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# Endoplasmic reticulum stress contributes to NMDA-induced pancreatic β-cell dysfunction in a CHOP-dependent manner

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ABSTRACT

*Aims:* Accumulating evidence suggest that endoplasmic reticulum (ER) stress is an important mechanism underlying the development of diabetes. We have reported that sustained treatment with *N*-methyl-D-aspartate (NMDA) results in apoptotic β-cell death and impairs insulin secretion. However, the molecular mechanism responsible for NMDA-induced β-cell dysfunction remains largely obscure. Thus, this study aimed to determine whether sustained activation of NMDA receptors (NMDARs) causes β-cell dysfunction through ER stress.

*Main methods:* Primary mouse islets and MIN6 mouse pancreatic β-cells were treated with NMDA for 24 h or high-glucose for 72 h. After the treatment, glucose-stimulated insulin secretion (GSIS) and the expression of ER stress markers were measured, respectively. *In vivo*, the expression of ER stress markers was measured in the pancreas of diabetic mice treated with or without NMDARs inhibitor Memantine.

*Key findings:* NMDA treatment caused an increase in the expression of ER stress markers (ATF4, CHOP, GRP78, and Xbp1s) in primary islets. While, tauroursodeoxycholic acid (TUDCA), an inhibitor of ER stress, significantly attenuated NMDA-induced β-cell dysfunction, including the loss of glucose-stimulated insulin secretion and reduction of pancreas duodenum homeobox factor-1 (*Pdx-1*) mRNA expression, a transcription factor regulating insulin synthesis. Besides, NMDA-induced ER stress strongly promoted pro-inflammatory cytokines synthesis (IL-1β and TNF-α) in β cells. Interestingly, knockdown of *CHOP* attenuated β-cell dysfunction evoked by NMDA. Furthermore, we demonstrated that blockade of NMDARs ameliorated high-glucose-induced ER stress *in vitro* and *in vivo*.

*Significance:* This study confirms that ER stress is actively involved in the activation of NMDARs-related β-cell dysfunction.

## 1. Introduction

Diabetes is one of the most common metabolic diseases worldwide. Patients with diabetes often require ongoing therapy throughout their lifetime. About 382 million people were affected by diabetes in 2013, and the prevalence of the disease is expected to rise to 592 million by 2035. Diabetes is characterized by hyperglycemia, which results from the loss of insulin secretion and insulin resistance [1]. Multiple mechanisms underlie defective insulin secretion and responses in type 2 diabetes [2]. However, the mechanisms underlying the β-cell dysfunction are far from completely understood.

Accumulating evidence indicate that endoplasmic reticulum (ER) stress is an essential mechanism contributing to the initiation, development, and progression of diabetes [3–6]. The ER is an organelle which fulfills vital biological roles, such as posttranslational modification, folding and assembly of newly synthesized secretory proteins and storing cellular calcium ($Ca^{2+}$) [7]. Pancreatic β-cells are particularly sensitive to ER stress due to their high rate of proinsulin biosynthesis in response to glucose stimulation [8,9]. Unfolded protein response (UPR), which is activated to restore ER homeostasis, is vital for the maintenance of the functional β-cell mass [10]. Three master ER sensors orchestrate the UPR: the pancreatic ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6), which induce markers of ER stress, such as activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), glucose-regulated protein 78 (GRP78), and splicing of X-box binding protein 1 (Xbp1s) [11,12]. If UPR activation fails to restore ER homeostasis, β-cell dysfunction and death occur through the increase of ER stress,

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which activates several pro-apoptotic and pro-inflammatory signaling pathways [13–15].

Recently, our group and others have found that blockade of *N*-methyl-D-aspartate receptors (NMDARs), glutamate receptors, by memantine or dextromethorphan improves islet function and enhances blood glucose control in an animal model of type 2 diabetes mellitus [16–18]. Furthermore, we demonstrate that sustained stimulation of NMDARs induces β-cell dysfunction through a decrease of insulin secretion and increase of apoptosis [19]. However, the underlying mechanism remains elusive. NMDARs play a crucial role in the central nervous system [20]. The excessive activation of the NMDARs disturbs intracellular $Ca^{2+}$ homeostasis, which triggers ER stress, contributing to overall excitotoxicity and neuronal death [21]. In peripheral non-neuronal cells, an ER stress mediates NMDA-induced retinal injury [22], indicating a relationship between NDMARs activation and ER stress. Besides, we found that prolong NMDA treatment causes an increase of intracellular [$Ca^{2+}$] in MIN6 β-cells [19]. Here, we propose that NMDA-induced β-cell dysfunction is partially mediated by ER stress.

CHOP, which is identified as an ER stress-induced transcription factor, regulates the various biological process and also contributes to many pathological processes, including obesity and diabetes [23–25]. CHOP expression is increased in β-cells from diabetic mice and humans [26,27]. Silenced CHOP ameliorates high-glucose-induced apoptosis in pancreatic β-cells [23]. Besides, CHOP deletion also increases the capacity of islets to produce insulin and curtails the progression of insulin resistance to diabetes [25], indicating that CHOP plays an essential role in β-cell survival and dysfunction.

Given that activation of NMDARs causes β-cell dysfunction [19], we hypothesized that the increase of ER stress was a potential mechanism underlying NMDA-induced β-cell dysfunction. Therefore, in this study, we investigated whether ER stress was actively involved in the activation of NMDARs-related β-cell dysfunction, and blockade of NMDARs could protect β-cells from high-glucose-induced ER stress.

## 2. Materials and methods

### 2.1. Ethics statement

The Ethics Committee of the Center for Scientific Research with Animal Models at Central South University (Changsha, China) approved the experiments, which were performed following the guidelines of the National Institutes of Health. Mice were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneal injection), and necessary efforts were taken to minimize suffering before operations.

### 2.2. Cell culture

MIN6 mouse pancreatic β-cells were cultured in Dulbecco's modification of Eagle's medium (DMEM; Gibco, Carlsbad, CA) containing 10% fetal bovine serum (Gibco) in 5% $CO_2$ at 37 °C.

### 2.3. Islet isolation

Primary islets were isolated from male 8-week-old Balb/c mice. The bile duct near the ampulla of Vater was ligated, and the common bile duct was cannulated and injected with 10–15 mL of Hanks' buffer containing collagenase V (1 mg/mL, Sigma-Aldrich, USA). The digested pancreas tissue was washed with cold Hanks' buffer, and the islets purified using cold Histopaque 1077 and Hanks' buffer (Sigma-Aldrich) with centrifugation at 3000*g* for 20 min [28]. Islets were collected and incubated at 37 °C [19].

### 2.4. Glucose-stimulated insulin secretion (GSIS)

For GSIS assays, MIN6 cells or islets were incubated for 1 h in KRB buffer (2.6 mM $CaCl_2 2H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $KH_2PO_4$, 4.9 mM KCl, 98.5 mM NaCl, and 25.9 mM $NaHCO_3$, all from Sigma-Aldrich) supplemented with 20 mM HEPES (Sigma-Aldrich) and 0.1% BSA (Sigma-Aldrich) and then incubated for 1 h in KRB buffer with 2.8 mM or 16.7 mM glucose. The insulin concentration in the supernatants was determined using ultrasensitive mouse insulin ELISA kits (Alpco, USA) [16,19].

### 2.5. CHOP siRNA transient transfection

*In vitro*, transient transfection of CHOP siRNA (sc-35438, Santa Cruz, USA) was carried to knockdown CHOP according to a previous study [29]. CHOP siRNA was transfected with plasmids using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). The efficiency of CHOP siRNA was assessed at 48 h after transfection.

### 2.6. Total RNA extraction and quantitative reverse-transcription (qRT)-PCR

Total RNA was extracted from MIN6 cells or islets using RNAiso reagent (Takara Bio Inc., Kusatsu, Japan). cDNA was reverse transcribed from total RNA (1 μg) using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio Inc.). qRT-PCR was run using SYBR Green Real-time PCR Master Mix (Takara Bio Inc.) on a real-time PCR system (CFX96 Touch™, Bio-Rad, Hercules, CA, USA). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sets were *Pdx-1* (acacagctctacaaggaccc, acttccctgctccagtgatc), *Atf4* (atccagcaaagccccacaac, caagccatcatccatagccg), *Chop* (ttcactactcttgaccctgcgtc, cactgaccactctgtttccgtttc), *Grp78* (aggacaagaaggaggatgtggg, accgaagggtcattccaagtg), *Xbp1s* (ctgagtccgaatcaggtgcag, gtccatgggaagatgttctgg), *Il-1β* (gggcctcaaaggaaagaatc, taccagttggggaactctgc), *Tnf-α* (ttcactactcttgaccctgcgtc, ggagtgcctcttctgccagt), and β-actin (tctttgcagctccttcgttg, tccttctgacccattcccac).

### 2.7. Animal model

Male Balb/c mice (8-week old) were divided into four groups ($n = 12$ each group): the control group, Memantine group, diabetes group, and Memantine + diabetes group. Mice were intraperitoneally injected with streptozotocin (STZ, Sigma-Aldrich) in a citrate-phosphate buffer (0.1 M, pH 4.2) at a dose of 100 mg/kg after fasting overnight. Hyperglycemia was assessed by the fasting serum glucose (FSG) 72 h after STZ injection. Mice with FSG levels higher than 13.89 mmol/L were selected for subsequent experiments. To evaluate the effects of NMDARs on ER stress in the islet *in vivo*, diabetic mice received an intraperitoneal injection with Memantine (an antagonist of NDMARs, 10 mg/kg/day) for 3 weeks. All mice were fed a high-fat diet for 9 weeks as our previous study [16].

### 2.8. Western blotting

Western blotting was conducted as described in our previous report [30,31]. Briefly, equal amounts of proteins were mixed with loading buffer and denatured at 100 °C for 10 min. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA, USA). The membranes were blocked with 5% fat-free milk for 2 h and then probed at 4 °C overnight with the following primary antibodies: anti-GAPDH (1:1000; Sigma-Aldrich), anti-GRP78 (1:1,000, ImmunoWay, Newark, DE, USA), anti-ATF4 (1:2,000; Abcam, Cambridge, MA, USA), or anti-CHOP (1:1,500, Abcam). After washes for three times, the membranes were incubated with secondary antibodies (1:7,500; Abcam) at room temperature for 1 h. Enhanced chemiluminescence (Millipore) was used to detect protein content. Images were obtained using ChemiDoc XRS system (Bio-Rad).

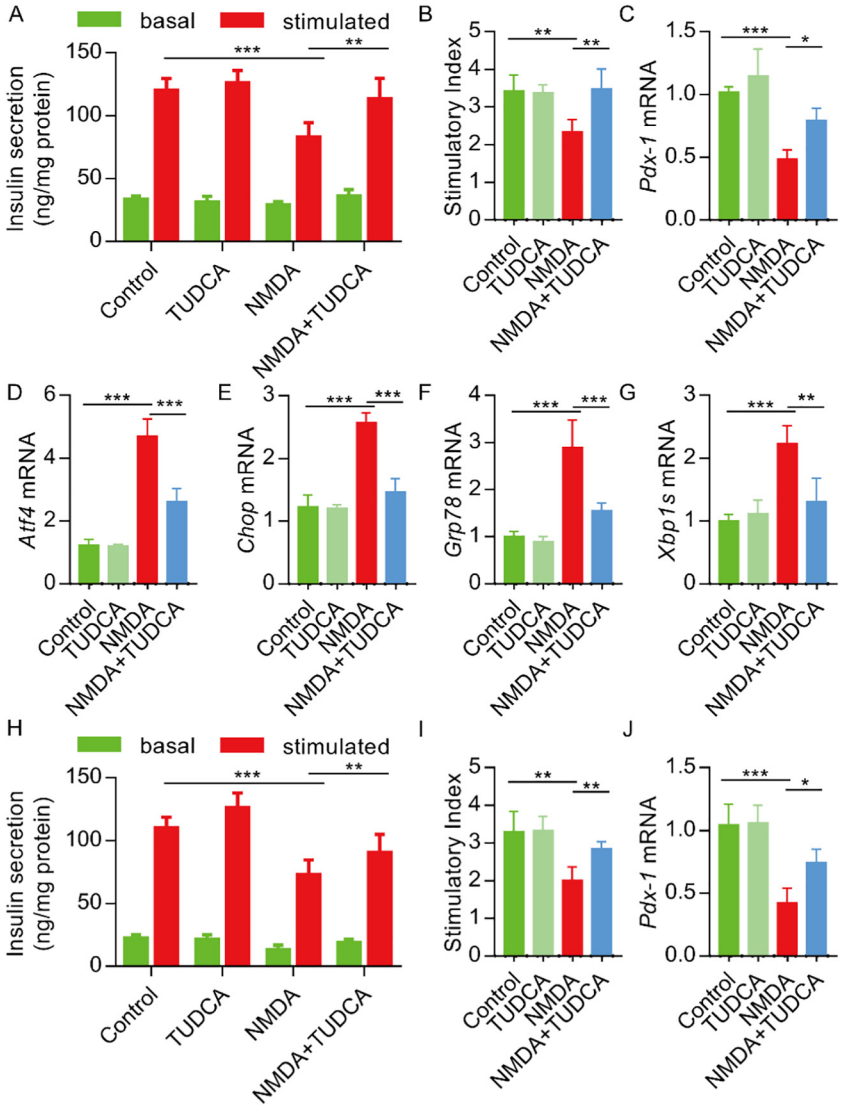


**Fig. 1.** NMDA induces β-cell dysfunction and ER stress. (A–B) Insulin secretion was determined after 1 h-incubation with 2.8 mM (basal) and 16.7 mM glucose (stimulated) from primary islets with NMDA (5 mM) and/or TUDCA (500 μg/mL). (C) qRT-PCR analysis of *Pdx-1* mRNA levels in primary islets treated with NMDA (5 mM) and/or TUDCA (500 μg/mL) for 24 h. (D–G) qRT-PCR analysis of ER stress-relative gene expression in primary islets treated with NMDA (5 mM) and/or TUDCA (500 μg/mL) for 24 h. (H–I) Insulin secretion was determined after 1 h-incubation with 2.8 mM (basal) and 16.7 mM glucose (stimulated) from MIN6 cells absent with NMDA (5 mM) and/or TUDCA (500 μg/mL). (J) qRT-PCR analysis of *Pdx-1* mRNA in murine β-cell line MIN6 treated with NMDA (5 mM) and/or TUDCA (500 μg/mL) for 24 h. Data are expressed as the mean ± SEM ($n = 4$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

### 2.9. Statistical analysis

All data analyses were conducted using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Data were presented as means ± SEM. Statistical differences were determined by ANOVA, followed by Tukey's *post hoc* test to assess significance. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. NMDA induces β-cell dysfunction and ER stress

To investigate whether NMDAR activation induces ER stress in β cells, we treated primary mouse islets with NMDA. NMDA treatment for 24 h impaired GSIS (Fig. 1A), reduced stimulatory index (Fig. 1B), and decreased the expression of pancreas duodenum homeobox factor-1 (*Pdx-1*) mRNA (Fig. 1C), a transcription factor regulating insulin synthesis, indicating the impaired β-cell function as our previous report [19]. Besides, NMDA treatment significantly increased the gene expressions of ER stress markers in primary islets, including *Atf4*, *Chop*, *Grp78*, and *Xbp1s* (Fig. 1D–G). To confirm the role of NMDA-induced ER stress in β-cell dysfunction, tauroursodeoxycholic acid (TUDCA), an inhibitor of ER stress [32,33], was employed. Our data showed that TUDCA restored the mRNA expression of *Atf4*, *Chop*, *Grp78*, and *Xbp1* as expected (Fig. 1D–G), and attenuated the β-cell dysfunction induced by NMDA treatment (Fig. 1A–C). To further confirm the effects of NMDA on β cells function, we stimulated a murine β-cell line MIN6 with NMDA and TUDCA for 24 h. Similar results had been found (Fig. 1H–J). Together, these findings confirm that NMDA induces ER stress in β-cells, subsequently impairs β-cell function.

### 3.2. ER stress precedes the induction of proinflammatory cytokines in NMDA-treated MIN6 cells

To confirm the effects of NMDA on β-cell function, we investigated the effects of NMDA on the ER stress in MIN6 cells. We found that NMDA treatment significantly increased the mRNA expression of *Atf4*, *Chop*, and *Grp78* in a time-dependent manner (Fig. 2A–C). Western blot results demonstrated the enhanced protein expression of ATF4, CHOP, and GRP78 in MIN6 cells after NMDA treatment (Fig. 2D–G). In type 2 diabetes, ER stress is a major contributor to inflammatory diseases [34]. So, we wondered whether NMDA-induced ER stress involved in pro-inflammatory cytokines release. Results showed that NMDA treatment enhanced the mRNA expression of *Il-1β* and *Tnf-α* in a time-dependent manner (Fig. 2H–I), which was partially revised by pretreatment of ER stress inhibitor TUDCA (Fig. 2J–K). Collectively, the occurrence of ER stress induced by NMDA precedes an increase in the expression of the pro-inflammatory cytokines in β-cells.

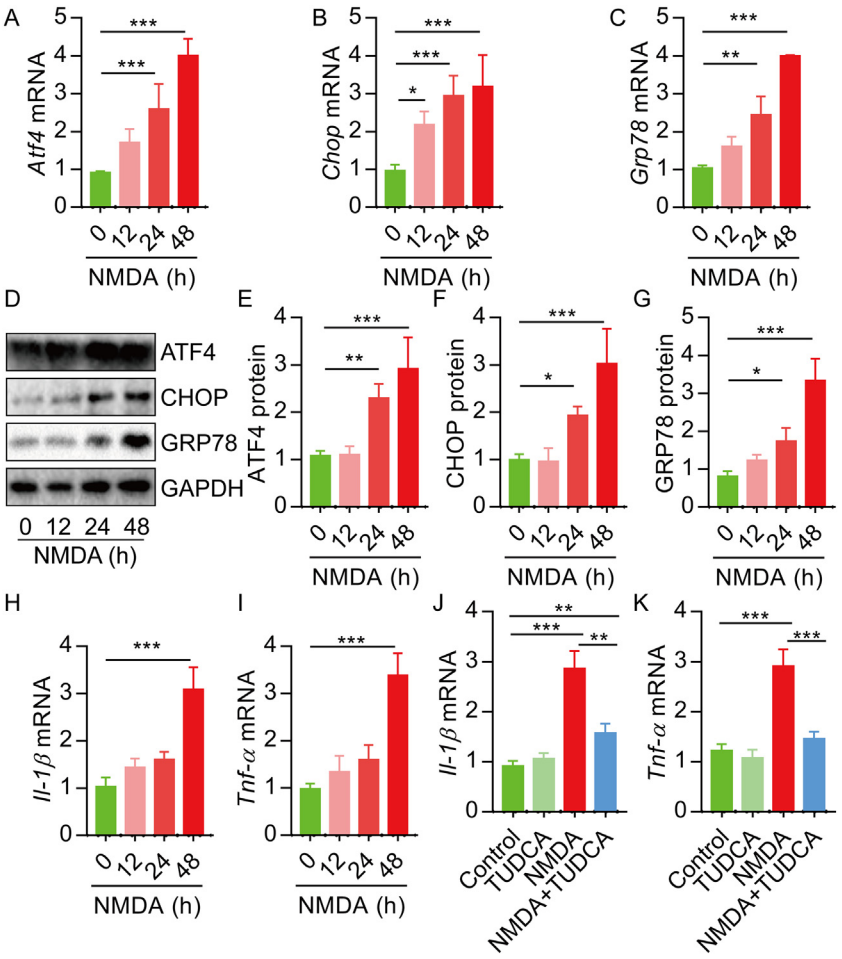


**Fig. 2.** ER stress precedes the induction of proinflammatory cytokines in NMDA-treated MIN6 cells. MIN6 cells were incubated with NMDA (5 mM) for 12, 24 or 48 h. (A–C) *Atf4*, *Chop*, and *Grp78* mRNA levels in MIN6 cells were determined by qRT-PCR. (D–G) ATF4, CHOP, and GRP78 protein in MIN6 cells were determined by western blot. (H–I) *Il-1β* and *Tnf-α* mRNA levels in MIN6 cells were determined. (J–K) MIN6 cells were treated with NMDA (5 mM) for 48 h with/without TUDCA (500 μg/mL). Then, *Il-1β* and *Tnf-α* mRNA levels in MIN6 cells were determined. Data are expressed as the mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

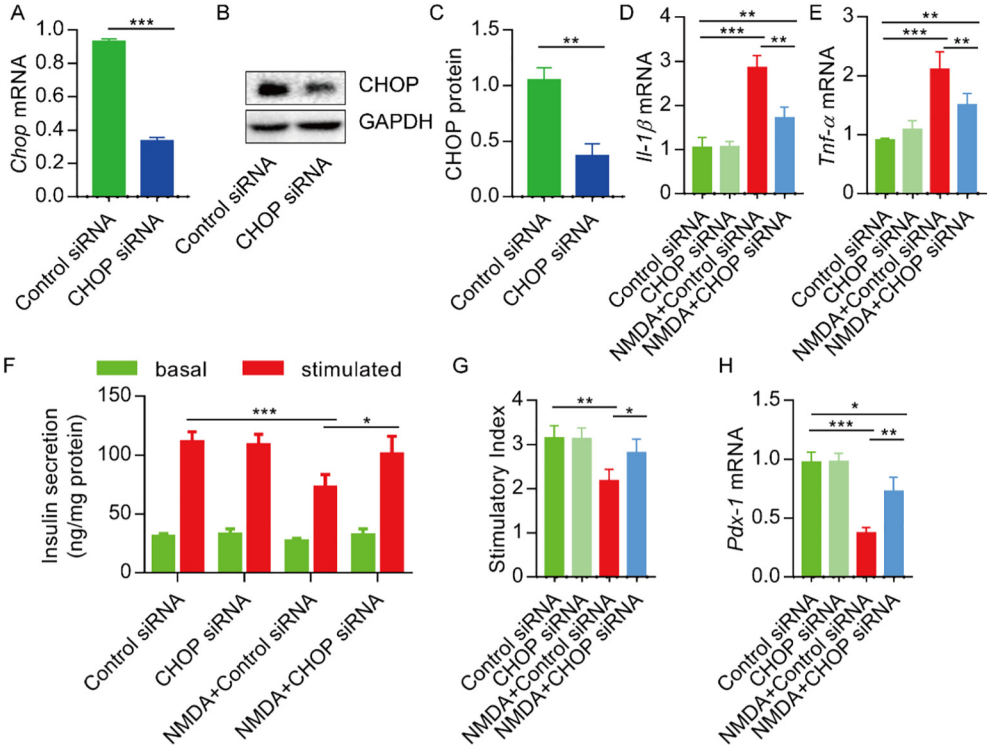


**Fig. 3.** NMDA-induced ER stress is dependent on CHOP. (A–C) The knockdown efficiency of CHOP mRNA and protein in MIN6 cells treated with CHOP siRNA. NMDA (5 mM) was added to MIN6 cells treated with CHOP siRNA or Control siRNA for 48 h. (D–E) *Il-1β* and *Tnf-α* mRNA levels in MIN6 cells were determined. (F) Insulin secretion was determined after 1 h-incubation with 2.8 mM (basal) and 16.7 mM glucose (stimulated) from MIN6 cells. (G) The insulin stimulatory index denotes the ratio of secreted insulin during 1 h-incubation with 16.7 mM to that secreted during 1-h incubation with 2.8 mM glucose in MIN6 cells. (H) *Pdx-1* mRNA levels in MIN6 cells were determined. Data are expressed as the mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

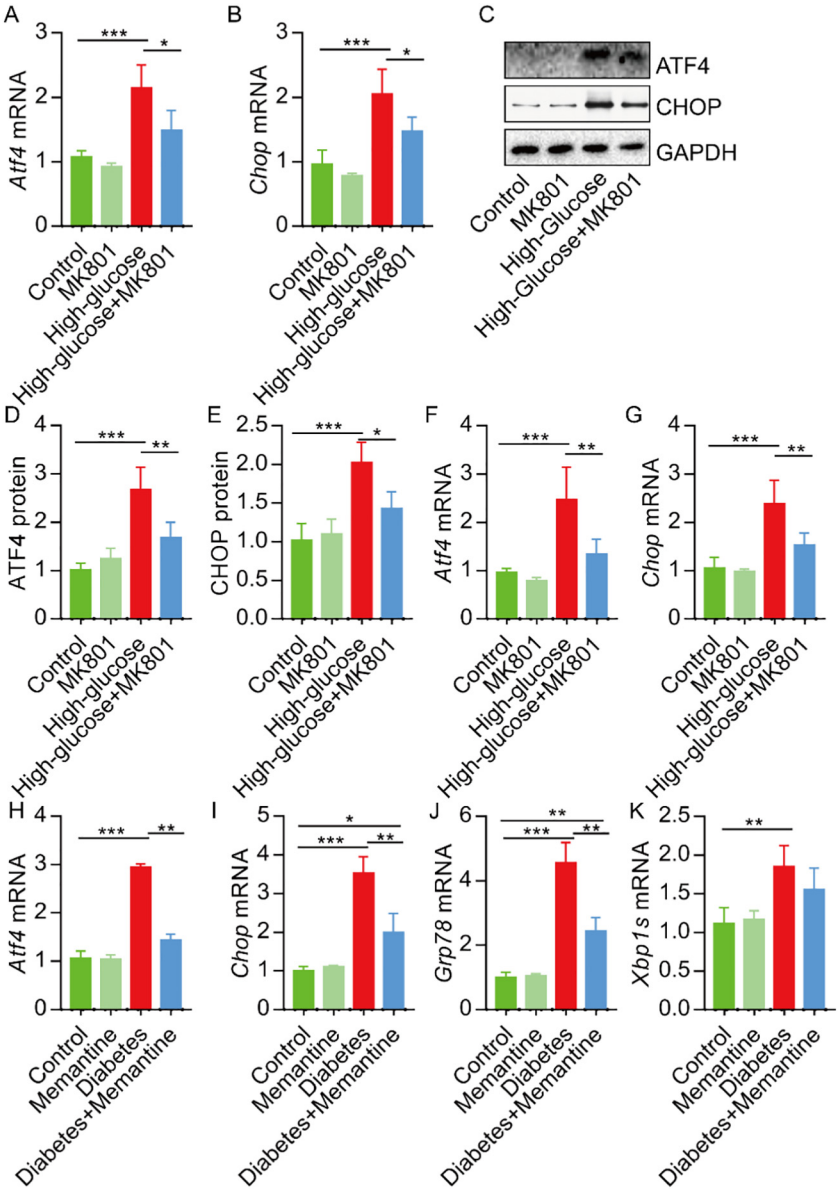


**Fig. 4.** Blockade of NMDARs ameliorates high-glucose-induced ER stress. MK801 (50 μM) was added to MIN6 cells or primary islets treated with high-glucose (33.3 mM) for 48 h. (A–B) *Atf4* and *Chop* mRNA levels in MIN6 cells were determined ($n = 4$). (C–E) ATF4 and CHOP protein in MIN6 cells were determined ($n = 4$). (F–G) *ATF4* and *CHOP* mRNA levels in primary islets were determined ($n = 4$). (H–K) Diabetic mice were treated with Memantine (10 mg/kg/day) for 21 days. The expression of *Atf4*, *Chop*, *Grp78*, and *Xbp1s* in pancreas tissues were determined ($n = 6$). Data are expressed as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

### 3.3. NMDA-induced ER stress is dependent on CHOP

To assess the potential mediatory role of CHOP in NMDA-induced ER stress, CHOP was knocked down using CHOP siRNA in MIN6 cells (Fig. 3A–C). As anticipated, siRNA-mediated knockdown of CHOP reduced NMDA-evoked increases of *Il-1β* and *Tnf-α* mRNA expression (Fig. 3D–E). Furthermore, CHOP siRNA partially restored the loss of GSIS and stimulatory index induced by NMDA in MIN6 cells (Fig. 3F–G), as well as *Pdx-1* mRNA expression (Fig. 3H). These results provide evidence that CHOP is required for the pro-inflammatory response induced by the activation of NMDARs.

### 3.4. Blockade of NMDARs ameliorates high-glucose-induced ER stress

High-glucose-induced β-cell dysfunction is closely related to ER stress [35]. In our previous study, we have found that blockade of NMDARs attenuated β-cell dysfunction induced by high-glucose [19]. Thus, we examined the effects of MK801, an NMDARs antagonist, on the ER stress induced by high-glucose in MIN6 cells and primary islets. High-glucose exposure for 72 h increased the mRNA and protein expression of ATF4 and CHOP in MIN6 cells, which were significantly reversed by treatment with MK801 in MIN6 cells (Fig. 4A–E). We also found that MK801 significantly reduced the *Atf4* and *Chop* mRNA levels induced by high-glucose in primary islets (Fig. 4F–G). *In vivo*, Memantine, another NMDARs antagonist, significantly reduced the expression of ER stress markers in the pancreas of diabetic mice, including *Atf4*, *Chop*, *Grp78*, and *Xbp1s* mRNA (Fig. 4H–K). Collectively, these data suggest that blockade of NMDARs ameliorates high-glucose-induced ER stress *in vitro* and *in vivo*.

## 4. Discussion

The sustained activation of NMDARs has been regarded as one of the critical mechanisms related to pancreatic β-cell dysfunction and death [16–19]. This hypothesis is supported by the fact that NMDARs antagonists protect from the reduction of insulin secretion and the increase of β-cell apoptosis in diabetes in our previous studies [16,19]. Recently, ER stress has been shown to play a vital role in β-cell dysfunction. To date, however, there has been no investigation concerning the involvement of ER stress in the pathogenesis of β-cell injury induced by activation of NMDARs. Here, we show that activation of NMDARs causes ER stress in β-cells both *in vitro* and *in vivo* and that this results in

a pro-inflammatory phenotype and β-cell dysfunction. The ER stress induced by activation of NMDARs is mediated by upregulation of CHOP. We also provide evidence that blockade of NMDARs ameliorates high-glucose-induced β-cell dysfunction through the decrease of ER stress.

Glutamate is the primary excitatory neurotransmitter in the CNS [20]. Functional GluRs, such as NMDARs, also have been demonstrated in nonneuronal tissues and cells as well, including in the pancreatic β-cells [16,18,36]. Our previous studies have shown that the sustained activation of NMDARs has deleterious effects on insulin secretion and apoptosis of β-cells [16,17]. ER functions as an intracellular calcium store and plays a vital role in $Ca^{2+}$ homeostasis [37]. It has reported that NMDA causes $Ca^{2+}$ efflux from the ER to cytosol, and results in ER $Ca^{2+}$ depletion and ER stress [22]. We have found that sustained treatment with NMDA increases intracellular [$Ca^{2+}$] in β-cells [19]. In the current study, we demonstrate that NMDA causes ER stress, while, ER stress inhibitor could attenuate the β-cell dysfunction induced by NMDA treatment. As mentioned above, we propose that the sustain activation of NMDARs impairs β-cell function partly dependent on ER stress.

CHOP plays a critical role in the progress and development in diabetes [23]. Recent studies have demonstrated that insulin resistance is associated with CHOP induction in murine and human islets [27,38]. CHOP deletion preserved the β-cell mass and improved β-cell function [25,26], indicating that CHOP deletion improves ER function and protects against oxidative stress in response to ER stress in β cells. A previous report on NMDA-induced retinal injury showed an up-regulation of CHOP [39]. And $CHOP^{-/-}$ mice are more resistant to NMDA-induced retinal damage [22]. Our results from a series of experiments showed that CHOP is up-regulated in pancreatic β-cell with NMDA-induced injury. In addition, silenced CHOP shows a protective role against β-cell dysfunction, suggesting that CHOP is a direct target of activation of NMDARs.

Many studies revealed complex mechanisms associated with the β-cell dysfunction behind diabetes. It has been reported that inflammation contributes to the initiation, development, and progression of diabetes [40]. Treatment with either anakinra, an IL-1 receptor antagonist, or anti-IL-1β antibodies improves β-cell function, improves glycemic control, and reduces inflammation [41]. Recently, a role for ER stress in inflammation has been shown in renal proximal tubular cells, bone marrow-derived cells, and β-cells [13,42,43]. We have found that NMDARs blockers decrease the expression of inflammatory cytokines in high-glucose-treated β-cells *in vitro* [16]. In this study, we demonstrate that NMDA-stimulated increase in pro-inflammatory cytokine expression in pancreatic β-cells is caused by ER stress as (1) pro-inflammatory cytokine (IL-1β and TNF-α) is inhibited by TUDCA; (2) IL-1β and TNF-α expressions are inhibited by reduced expression of CHOP.

In summary, our results indicate that the over-activation of NMDARs induces ER stress-CHOP pathway, subsequently results in β-cell dysfunctions, enriching the mechanism underlying NMDARs-induced β-cell dysfunction.

## Author contributions

XTH designed and performed most of the experiments, analyzed and interpreted the data, and wrote the manuscript. WL, YZ, MS, CCS, and CYZ assisted during the acquisition, analysis, and interpretation of data and revised the manuscript. WL and YZ assisted with data acquisition and revision of the manuscript. SYT is responsible for the integrity of the work as a whole. All authors reviewed and approved the final version of the manuscript.

## Declaration of Competing Interest

The authors declare no competing financial interests.

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