Pharm Res (2013) 30:2584–2595
DOI 10.1007/s11095-013-1049-9

RESEARCH PAPER

Knocking Down HMGB1 Using Dendrimer-Delivered siRNA Unveils Its Key Role in NMDA-Induced Autophagy in Rat Cortical Neurons

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Received: 20 September 2012 / Accepted: 2 April 2013 / Published online: 19 April 2013

ABSTRACT

Purpose To explore the role of the High Mobility Group Box 1 (HMGB1) protein in NMDA-mediated excitotoxicity in rat cortical neurons.

Methods We knocked down HMGB1 using small-interfering RNA (siRNA) delivered into neurons by means of a dendrimer. We determined autophagy activation by measuring the ratio of light chain 3 protein isoforms (LC3B-I)/LC3B-II and by determining autophagolysosome labeling using the specific marker monodansyl cadaverine. Neuronal toxicity was induced by exposing the neurons to N-methyl-D-aspartate (NMDA) and it was determined by measuring Lactate dehydrogenase and MTT reduction.

Results We found that NMDA receptor stimulation induced both neuronal death and autophagy in rat cortical neurons. In addition, NMDA also caused HMGB1 translocation from the neuronal nucleus to the cytoplasm where it formed a complex with Beclin1. HMGB1 was efficiently knocked down using a specific siRNA causing a blockade of NMDA-induced autophagy and potentiating NMDA-induced neuronal death.

Conclusions Our study demonstrates that HMGB1 plays a relevant role in neuronal autophagy regulation and suggest a protective role of autophagy during excitotoxicity. In addition, the dendrimer that we have used here is a good vector for siRNA delivery to neurons allowing lack-of-function studies.

Electronic supplementary material The online version of this article (doi:10.1007/s11095-013-1049-9) contains supplementary material, which is available to authorized users.

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KEY WORDS autophagy · dendrimer · excitotoxicity · neurons · siRNA

ABBREVIATIONS

DTT	Dithiothreitol
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2- ethanesulphonic acid
HMGB1	High Mobility Group Box 1
HRP	Horseradish peroxidase
LC3	Microtubule-associated light chain 3
LDH	Lactate dehydrogenase
MDC	Monodansylcadaverine
MTT	2,5-diphenyl-3-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide
NMDA	N-methyl-D-aspartate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
siRNA	Small-interfering RNA
TGD	Transgeden dendrimer

INTRODUCTION

Autophagy is a widely distributed process by which neurons degrade cellular macromolecules and recycle cytosolic organelles by sequestering cytoplasmic organelles within cytoplasmic vesicles called autophagosomes, which later form autophagolysosomes (1). However, autophagy is also involved in other cellular functions, such as differentiation, cell proliferation and excitotoxicity (2–4). Autophagy is very important in neurons, because they do not divide and cannot redistribute damaged organelles (5).

Excitotoxicity is initiated by a large Ca^{2+} influx. Calcium overload initiates a series of cytosolic events that include reactive oxygen species generation (6) and mitochondrial

depolarization (7) leading to the release of proapototic proteins that trigger neuronal death (8). Interest in autophagy has increased because it is activated in neurons during hypoxia (9) or excitotoxicity (4,10).

High-mobility group box-1 (HMGB1) is a multifunctional molecule, widely expressed in various tissues including the brain. In neurons, HMGB1 can up-regulate synaptic proteins, mediate neurite outgrowth and regulate cell survival (11,12). In addition, it has been proposed that HMGB1 might act as an extracellular signaling molecule that plays important roles in inflammation, cell differentiation, cell migration and survival (13–15). On the other hand, HMGB1 is secreted by inflammatory cells after pro-inflammatory stimuli (16) or oxidative stress, such as that produced by H_2O_2 (17). In these situations, HMGB1 might act as a pro-inflammatory cytokine mediating different physiological and pathological responses through membrane receptors (18,19). Thus, it has been proposed that HMGB1 might regulate the neuroimmune system and the progression of certain neurologic diseases, mainly those involving changes in oxidative stress (20). However the role of HMGB1 in neuronal autophagy induced by excitotoxicity remains unclear.

Small interfering RNA (siRNA) is a gene-silencing mechanism through specific mRNA degradation (21). The specificity of siRNA has converted it into a powerful tool for specific knockdown of selected proteins of interest for therapeutic applications (22). However, one of the main problems with the use of siRNA in neurons is the low transfection efficiency achieved using various non-viral vectors for siRNA delivery to neurons (23). Recently, we described a dendrimeric vector known as Transgeden (TGD) that showed high transfection efficiency to deliver siRNA into cerebellar granular neurons (24) and cortical neurons (4,25).

The aim of this work was to explore the role of the HMGB1 protein in NMDA-mediated excitotoxicity in rat cortical neurons. To achieve this goal, we knocked down HMGB1 using siRNA delivered into neurons by means of the non-viral dendrimeric vector TGD, and we studied the effect of this protein knockdown on autophagy activation and excitotoxic death. We found that NMDA receptor stimulation induces HMGB1 translocation to the neuronal cytoplasm. In addition, HMGB1 knock down decreases NMDA-mediated autophagy induction and potentiates NMDA-induced neuronal death, suggesting that autophagy plays a neuroprotective role during excitotoxicity.

MATERIALS AND METHODS

Cell Culture

Rat cortical neuronal culture was prepared as described elsewhere (26). Briefly, the cortical lobes were dissected from Sprague–Dawley fetuses (embryonic day 17) and mechanically dissociated in Hanks' balanced solution. Following isolation, cells were cultured in serum-free Neurobasal medium supplemented with B27, containing 2 mM L-glutamine, penicillin (20 units/mL) and streptomycin (5 μg/mL). Afterwards, isolated cells were plated on poly-L-Lysine-coated culture plates or on poly-L-Lysine-coated glass coverslips and maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO_2 and were used for experiments after 7–11 days *in vitro* (DIV). Experimental procedures were carried out according to the guidelines of the European Community on Welfare of Research Animals (directive 2003/65/CE) and the Castilla–La Mancha University Animal Research Ethics Committee.

Cytotoxicity Studies

Lactate dehydrogenase (LDH) assays were performed as previously reported (27). Neuronal culture supernatants were collected at the times indicated. Neurons attached to the culture plate were washed with phosphate-buffered saline (PBS) and lysed using 0.9% Triton X-100 in (v/v) in saline. Both LDH released to the culture media by neurons as well as LDH content in the cells were measured spectrophotometrically at 490 nm on a 96-well plate reader using the CytoTox 96® Kit (Promega, Madison, USA) following the manufacturer's instructions. Toxicity was expressed as the percentage of LDH released, which is defined as the ratio LDH released/total LDH present in the cells.

MTT assays were performed as previously described (26). After treatments, 2,5-diphenyl-3-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT, Sigma) at 0.2 mg/mL was added to the medium and the neurons were incubated at 37°C for 30 min. Next, the culture medium was removed and the insoluble formazan crystals were dissolved in 300 μL DMSO (Sigma, Barcelona, Spain). The plate was incubated for 10 min under agitation and 200 μL from each well were then transferred to a 96-well microplate. The absorbance of each well was measured by spectrophotometry at reference wavelengths of 570 and 630 nm on a 96-well plate reader (Infinite 2000, Tecan).

Extraction of Total Cellular Lysates

Cortical neurons were cultured in poly-L-lysine-coated 6-well plates for 7 to 11 days and treated with either vehicle or NMDA (150 μM) alone or in the presence of the indicated treatments. The cells were then washed twice with cold PBS and resuspended in a buffer containing:

Tris (25 mM), NaCl (25 mM), deoxycholic acid, Triton X-100 (1%) (v/v), phenylmethylsulfonyl fluoride (0.5 mM), DTT (1 mM), leupeptine (1 μg/mL) and aprotinine (0.1 μg/mL), pH 7.4. Then, the homogenates were centrifuged at 13,200 ×*g* for 15 min and supernatants removed and stored at −80°C until analyzed by gel electrophoresis.

Protein Precipitation

Cortical neurons were seeded on poly-L-lysine-coated 6-well plates and treated with vehicle or NMDA (150 μM) for various time periods. Next, proteins in the culture medium were concentrated using 2,2,2-trichloroacetic acid (TCA), as described elsewhere (28). The culture media were treated with TCA to achieve a final TCA concentration of 10%. Then, the samples were incubated at 4°C for 1 h. The precipitated proteins were centrifuged at 13,200 ×*g* for 20 min. The precipitated products were washed with cold acetone, incubated at 4°C for 30 min and then centrifuged at 13,200 ×*g* for 10 min. Samples were then resuspended in a protein buffer containing: Tris (25 mM), NaCl (25 mM), PMSF (0.5 mM), DTT (1 mM), leupeptine (1 μg/mL) and aprotinine (0.1 μg/mL), pH 7.4 and stored at −80°C until they were analyzed by gel electrophoresis.

Subcellular Fractionation

Cortical neurons were cultured in poly-L-lysine-coated 6-well plates and treated with vehicle or NMDA (150 μM) for different times. Then the medium was removed and the cells were subsequently washed twice with cold PBS, detached from the plate and collected by centrifugation at 1,500 ×*g* for 5 min. Cell pellets were resuspended in an extraction buffer containing HEPES (10 mM), EDTA (1 mM), EGTA (1 mM), KCl (10 mM), phenylmethylsulfonyl fluoride (0.5 mM), DTT (1 mM), leupeptine (1 μg/mL), aprotinine (0.1 μg/mL), NaF (5 mM), Na_3VO_4 (1 mM) and NP-40 (0.1%) (pH 8) and homogenized. The homogenate was incubated at 4°C for 30 min and centrifuged at 13,200 ×*g* for 30 s. The supernatants were considered the cytosolic fraction and the pellets were resuspended in a extraction buffer composed of HEPES (10 mM), EDTA (1 mM), EGTA (1 mM), NaCl (0.4 M) (pH 8), phenylmethylsulfonyl fluoride (0.5 mM), DTT (1 mM), leupeptine (1 μg/mL), aprotinine (0.1 μg/mL), NaF (5 mM) and Na_3VO_4 (1 mM) (pH 8). Pellets were rocked for 30 min at 4°C and then centrifuged at 13.200 ×*g* for 5 min. The supernatants were considered as nuclear fraction. All supernatants were stored at −80°C until analysis by gel electrophoresis.

Beclin I Inmunoprecipitation

Cortical neurons were cultured in poly-L-lysine-coated 6-well plates and treated with vehicle or NMDA (150 μM) for various times, after 7–11 DIV. Next, cells were washed twice with cold PBS and resuspended in a buffer containing Tris (25 mM), NaCl (25 mM), deoxycholic acid, Triton X-100 (1%) (v/v), phenylmethylsulfonyl fluoride (0.5 mM), DTT (1 mM), leupeptine (1 μg/mL) and aprotinine (0.1 μg/mL), pH 7.4. Then, the homogenates were centrifuged at 13.500 xg for 15 min. The supernatants were mixed with anti-Beclin 1 antibody (1 μg) and Protein A-sepharose (50 μL). After rocking overnight at 4°C, samples were centrifuged. The resulting pellets were washed and resuspended in sample buffer containing 1% β-mercaptoethanol, boiled for 5 min at 95°C and then frozen at −80°C until analysis by gel electrophoresis.

Monodansylcadaverine (MDC) Staining

Cortical neurons were plated on poly-L-Lysine-coated glass coverslips and cultured for 7 DIV before being exposed to the appropriate treatments. For labeling with the fluorescent agent MDC, cortical neurons were incubated for 10 min in the presence of 50 μM MDC at 37°C. Cells were observed under a fluorescence microscope (excitation wavelength 380 nm, emission filter 525 nm). The level of autophagy was determined as the percentage of MDC-labeled cells out of 200 cells from each treatment group, as previously described (29).

Western-Blot Analysis

Western blots were performed as described elsewhere (30). Protein content from cytosolic and nuclear extracts, extracellular medium and total cellular lysates was determined using the commercial kit "BCA protein assay" (Pierce, Rockfork, IL, USA). Protein samples (30–40 μg for cytosolic fraction and total cellular lysates and 10–15 μg for nuclear fraction and proteins precipitated from culture medium) were solubilized in sampling buffer containing (Tris–HCl (313 mM, pH 6.8), SDS (10%), Bromophenol Blue (0.05), Glycerol (50%) and 2-mercaptoethanol (5%), heated at 95°C for 5 min and loaded on 15% PAGE-SDS gels. Gels were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked in PBS-Tween 20 (0.1%) containing 5% non-fat dry milk and 0.1% BSA for 1 h at 4°C, and subsequently incubated overnight at 4°C with either a polyclonal anti-HMGB1 antibody (1:1,000) (Cell Signaling Technology, Beverly, MA), a monoclonal anti-LC3B antibody (1:500) (Enzo Life Sciences, Faimingdale, NY, USA), polyclonal anti-Bcl-2 antibody (1:2,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-β-actin antibody (1:4,000) (Sigma Chemical Co., St. Louis,

MO, USA) or polyclonal anti-histone H_2A (1:1,000) (Cell Signaling Technology, Beverly, MA). The blots were then washed with PBS-Tween 20 (0.1%) and incubated with horseradish peroxidase (HRP)-anti-mouse Ig G (1:10,000) (Millipore, Bedford, MA, USA) or horseradish peroxidase (HRP)-anti-rabbit IgG (1:10,000) (Millipore, Bedford, MA, USA) for 2 h at 4°C. Immunocomplexes were visualized using an enhanced chemiluminiscence system (Millipore, Bedford, MA, USA). Densitometric analysis of immunoreactive bands was performed by using Quantity One Software (Bio-Rad Laboratories).

Dendrimer Preparation and siRNA-TGD Complex Formation

The TGD dendrimer synthesis and siRNA-TGD dendriplex formation was performed as described elsewhere (24). Briefly, pre-designed siRNA targeting rat HMGB1 (Rn_Hmgb1_5) and a control scrambled RNA targeting a sequence not sharing homology with the rat genome (AllStars Negative Control) were obtained from Qiagen (Crawley, UK). Dendriplexes containing siRNA or scrambled siRNA solutions were prepared 30 min prior to be added to the cell culture. The molar ratio of siRNA to the TGD dendrimer was 100 nM siRNA to 3 μM TGD dendrimer. Just before transfection, the culture medium was replaced with fresh medium and the siRNA-TGD dendriplex was added to cells that were kept at 37°C in a humidified atmosphere containing 5% CO_2.

Dendriplex Uptake Assays

For transfection assays, cortical neurons were plated on poly-L-Lysine-coated glass coverslips and cultured for 7 DIV. Then, cortical neurons were incubated with 100 nM fluorescein-labeled siRNA (FAM-siRNA) alone or 100 nM FAM-siRNA/3 μM TGD dendriplex for 24, 48, 72 and 96 h. Subsequently, cortical neurons were washed with PBS and examined using a Leica DMRXA microscope (Leica, Wetzlar, Germany) with suitable fluorescence filters (excitation wavelength of 490 nm and emission wavelength of 520 nm). Image capture was performed using a Leica DC500 camera (Leica, Wetzlar, Germany). Transfection efficiency was determined by counting the number of fluorescein-positive cells over the total cell number in 9 randomly selected regions from three independent experiments, as described elsewhere (31).

Neuronal Transfection

To explore the effect of HMGB1 knockdown, cells were transfected with either the dendrimer TGD alone (3 μM), the dendriplex containing TGD (3 μM) plus HMGB1 siRNA (100 nM) or the dendriplex containing TGD (3 μM) plus scramble siRNA (100 nM) for 72 h. Next, the medium was replaced by fresh medium and neurons were treated with NMDA (150 μM) or vehicle for 24 h. In one set of experiments, samples were studied by Western-Blot or real-time RT-PCR analysis and in another set of experiments, LDH activity was determined after the incubation period, as described previously (26).

Real-Time RT-PCR Analysis

Real-time RT-PCR analysis was performed as previously described (32). Briefly, total RNA was extracted using the commercially available reagent Tripure (Sigma Chemical Co., St. Louis, MO, USA) and cDNA was synthesized from the purified total RNA using the kit RevertAid™ First Strand cDNA Synthesis (Fermentas, Themo Fisher Scientific, MA, USA) according to the manufacturer's instructions. For real-time RT-PCR, cDNA was amplified using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies, Foster City, CA) with the StepOne Real-Time PCR System and the StepOne v2.0 software (Applied Biosystems, Life Technologies, Foster City, CA). The primers used to amplify the HMGB1 gene were 5′-CTGAGTACCGCCCAAAAATCA-3′ (forward) and 5′-TCGCAACATCACCAATGGATA-3′ (reverse). To normalize the data, β-actin RNA was used as an internal control using the following primers: 5′-CGGAACCGCTCATTGCC-3′ (forward) and 5′-ACCCACACTGTG CCCATCTA-3′ (reverse). To ensure the reliability of the results obtained, all samples were processed in duplicate. Quantification was performed by the comparative cycle threshold (*Ct*) method (33).

Statistical Analysis

All data are expressed as means ± SEM of at least 3 independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Bonferroni's test for multiple comparisons using the GraphPad software program. p-values less than 0.05 were considered statistically significant. Statistical results are provided in the figure legends.

RESULTS

NMDA Induces Mitochondrial Toxicity and Cell Death in rat Cortical Neurons

First, the characterization of NMDA-induced neuronal death was determined by LDH assay, resulting in a concentration-response curve (Supplementary Material Figure S1a). Treatment of rat cortical neurons with NMDA at 10, 50, 150 and 300 μM for 24 h caused a concentration-dependent

neuronal death. To further explore this issue, the NMDA concentration was established at 150 μM and a time-course from 1 to 72 h was performed to explore toxicity (Supplementary Material Fig. S1b, c). The data indicated that the exposure of cortical neurons to NMDA (150 μM) resulted in concentration- and time-dependent neuronal damage that amounted to approximately 22% of the neurons after 24 h of treatment. These conditions were selected for all experiments involving excitotoxicity.

NMDA Promotes HMGB1 Translocation and Release to Extracellular Medium in rat Cortical Neurons

To investigate the possible role of HMGB1 in NMDA-induced autophagy, we analyzed HMGB1 protein expression and location in cortical neurons exposed to NMDA (150 μM) for various time periods. Western-Blot analysis showed that NMDA caused a slightly reduction in neuronal HMGB1 protein levels after 48 h (Fig. 1a) which was accompanied by HMGB1 release into the extracellular medium. This release was evident after 24 h of NMDA exposure, reaching a value of 54.9±19% of total neuronal HMGB1 protein content following 48 h of treatment (Fig. 1b).

On the other hand, Western-Blot analysis of nuclear and cytosolic fractions showed that in the absence of treatment, the HMGB1 was located mainly in the nucleus of rat cortical neurons (Fig. 1c) with very low levels in the cytoplasm. However, following NMDA exposure, cytosolic levels increased at times as early as 3 h and remained high until 24 h (Fig. 1d) where they declined at the same time as nuclear levels, likely because the protein was secreted into the culture medium (Fig. 1b). These results suggest that NMDA addition induced HMGB1 translocation from the nucleus to the cytosol in rat cortical neurons followed by extracellular release into the culture medium.

Cytosolic HMGB1 May Mediate NMDA-Induced Autophagy in rat Cortical Neurons

In a previous study, we found that NMDA induced autophagy in rat cortical neurons (4). To evaluate the possible relationship between HMGB1 translocation from the nucleus to the cytosol and autophagy activation, we decided to correlate HMGB1 cytosolic expression with the increase of LC3B-II/LC3B-I ratio, which happens under excitotoxic conditions. During autophagy, a cytosolic form of light chain 3 (LC3B-I) is cleaved and then conjugated to phosphatidylethanolamine to form LC3-B-II, which is recruited to the autophagosomal membranes. Analysis of LC3B-I/LC3B-II conversion has become a widely used method for monitoring autophagy and autophagy-related processes (34).

When cortical neurons were exposed to NMDA (150 μM) for various time periods, we found that the LC3B-II/LC3B-I ratio increased in a time-dependent manner, from 0.20±0.05 in untreated neurons, to 1.95±0.05 after 24 h, and up to 2.25 ±0.15 following 48 h of treatment (Fig. 2a), suggesting that autophagy was up-regulated. To further asses NMDA induction of autophagy, we incubated the neurons with the fluorescent agent MDC (Sigma, Barcelona, Spain) which is a specific marker for acidic vesicles such as autophagosomes or autophagolysosomes (35). Exposure of cortical neurons to NMDA (150 μM) increased, in a time-dependent manner, the number of neurons labeled by MDC (Fig. 3). The time-course of HMGB1 translocation from neuronal nucleus to cytosol is very similar to NMDA-induced autophagy (Fig. 2b), suggesting that HMGB1 might participate in excitotoxicity-induced autophagy.

HMGB1 Is Associated with Beclin 1 When Autophagy is Activated by NMDA

To elucidate whether HMGB1 participates in NMDA-mediated autophagy, we explored the possible interaction between HMGB1 and the autophagic protein Beclin 1. Total cellular lysates obtained from cortical neurons treated with vehicle or NMDA (150 μM) were used to immunoprecipitate the Beclin 1 protein. The results showed that, as we previously reported (4), Beclin 1 protein levels were increased in response to NMDA. However, we found that Beclin 1 co-precipitated with HMGB1 in a time-dependent manner. This association was specific because when a non-specific IgG was used to immunoprecipitate the neuronal cytosol, neither Beclin 1 or HMGB1 were found in the precipitate (data not shown). The association between Beclin 1 and HMGB1 was evident 1 h after NMDA addition, reaching maximum levels after 24 h of NMDA treatment (Fig. 4). However, this association disappeared after 48 h and the levels of both proteins returned to almost basal levels. The fact that the time-course of this interaction was similar to HMGB1 cytosolic translocation and autophagy activation mediated by NMDA suggested that HMGB1, once localized in the neuronal cytosol, may be involved in activation of the autophagic pathway.

TGD Dendrimer Lacks Toxicity in rat Cortical Neurons

To explore TGD dendrimer toxicity on rat cortical neurons, cells were exposed to TGD dendrimer alone at concentrations ranging from 1 to 8 μM for 72 h. Neuronal toxicity was determined using MTT and LDH assays. These results showed no toxic effects for up to 6 μM TGD concentration (Fig. 5a and b). In addition, neurons were also transfected with a dendriplex containing 3 μM TGD and 100 nM scramble siRNA for different times. As it can be observed in Fig. 5c and

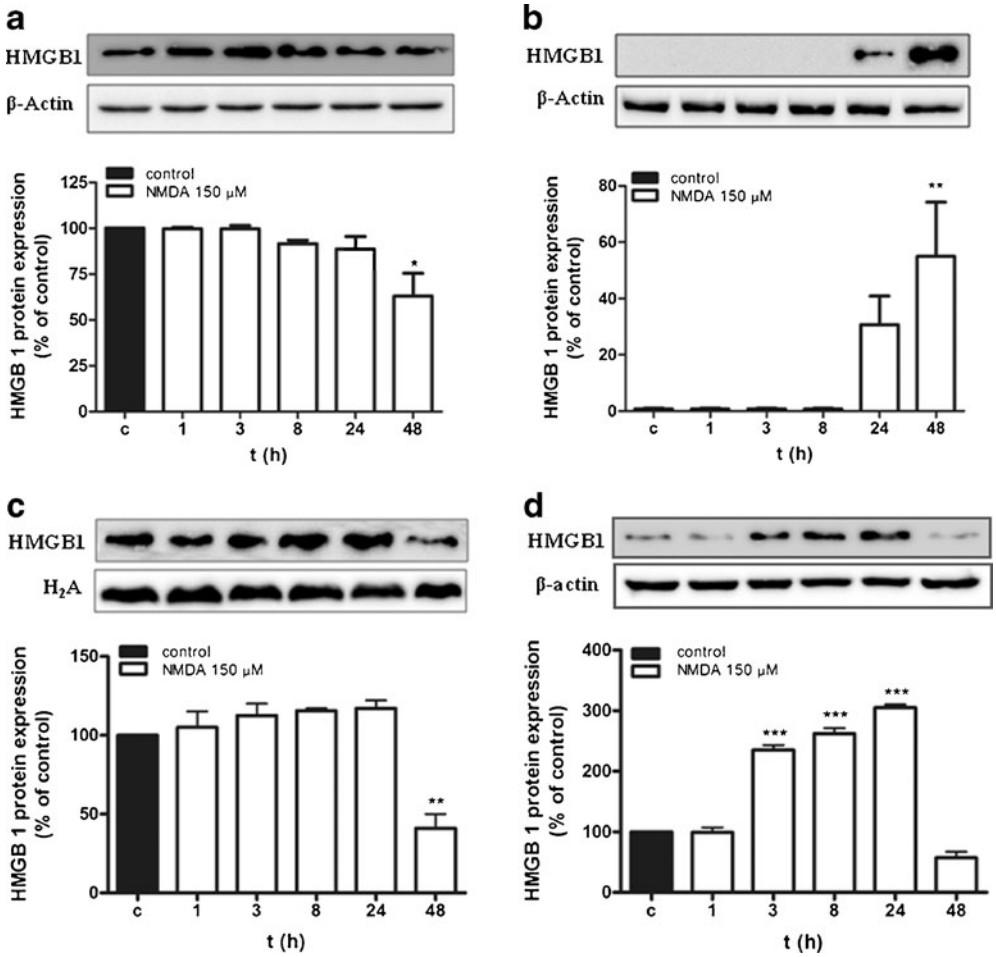

Fig. 1 NMDA promotes HMGB1 translocation from nucleus to cytosol and extracellular release in rat cortical neurons. Cells were incubated with vehicle or NMDA (150 μM) for different times and total cellular lysates, protein precipitation of culture medium and nuclear and cytosolic fraction were obtained. (**a**) Levels of HMGB1 and β-actin proteins in total cellular lysates. Graph represent densitometric analysis of HMGB1 levels related to β-actin protein levels. Data are expressed as mean ± SEM ($n = 3$). *$p < 0.05$ as compared with control untreated neurons. (**b**) Levels of HMGB1 protein released into the culture medium. Graph represent densitometric analysis of HMGB1 levels related to β-actin protein levels in the cultured neurons. Data are expressed as mean ± SEM ($n = 3$). **$p < 0.01$ as compared with control untreated neurons. (**c**) Levels of HMGB1 and histone H_2A in nuclear fraction. Graph represent densitometric analysis of HMGB1 levels related to hystone H_2A protein levels. Data are expressed as mean ± SEM ($n = 3$). **$p < 0.01$ as compared with control untreated neurons. (**d**) Levels of HMGB1 and β-actin in the cytosolic fraction. The graph represents densitometric analysis of HMGB1 levels related to β-actin protein expression. Data are expressed as mean ± SEM ($n = 3$). ***$p < 0.001$ as compared with control untreated neurons.

5d, no toxic effect was observed as measured by MTT and LDH assays. These results indicate that neither the TGD dendrimer alone or the TGD/siRNA dendriplex were toxic at the doses and the incubation periods used to perform loss-of-function studies in cultured neurons.

Transfection Efficiency in Rat Cortical Neurons

To optimize the transfection efficiency, neurons were incubated with FAM-siRNA alone (100 nM) or with dendriplex containing TGD (3 μM)/FAM-siRNA (100 nM) for different times. We found that neurons exposed to naked FAM-siRNA alone showed very few fluorescein-positive cells (data not shown). However, incubations of neurons with dendriplex TGD/FAM-siRNA revealed an increase in the number of fluorescein-positive neurons with time reaching a maximum at 72 h (Fig. 6).

HMGB1 Knock Down in Rat Cortical Neurons

To evaluate whether HMGB1 plays an important role in NMDA-mediated neuronal autophagy, we performed loss-of-function studies using a specific HMGB1 siRNA delivered to the neurons by the TGD dendrimer. Cortical neurons were treated with either the TGD dendrimer alone, the dendriplex formed upon complexing the TGD dendrimer with HMGB1 siRNA or the dendriplex formed upon complexing the TGD dendrimer with scramble siRNA. Real-time RT-PCR analysis, using total RNA extracted from rat cortical neurons, indicated that HMGB1 mRNA levels were markedly decreased to 34.07

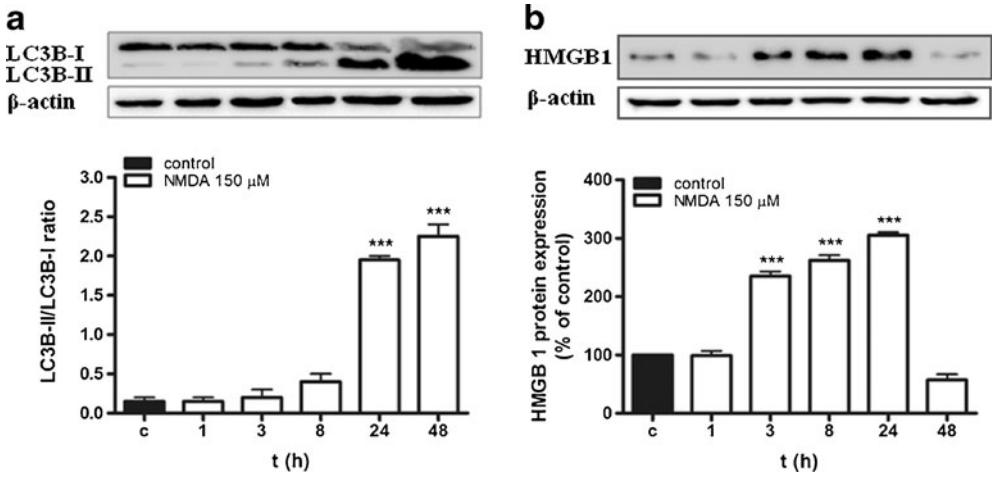

Fig. 2 Autophagy activation correlates with HMGB1 cytosolic translocation. Cells were incubated with vehicle or NMDA (150 μM) for different times. (**a**) LC3B-II/LC3B-I ratio in total cellular lysates. Graph represents a densitometric analysis of LC3B-I and LC3B-II proteins levels related to β-actin protein levels. Data are expressed as mean ± SEM ($n=3$). ***$p<0.001$ as compared with control untreated neurons. (**b**) Levels of HMGB1 and β-actin in the cytosolic fraction. Graph represents a densitometric analysis of HMGB1 levels related to β-actin protein levels. Data are expressed as mean ± SEM ($n=3$). ***$p<0.001$ as compared with control untreated neurons.

±7.6% as compared to control neurons (Fig. 7a). This effect was specific because treatment with the TGD dendrimer alone or TGD (3 μM)/scramble siRNA (100 nM) dendriplex did not show any effect. Moreover, Western-Blot analysis of total cellular lysates showed similar results upon exposition to a TGD (3 μM)/HMGB1 siRNA (100 nM) dendriplex markedly reducing HMGB1 protein levels to about 20% of control values after 72 h of treatment (Fig. 7b).

HMGB1 Down-Regulation Limits NMDA-Mediated Autophagy in Rat Cortical Neurons

To elucidate a possible role of HMGB1 in NMDA-induced autophagic activity, we evaluated the LC3B-II/LC3B-I ratio, which is considered a very good indicator of autophagy. Cortical neurons were treated with either vehicle, TGD (3 μM)/scramble siRNA (100 nM) dendriplex or TGD

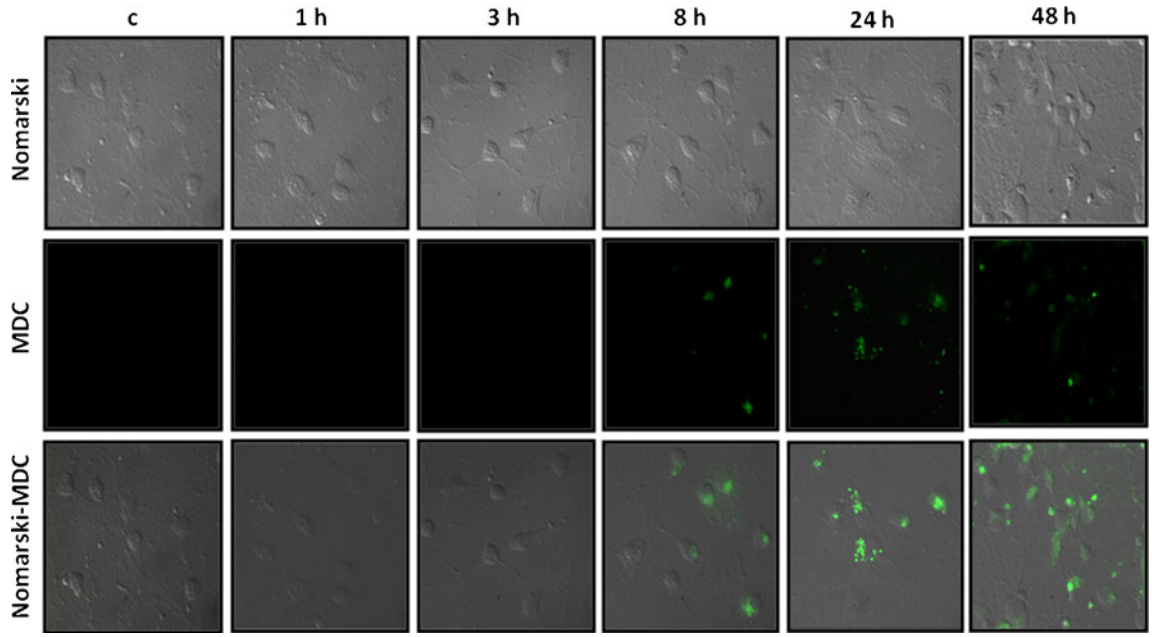

Fig. 3 NMDA-induced autophagy in rat cortical neurons. The neurons were labeled with MDC as indicated in Materials and Methods and exposed to NMDA (150 μM) for 0, 1, 2, 8, 24 and 48 h. The figure represents Nomarski and fluorescence images and the overlay between them. The figure shows a representative experiment repeated 3 times with similar results.

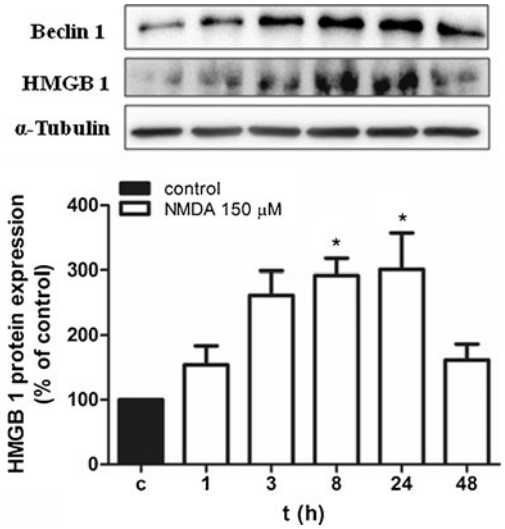

Fig. 4 NMDA-induced HMGB1-Beclin 1 interaction and dissociation of Bcl-2-Beclin 1 complex. Cells were incubated with vehicle or NMDA (150 μM) for different times and total lysates were obtained and inmunoprecipitated with anti-Beclin 1 antibody as indicated in Material and Methods. HMGB1 was immunoprecipitated with Beclin 1 after NMDA treatment. A gel showing immunoreactivity for β-actin is shown to indicate an equal amount of protein in each lane. Graph bars represent the ratio HMGB1/β-actin protein levels in the inmunoprecipitates. Changes in Beclin 1 levels in a representative Western blot are also shown for comparison. Data are expressed as mean ± SEM ($n = 3$). *$p < 0.05$ as compared with control untreated neurons.

(3 μM)/HMGB1 siRNA (100 nM) dendriplex for 72 h and then exposed to vehicle or NMDA (150 μM) for an additional 24 h. HMGB1 levels were markedly decreased in the neurons treated with the dendriplex containing the specific HMGB1 siRNA both in the presence or the absence of NMDA treatment (Fig. 8a). Next we examined whether NMDA-induced autophagy, determined as an increment in the LC3B-II/LC3B-I ratio, would be affected by knocking down HMGB1. We found that HMGB1 knock down blocked NMDA-mediated conversion of LC3B-I in LC3B-II (Fig. 8b), suggesting an inhibition of the autophagic pathway. Once more, the effect was specific and no inhibition of NMDA-induced autophagy was observed in the presence of the dendriplex containing TGD and scramble siRNA.

Autophagy Inhibition, Through HMGB1 Down-Regulation, Increases Excitotoxic Neuronal Death

To explore whether knocking down HMBG1 and the subsequent autophagy inhibition had any effect on NMDA-induced neuronal death, cells were treated with either vehicle, TGD (3 μM)/scramble siRNA (100 nM) dendriplex or TGD (3 μM)/HMGB1 siRNA (100 nM) dendriplex for 72 h and later exposed to vehicle or NMDA (150 μM) for 24 h. Neuronal viability was studied after the treatments using LDH assays. The results showed that the loss of autophagy, through HMGB1 knock down, potentiated the toxic effect of NMDA on rat cortical neurons (Fig. 9). Moreover, this result was

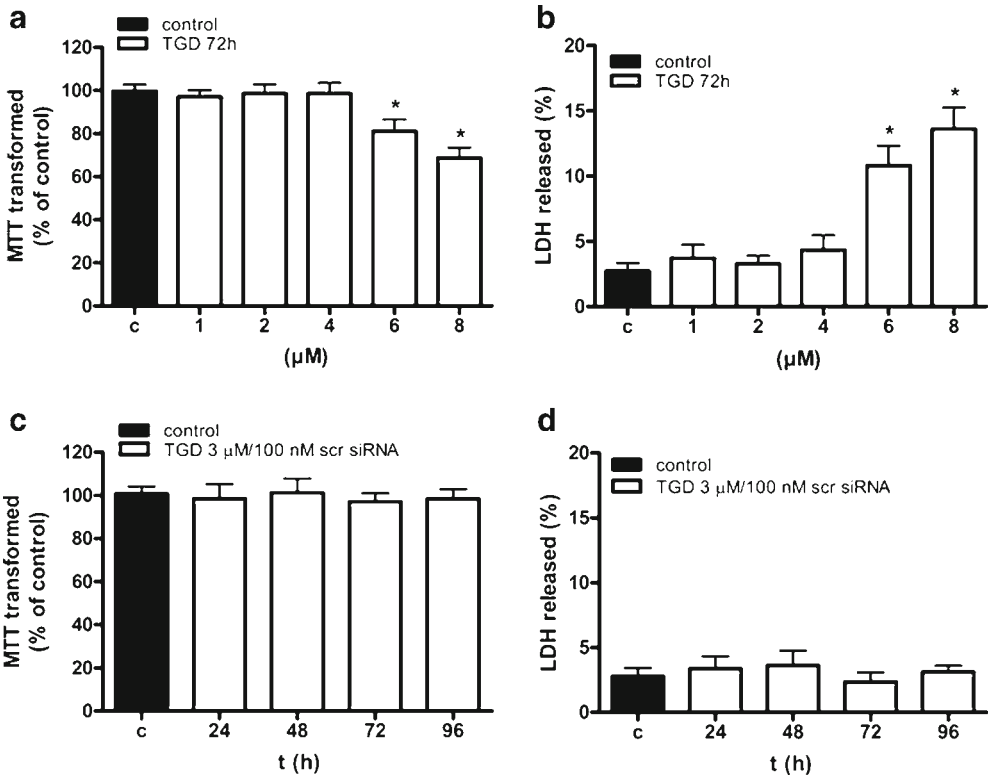

Fig. 5 Toxicity analysis of TGD and TGD/scramble siRNA dendriplex in rat cortical neurons. Neurons were treated with vehicle or different concentrations of TGD dendrimer for 72 h. (**a**) Cell viability was determined by MTT assay. The percentage of MTT transformed was quantified as an index of mitochondrial neuronal toxicity. Data are expressed as mean ± SEM ($n = 12$). *$p < 0.05$ as compared with control neurons. (**b**) The percentage of LDH released was quantified as an index of cellular mortality. Data are expressed as mean ± SEM ($n = 12$). *$p < 0.05$ as compared with control neurons. The neurons were also treated with vehicle or TGD/scramble siRNA dendriplex for 24–96 h and (**c**) cell viability was determined by the MTT assay or (**d**) by the percentage of LDH released. Data are expressed as mean ± SEM ($n = 12$).

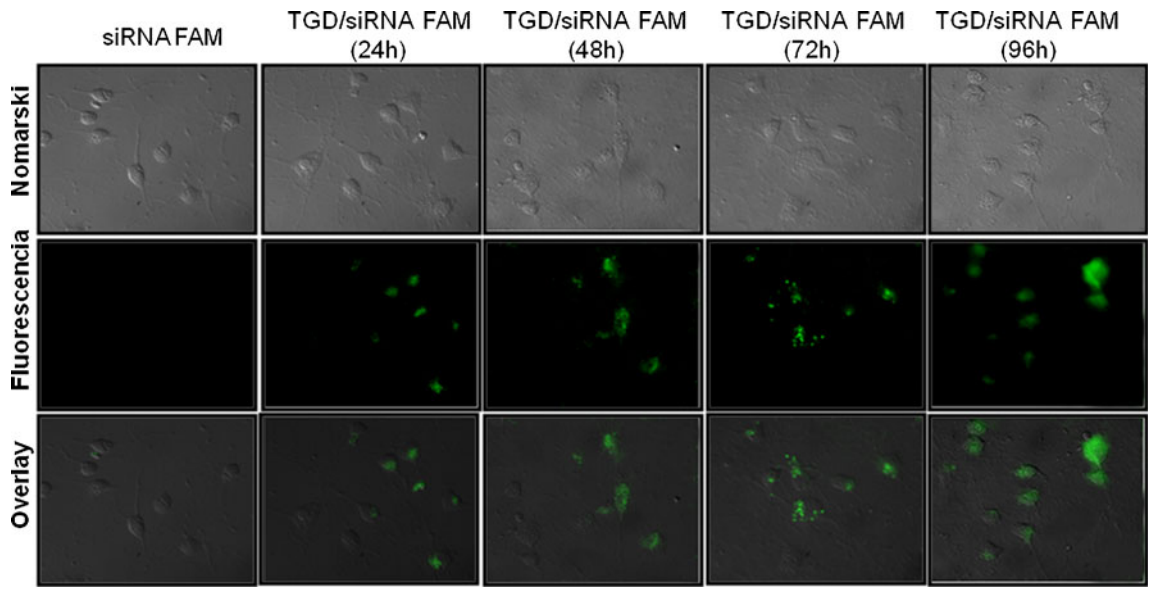

Fig. 6 Transfection efficiency of TGD dendriplex in rat cortical neurons. Nomarski (top images) and fluorescence images (middle images), as well as, the overlay between both images (bottom images) of cortical neurons treated with either FAM-siRNA (100 nM) or TGD (3 μM)/FAM-siRNA (100 nM) dendriplex for 24–72 h. The figure shows a representative experiment that was repeated 3 times with similar results.

specific for HMGB1 silencing because neurons treatment with a dendriplex containing scramble siRNA did not produce any effect.

Taken together, these results indicate that HMGB1 plays a key role in autophagy activation against excitotoxicity and lead us to confirm the hypothesis that autophagy acts as a defense mechanism against excitotoxic damage in cortical neurons.

DISCUSSION

Excitotoxic neuronal death due to the over-activation of NMDA receptors is considered the major mechanism responsible for cell death associated with several neurological diseases such as trauma and several neurodegenerative diseases (36). NMDA induces neuronal toxicity in a concentration and time-

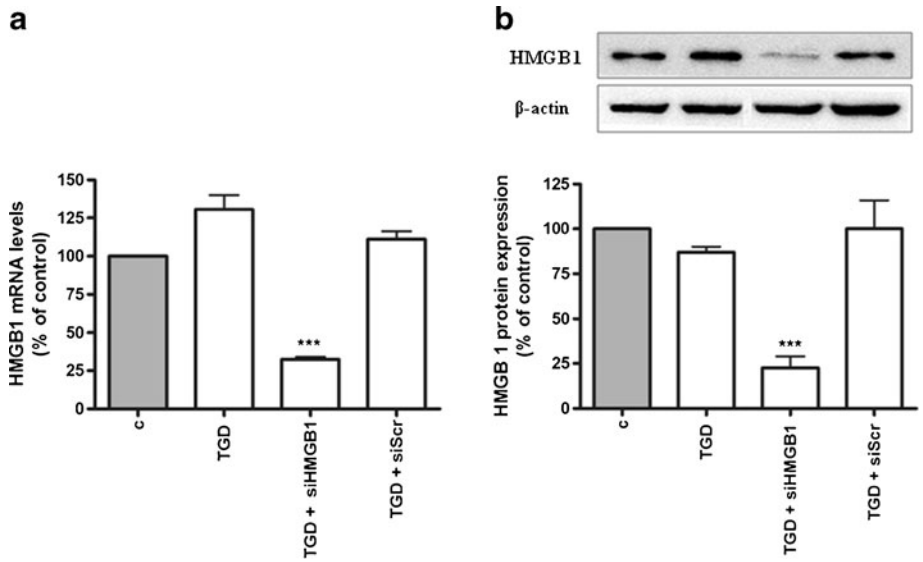

Fig. 7 HMGB1 knock down in rat cortical neurons. Neurons were incubated with TGD (3 μM), TGD (3 μM)/Beclin 1 siRNA (100 nM) dendriplex and TGD (3 μM) and TGD (3 μM)/scramble siRNA (100 nM) dendriplex for 72 h and HMGB1 protein levels were determined by western blot. (**a**) HMGB1 mRNA levels related to β-actin mRNA levels. Data are expressed as mean ± SEM ($n = 2$). ***$p < 0.001$ as compared with control neurons. (**b**) Protein levels of HMGB1 and β-actin in whole cellular lysates. The graph represents a densitometric analysis of HMGB1 levels related to β-actin protein levels. Data are expressed as mean ± SEM ($n = 3$). ***$p < 0.001$ as compared with control neurons.

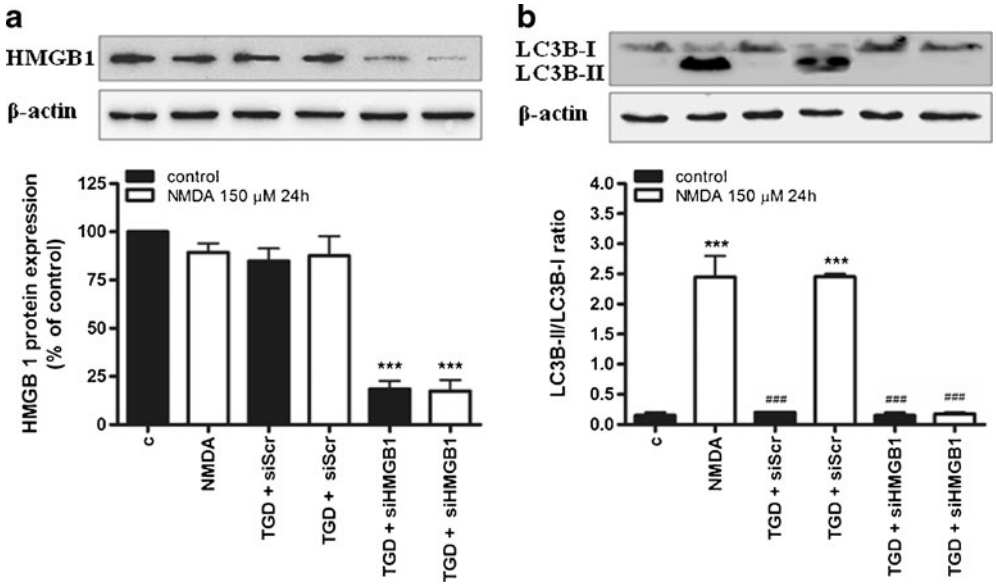

Fig. 8 HMGB1 knock down prevents NMDA-induced autophagy. Cortical neurons were left untreated or treated with TGD (3 μM), TGD (3 μM)/Beclin 1 siRNA (100 nM) dendriplex and TGD (3 μM) and TGD (3 μM)/scramble siRNA (100 nM) dendriplex for 72 h and NMDA (150 μM) was then added, when indicated, for 24 h. (**a**) Levels of HMGB1 and β-actin proteins in total cellular lysates. Graph represents densitometric analysis of HMGB1 levels related to β-actin protein levels. Data are expressed as mean ± SEM ($n = 3$). $^{***}p < 0.001$ as compared with control neurons. (**b**) LC3B-II/LC3BI ratio. Graph represents the densitometric analysis of the ratio LC3B-II/LC3BI normalized by β -actin protein levels (LC3B-I/β-actin)/(LC3B-II/β-actin). Data are expressed as mean ± SEM ($n = 3$). $^{***}p < 0.001$ as compared with control neurons and $^{\#\#\#}p < 0.001$ as compared with NMDA-treated neurons.

dependent manner, through a mechanism that includes calcium overload, changes in mitochondrial activity, free radical production and caspase activation (37,38).

HMGB1 is usually located in the cell nucleus and is diffusely distributed in the cell cytoplasm (39). However, following different stimuli, HMGB1 can be released from the nucleus into the extracellular milieu as previously reported (40). Translocation of HMGB1 to the cytosol and extracellular space occurs during trauma, oxidative and metabolic stress, cellular death (18) including excitotoxic cell death (41) and inflammation. In agreement with previously published results, our data indicate that NMDA treatment induces a progressive HMGB1 translocation from the nucleus to the neuronal cytosol and subsequent extracellular release into the culture medium. On the other hand, excitotoxicity increased autophagic activity in rat cortical neurons as previously described by us and by others (4,42). The similarity between the time-courses of NMDA-induced HMBG1 translocation from the nucleus to the cytosol and autophagy induction suggest that HMGB1 may participate in this phenomenon. HMGB1 may regulate autophagy through its interaction with the autophagic protein Beclin 1. We found that following NMDA treatment, HMGB1 binds Beclin 1, likely by displacing the antiapoptotic protein Bcl-2 from its binding to Beclin-1 (43) leading to autophagy activation.

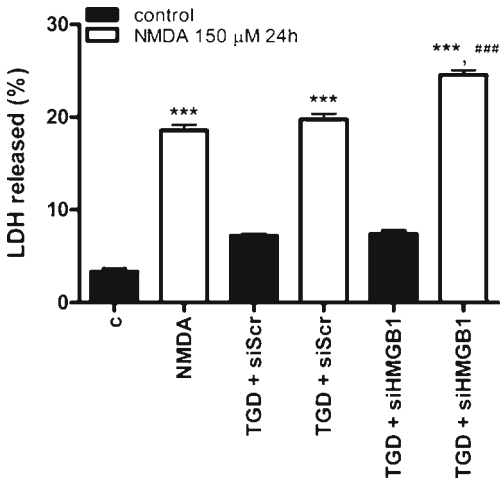

Fig. 9 HMGB1 knock down increases NMDA-mediated neuronal death. Cortical neurons were untreated or treated with TGD (3 μM), TGD (3 μM)/Beclin 1 siRNA (100 nM) dendriplex and TGD (3 μM) and TGD (3 μM)/scramble siRNA (100 nM) dendriplex for 72 h and then NMDA (150 μM) was added, when indicated, for an additional 24 h. The percentage of LDH released was quantified as an index of cellular mortality. Data are expressed as mean ± SEM ($n = 12$). $^{***}p < 0.001$ as compared with untreated neurons and $^{\#\#\#}p < 0.001$, as compared with NMDA-treated neurons.

To study the function of neuronal proteins it is necessary to carry out loss-of-function studies. siRNA technology is a very powerful tool for acute knockdown of selected proteins (44). However, the use of this technology in neurons has been scarce due to the low efficiency of the various non-viral vectors used to deliver siRNA to neurons and the high complexity of preparation and toxicity of viral vectors (23). To explore the role of HMGB1 in NMDA-mediated autophagy, we used a

recently described dendrimer (TGD) which combines a conjugated rigid polyphenylenevinylene core with flexible polyamidoamine branches (24), to deliver a specific siRNA to cortical neurons to knock down HMGB1 protein expression. Our results show that the TGD dendrimer efficiently delivers siRNA to neurons at concentrations that are not toxic and decreases HMGB1 mRNA and protein levels to about 20% of control values. Moreover, this effect is very specific because the dendriplex containing a scramble siRNA had no effect. This result suggests that dendrimers are promising vectors to deliver both genetic material and/or drugs to neuronal cells.

Autophagy plays a key role in removing altered proteins and damaged organelles in neurons and other cell types. We have demonstrated here that knocking down the HMGB1 protein leads to a complete blockade of NMDA-induced autophagy and to a potentiation of NMDA-induced neuronal death. These results suggest that autophagy plays a neuroprotective role by attempting to counterbalance the extensive cell damage that excitotoxicity causes, as described elsewhere (4). This suggests a key role for HMGB1 in NMDA-induced autophagy, likely through its oxidation and translocation from the nucleus to the cytosol, as it has been described elsewhere (40). It has been also proposed that autophagy mediates excitotoxic death (42). The reasons for this discrepancy may be explained by the use in that report of 3-methyl adenine (3-MA), a known blocker of autophagy, to inhibit NMDA-mediated autophagy. In addition to inhibiting autophagy, 3-MA also blocks NMDA-induced Ca^{2+} influx, which would prevent the initial steps of excitotoxicity. The idea of a neuroprotective role of autophagy during excitotoxicity fits with previous reports showing that mice lacking brain expression of the autophagy-related genes Atg5 or Atg7 quickly develop neurodegenerative phenotypes early in post-natal life (45). Moreover, reduced levels of Beclin 1, a key protein in autophagy activation, have been found in brain tissues obtained from elderly humans as well as from patients with Huntington's and Alzheimer's disease (46,47).

CONCLUSIONS

Our study demonstrates that the TGD dendrimer that we used here is a good vector for siRNA delivery to neurons, achieving a reduction in the target protein of about 80% and permitting loss-of-function studies. We have taken advantage of this property to study the role of the protein HMGB1 in NMDA-induced autophagy. We found that HMGB1 played a relevant role in neuronal autophagy regulation. Following NMDA-mediated excitotoxicity, autophagy was induced and HMGB1 was translocated from the neuronal nucleus to the cytosol, where it formed a complex with Beclin1. Knockdown of HMGB1 using a dendrimer/siRNA dendriplex blocked NMDA-induced autophagy in rat cortical neurons and also potentiated NMDA-induced neuronal death. These results suggest a protective role of autophagy during excitotoxicity and define a new function for HMGB1 in promoting neuronal survival by sustaining autophagy in response to excitotoxicity.

ACKNOWLEDGMENTS AND DISCLOSURES

We thank Ana B. García for her technical assistance. M.D.P-C. is a recipient of a Torres Quevedo contract from Ministerio de Ciencia e Innovación (Spain) and NanoDrugs, S.L. This work has been supported, in part, by grants PI081434 from Fondo de Investigaciones Sanitarias, BFU2011-30161-C02-01 from Ministerio de Ciencia e Innovación and PII1I09-0163-4002 and POII10-0274-3182 from Consejería de Educación, JCCM and EuronanoMed projects DENANORNA and DENPEPTHIV to V.C.

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