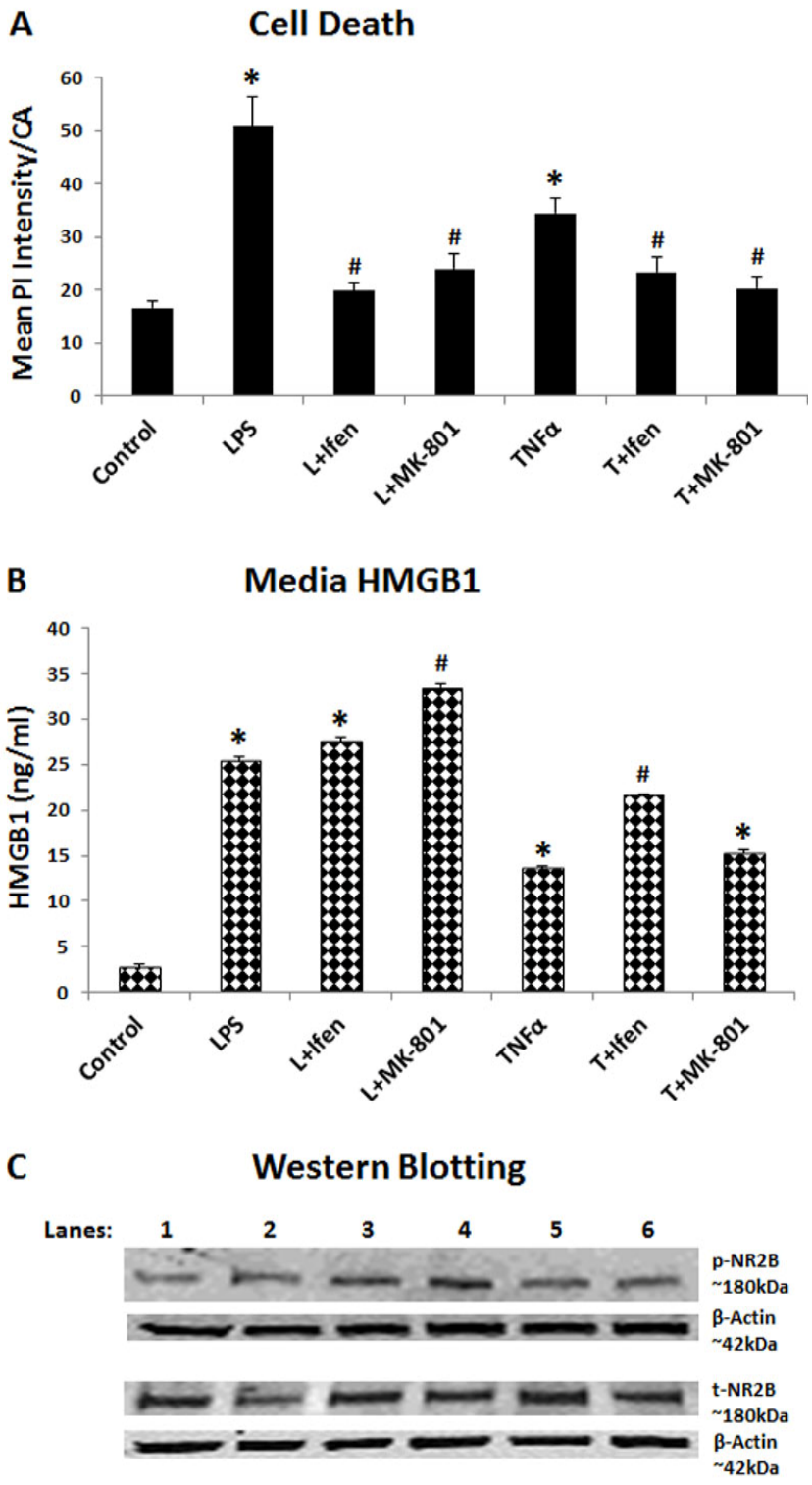

Figure 9. Ifenprodil blocks neuronal cell death induced by LPS and TNFα. A: Shown are mean ± SEM of PI intensity measured from CA1 after 48 hrs treatment with LPS and TNFα. Both ifenprodil and MK-801 blocked neuronal death induced by LPS and TNFα.treatment (*p < 0.001 compared to control; #p < 0.01compared to corresponding LPS or TNFα; n = 8–10). B: Shown are mean ± SEM of media HMGB1 levels corresponding to each group in A. Both ifenprodil and MK-801 failed to reduce

media HMGB1 levels induced by LPS or TNFα as shown (*p < 0.001 compared to control; #p < 0.01compared to corresponding LPS or TNFα; n = 3). C: Western blotting of total and phospho-NR2B from groups indicated in lanes: 1-control; 2-HMGB1 (500 ng/mL); 3-LPS (50 ng/mL); 4-TNFα (50 ng/mL); 5-LPS+Gly 100 μM and 6-TNFα+ Gly 100 μM. Increase expression of phospho-NR2B by LPS or TNFα was reduced by HMGB1 inhibitor glycyrrhizin.

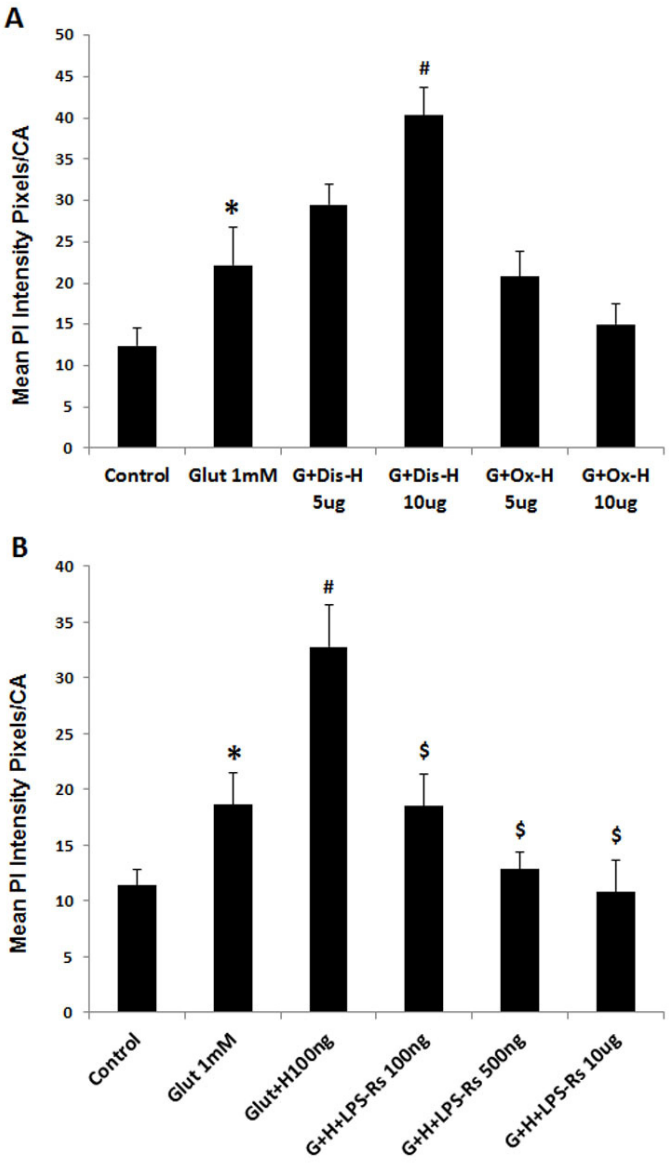

Figure 10. Disulfide-HMGB1 and TLR4 antagonist. A: Shown are mean ± SEM of PI intensity measured from CA1 field indicating disulfide-HMGB1 (Dis-H), not oxidizable HMGB1 (Ox-H) has capability of potentiating glutamate neurotoxicity (*p < 0.001 compared to control; #p < 0.01compared to glutamate; n = 8–10). B: Potentiation of glutamate-induced neuronal death by exogenous HMGB1 is blocked by TLR4 antagonist Lps-Rs, suggesting HMGB1 activation of TLR4 signaling is involved (*p < 0.001 compared to control; #p < 0.01compared to glutamate; \$$p$ < 0.001 compared to corresponding Glut+H100ng; n = 8–10).

3.6. TLR4 receptors mediate HMGB1 potentiation of glutamate neurotoxicity

It has been found that the redox state of HMGB1 alters signaling with only disulfide-HMGB1 being an agonist at TLR4 [32]. To investigate the form of HMGB1 involved in excitotoxicity we used chemically modified HMGB1; non-oxidizable HMGB1 (Ox-HMGB1) with a triple cysteine-to-serine replacement (3S-HMGB1) which is a RAGE agonist, but not a TLR4 agonist, and the disulfide-containing form of HMGB1 (Dis-HMGB1), which is a TLR4 agonist with cytokine activity. HEC slices were treated with glutamate alone or in combination with Ox-HMGB1 or Dis-HMGB1 and the results indicate that glutamate (1 mM) neurotoxicity is potentiated by Dis-HMGB1, with a 32% and 82% increase at concentrations of 5 and 10 ug/mL, respectively (Figure 10A). Non-oxidizable HMGB1 at 5 μM has no effect on glutamate-induced neurotoxicity, but at 10 μM results in a 32% decrease in glutamate neurotoxicity (Figure 10A). We evaluated the effects of TLR4 antagonist Lps-Rs on HMGB1 potentiation of glutamate-induced neurotoxicity. We co-treated HEC slices with TLR4 antagonist Lps-Rs (pretreatment for 2 hrs) during HMGB1-glutamate treatment. HMGB1 (100 ng/mL) potentiated glutamate (1 mM)-induced neuronal death by 76% and TLR4 antagonist Lps-Rs blocked HMGB1 potentiation of glutamate neurotoxicity (Figure 10B). Together, these results suggest that activation of TLR4 receptors plays a prominent role in mediating HMGB1 potentiation of glutamate neurotoxicity.

4. Discussion

We report here that glutamate/NMDA excitotoxicity and neuroimmune toxicity share common elements that contribute to neurodegeneration. Glutamate and NMDA concentration response curves and time courses find release of HMGB1 at lower concentrations and earlier time points than those inducing neuronal death. For example, glutamate/NMDA treatment for 4 hrs finds media HMGB1 increased 6–7 fold with no apparent neuronal cell death. Further, treatment of slices for 48 hrs with concentrations of glutamate < 1 mM and NMDA < 1 μM increase HMGB1 release into the culture media many fold without causing measurable neuronal death. Neuronal death was associated with massive release of HMGB1 at excitotoxic concentrations of glutamate/NMDA. Further, we found glutamate/NMDA stimulation increased media acetyl-HMGB1, consistent with active neuronal release of HMGB1 [11]. Our data strongly suggest that glutamate stimulated HMGB1 release precedes neuronal death. These findings are consistent with glutamate stimulated HMGB1 release contributing to delayed neuronal cell death induced by glutamate/NMDA excitotoxicity. Other studies find glutamate stimulation of neuronal firing releases HMGB1 [19] and increases NMDA stimulated neuronal calcium influx [29], the key signal mediating excitotoxicity [35]. Further, previous studies in primary neuronal cultures have found HMGB1 can mediate neuronal cell death through apoptosis [34] and potentiate NMDA-mediated necrotic cell death [32]. We found HMGB1 neutralizing antibodies and the inhibitor glycyrrhizin as well as blocking TLR4 activation all reduce glutamate/NMDA induced cell death. These findings support an important role for neuronal excitation releasing and increasing extracellular HMGB1 that potentiates excitotoxic neuronal death.

Neuroimmune gene expression is associated with most neurodegenerative diseases [35]. We report here that LPS and TNFα stimulation of neuronal death is reduced by ifenprodil and MK801, glutamate/NMDA receptor antagonists, consistent with glutamate release from neurons in response to LPS-TLR4 receptor activation contributing to the neuronal death. Release of HMGB1 leads to

activation of TLR4 receptors and subsequently triggers more release of HMGB1. We found HMGB1 release increased phosphorylation of NR2B, an NMDA receptor subunit, that is associated with increased NMDA receptor signaling and increased NMDA mediated calcium flux [29]. The finding that blocking glutamate/NMDA signaling reduces LPS-TLR4 and TNFα toxicity indicates both processes contribute to neuronal death triggered through the respective signaling mechanisms. HMGB1-TLR4 signaling increases excitability through phosphorylation of glutamate/NMDA receptor NR2B, that increases excitability and risks of seizures [19, 29] and glutamate release increasing excitotoxicity. These studies are consistent with HMGB1 and neuroimmune signaling increasing glutamate release and sensitivity to glutamate, with both signaling processes contributing to neuronal cell death.

Consistent with several previous studies [11,22,36], our results indicate that HMGB1 is predominantly released from neuronal cells in response to glutamate/NMDA and neuroimmune activation, though microglia and/or astrocytes may also be involved. Rapid release of HMGB1 from neuronal cells has been reported within 1 hr after the onset of middle cerebral artery occlusion in animal models of brain ischemia [37,38] and media HMGB1 increased 3 hrs following NMDA stimulation in the primary cultures of neuronal cells [34]. Consistent with these reports, the present study finds that the direct stimulation of glutamate receptors with glutamate, NMDA or mGluR agonists triggers rapid nucleocytoplasmic translocation and subsequent release of neuronal HMGB1, with 3 fold increase of media HMGB1 at 30min after applying neurotoxic concentration of NMDA (Figure 3). In general, at 4 hrs treatments with sub toxic concentrations of glutamate/NMDA, media HMGB1 has increased 6–7 fold while neuronal cell death has not been evident, whereas massive release of HMGB1 occurs with neuronal cell death. Our data strongly suggest that nuclear HMGB1 release proceeds neuronal death. Excitotoxicity is a leading cause of neuronal cell death in acute or chronic neurological conditions. Under pathological conditions, excessive activation of glutamate receptors causes Ca^{2+} influx and subsequent excitotoxicity [39]. Putative blockade of glutamate receptors in order to prevent neuronal cell death in neurodegenerative disorders has been disappointing in clinical trials due in part to the complex nature of excitotoxicity-induced neuronal death. The danger molecule-like actions of extracellular HMGB1 contributes stimulating glutamate release, which in turn increases neuronal toxicity [19]. In primary culture of neuronal cells, HMGB1 directly potentiates NMDA-stimulated calcium influx [32]. Animal models find that extracellular HMGB1 can worsen ischemic neurodegeneration [18]. Direct injection of HMGB1 into the hippocampus prior to injection of kainic acid significantly enhances the severity of seizures [22]. On the other hand, many studies have shown that targeting HMGB1 offers effective results in reducing neuronal death under neuropathological conditions. In animal models of ischemia, antagonizing HMGB1 by using HMGB1 neutralizing antibodies, antagonists or siRNA confer significant neuroprotection [23,40,41]. The present study further supports the notion that extracellular HMGB1 contributes to delayed neuronal death induced by excitotoxicity. Release of HMGB1 precedes neuronal cell death in response to glutamate/NMDA stimulation. Enhanced neuronal death can be effectively blocked by application of HMGB1 neutralizing antibodies and inhibitor glycyrrhizin. Our data in concert with others strongly suggest that HMGB1 may be mediating components of neuronal death. It is conceivable that underlying initial insults to brain such as inflammation, trauma and ischemia can cause release of glutamate and the latter in turn triggers mobilization of nuclear HMGB1 and subsequent release. To our knowledge, the present study is the first to show that blockade of glutamate receptor activation with antagonists including MK-801 and NBQX as well as

mGluR antagonists prevent HMGB1 release into culture medium and reduce neuronal death. Confocal analysis indicates that MK-801 maintains nuclear localization of HMGB1 blocks HMGB1 release and neuronal cell death induced by glutamate/NMDA. These results suggest the possibility that stabilizing nuclear HMGB1 during brain insults may protect neuronal cells from excitotoxicity.

HMGB1-TLR4 signaling has been implicated in mediating neuroimmune responses under many neuropathological conditions inducing brain ischemia [42,43], post-traumatic brain edema [44] and ethanol-induced neuroinflammation [11]. Recent studies indicate that activation of TLR4 by HMGB1 is related to HMGB1 redox state. TLR4-mediated NMDA-induced Ca^{2+} influx and excitotoxicity are potentiated only by the disulfide-containing form of HMGB1, but not by non-oxidizable HMGB1 [32]. The present study further supports that HMGB1-TLR4 signaling contributes to glutamate/NMDA excitotoxicity, which is blocked by selective TLR4 antagonist Lps-Rs. We did not try to distinguish necrotic or apoptotic cell death, excitotoxicity often involves necrosis or a broader spectrum, e.g. necroptosis, consistent with release of TLR4 active HMGB1 contributing to glutamate necrotic neuronal death [16, 29], however, actively released HMGB1 has been reported to induce neuronal apoptosis through RAGE activation [31]. RAGE activation by HMGB1 can occur with a partially oxidized HMGB1that does not have TLR4 activity [17]. We found that disulfide- HMGB1, the TLR4 agonist form of HMGB1, but not oxidized HMGB1 that is inactive at TLR4 and RAGE receptors, potentiates glutamate/NMDA-induced neuronal cell death. Studies have found that HMGB1 contributes to neuronal death in models of ischemic-stroke [33,34,38], hemorrhagic stroke [37] and traumatic brain injury [40]. We found that Lps-Rs, the TLR4 antagonist, blocks glutamate/HMGB1 toxicity consistent with activation of TLR4 receptors contributing to excitotoxicity. Although we cannot rule out a contribution of HMGB1-RAGE signaling, these results suggest blocking HMGB1-TLR4 signaling increases excessive glutamate/NMDA receptor activation and that blocking HMGB1/TLR4 signaling could protect against neurodegeneration.

We further extend the study into the role of HMGB1 in neuroimmune signaling induced neurodegeneration. These studies indicate that neuroimmune signaling is a component of glutamate/NMDA mediated excitotoxic neuronal death. Treatments of endotoxin LPS and cytokine TNFα induce delayed (after 48 hrs) neuronal cell death in HEC slices, which is enhanced by HMGB1. Time courses indicate that media HMGB1 is rapidly elevated in response to LPS and TNFα stimulation. At the 8 hrs time point, media HMGB1 levels were 10 and 7 fold increase with LPS and TNFα stimulation respectively while index of neuronal cell death is not increased (Figure 9). Media HMGB1 levels after 48 hrs correlated with increased neuronal death (Figure 9). Acetyl-HMGB1 release is increased indicating at least a portion of the HMGB1 release appears to be active release from excited neurons (These results suggest that active release of HMGB1 proceeds neuronal cell death induced by LPS and TNFα. HMGB1 likely increases NR2B-containing NMDA receptors increasing excitability [45] by enhancing NMDA function [32]. NMDA antagonists MK-801 and selective NR2B antagonist ifenprodil significantly reduced neuronal cell death induced by LPS and TNFα, suggesting glutamate increased activation of NR2B may be the molecular target modulated by HMGB1 to potentiate LPS and TNFα neurotoxicity through increased glutamate excitation. Interestingly the presence of ifenprodil has no inhibitory effects on media HMGB1 release while neuronal death is abrogated. This may be interpreted as the release of neuronal HMGB1 persists throughout treatment with LPS and TNFα involving distinct mechanisms independent of neuronal excitation and glutamate stimulation. These findings suggest HMGB1-TLR4 signaling and glutamate-NMDA signaling share common pathways to neuronal death.

5. Conclusions

In conclusion, the present study identifies danger signal HMGB1 as a molecule mediating delayed neuronal cell death induced by glutamate/NMDA as well as by LPS and TNFα. HMGB1 activation of TLR4 or modulation of NR2B represents molecular targets involving in HMGB1-mediated neuronal cell death. Targeting HMGB1 and its molecular targets may offer effective approaches to prevent neuronal death under neurological conditions.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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