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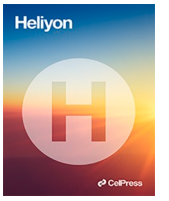

Research article

Expression of ITPR2 regulated by lncRNA-NONMMUT020270.2 in LPS-stimulated HT22 cells

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ABSTRACT

Background: Long non-coding RNA (lncRNA)-NONMMUT020270.2 is downregulated and co-expressed with inositol 1,4,5-trisphosphate receptor type 2 (ITPR2) in the hippocampus of Alzheimer's disease (AD) mice. However, whether the expression of ITPR2 was regulated by lncRNA-NONMMUT020270.2 remains unclear. we aimed to investigate regulating relationship of lncRNA-NONMMUT020270.2 and ITPR2.

Methods: HT22 cells were firstly transfected with the pcDNA3.1-lncRNA-NONMMUT020270.2 overexpression plasmid or with the lncRNA-NONMMUT020270.2 smart silencer, and then were stimulated with lipopolysaccharide (LPS) for 24h. The mRNA expression levels of lncRNA-NONMMUT020270.2 and ITPR2 were measured by reverse transcription-quantitative PCR. Cell viability was assessed using a Cell Counting Kit 8 assay. The expression of $A\beta_{1\text{-}42}$ was detected by ELISA. The expression levels of p-tau, caspase-1, and inositol trisphosphate receptor (IP3R) proteins were detected by western-blotting. Nuclear morphological changes were detected by Hoechst staining. Flow cytometry and Fluo-3/AM were carried out to determine cell apoptosis and the intracellular Ca^{2+}.

Results: LPS significantly decreased cell viability, and ITPR2 mRNA and IP3R protein expression levels. While it markedly enhanced the expression levels of p-tau and $A\beta_{1\text{-}42}$, cell apoptosis rate, as well as intracellular Ca^{2+} concentration ($P < 0.05$). In addition, lncRNA-NONMMUT020270.2 overexpression significantly increased the expressions levels of ITPR2 mRNA and IP3R protein ($P < 0.05$), and inhibited expression of p-tau and $A\beta_{1\text{-}42}$, cell apoptosis rate, and reduced intracellular Ca^{2+} concentration ($P < 0.05$). By contrast, lncRNA-NONMMUT020270.2 silencing notably downregulated expressions levels of ITPR2 mRNA and IP3R protein ($P < 0.05$), and elevated expression levels of p-tau and $A\beta_{1\text{-}42}$, cell apoptosis rate, and intracellular Ca^{2+} concentration ($P < 0.05$).

Conclusion: lncRNA-NONMMUT020270.2 was positively correlated with ITPR2 expression in LPS-induced cell. Downregulating the lncRNA-NONMMUT020270.2 and ITPR2 may promote cell apoptosis and increase intracellular Ca^{2+} concentration.

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1. Introduction

Alzheimer's disease (AD) is a common age-related neurodegenerative disease, which is characterized by progressive cognitive impairment [1]. It is the most common cause of dementia in elderly people. The pathology of AD is characterized by progressive loss of synapses and neurons in the hippocampus and cerebral cortex [2]. The typical pathological signs of AD are senile plaques formed by the deposition of insoluble β-amyloid (Aβ) proteins outside neurons and nerve fiber tangles formed by the aggregation of highly phosphorylated Tau (p-tau) protein inside neurons [3,4]. There are several hypotheses on the etiology and pathogenesis of AD. Among them, it has been reported that the imbalance of intracellular Ca^{2+} homeostasis serves a significant role in the development of AD [5–9].

Increased Ca^{2+} concentration in neurons may lead to cell injury and provides a common pathway for the eventual occurrence of AD [10]. Endoplasmic reticulum (ER) is considered as the main intracellular Ca^{2+} storage, buffering and signaling organelle. Its release depends on the binding of the second messenger inositol 1,4,5-trisphosphate (IP3) to the IP3 receptor (IP3R). IP3R protein, encoded by the inositol 1,4,5-trisphosphate receptor type 2 (ITPR2) gene, is primarily responsible for regulating intracellular calcium concentrations in neurons [11]. After stimulation, IP3 is released and then binds to IP3R in neurons [12,13]. Altered IP3R function can increase vulnerability to high intracellular calcium concentrations, which may result in cell apoptosis and selective degeneration of neurons. Calcium release from the ER by IP3R seems to be crucial to both the extrinsic death receptor and the intrinsic apoptosis pathways [14]. Our previous study has demonstrated that ITPR2 was significantly downregulated in an LPS-induced AD mouse model [15], thus further supporting the hypothesis that the abnormal function of ITPR2/IP3R could be closely associated with the onset of AD. However, the regulatory mechanism of ITPR2 remains unclear.

Long non-coding RNAs (lncRNA) are known to play a significant role in chromatin remodeling, transcriptional regulation, mRNA stability, translation, post-translation and protein degradation [16,17]. Emerging evidence has suggested that lncRNAs, such as BACE1-AS, 51A, 17A, BC200 and NDM29, can increase the expression of several key proteins involved in the pathogenesis of AD via regulating the related proteins [18–24], thus suggesting that lncRNAs may play a significant role in the pathological process of AD. Our previous study also identified numerous lncRNAs that were notably down/upregulated in an LPS-induced AD mouse model. Bioinformatics analysis revealed that lncRNA-NONMMUT020270.2 was expressed at low levels and co-expressed with ITPR2 in LPS-induced AD mouse models [15]. While, whether the expression of ITPR2 is regulated by lncRNA-NONMMUT020270.2 remains unknown.

To investigate whether the ITPR2 was regulated by lncRNA-NONMMUT020270.2 in LPS-stimulated HT22 cells, we firstly transfected HT22 cells with pcDNA3.1-lncRNA-NONMMUT020270.2 overexpression plasmid and the lncRNA-NONMMUT020270.2 smart silencer, respectively. And cell viability, expression levels of lncRNA-NONMMUT020270.2 and ITPR2 mRNAs, $A\beta_{1\text{-}42}$, P-tau, IP3R, and caspase-1 proteins, as well as cell apoptosis rate and intracellular Ca^{2+} concentration were detected. The research may enrich the research on the pathogenesis of AD and lay the foundation for the diagnosis and treatment of AD.

2. Experimental procedure

2.1. Cell culture and treatment

HT22 cells were cultured in DMEM supplemented with 15 % fetal bovine serum (FBS) and 1 % antibiotics (penicillin 10^5 μl^{-1}, streptomycin 100 mg^{-1}) in a flask at 37 °C in a 5%-CO_2 incubator. Following incubation for 24 h, cells were digested with trypsin-EDTA prior to subsequent use. HT22 cells were divided into the 15 following groups.

(1) LPS 0 h, 12 h, and 24 h groups: HT22 cells were treated with 20 μmol/L LPS for 0 h, 12 h and 24 h, respectively.
(2) Inhibitory-expression groups: mock group, mock + LPS group, smart silencer-negative control (NC) group, smart silencer-NC + LPS group, smart silencer lncRNA NONMMUT020270.2 and smart silencer lncRNA NONMMUT020270.2+LPS group.
(3) Overexpression groups: mock group, mock + LPS group, pcDNA 3.1(+) group, pcDNA 3.1(+) + LPS group, pcDNA 3.1 (+)-lncRNA NONMMUT020270.2 and pcDNA 3.1(+)-lncRNA NONMMUT020270.2+LPS group.

2.2. Construction of vectors and smart silencer lncRNA, and transfection

HT22 cells were seeded into a 6-well plate at a density of 10^5 cells/ml and the culture medium was changed when cell confluence reached ~80 %. The synthesized smart silencer lncRNANONMMUT020270.2 sequences (position 1073) (sense, 5′-CAGUGGCUGUUUCACUGACCGACUA-3' (targeted sequence: CAGTGGCTGTTTCACTGACCGACTA); antisense 5′-UAGUCGGUCAGUGAAACAGCCACUG-3' (targeted senquence: TAGTCGGTCAGTGAAACAGCCACTG); smart silencer NC sequence: 5′-GTTGTATCACACGCACCTAGTGGTC-3'; concentration, 20 nM) and the pcDNA3.1(+)-lncRNANONMMUT020270.2 overexpression plasmid were diluted in 100 μl serum-free medium, followed by gently mixing. In another tube, 7.5 μl Lipofectamine 2000 (Invitrogen, USA) was supplemented in 100 μl serum-free medium and mixed gently. The two mixtures were added together, gently mixed, incubated at room temperature for 20 min and the smart silencer- or lncRNA-liposome complexes were then added into a pore plate. Following incubation for 48 h, cells were collected for the subsequent experiments. Cells were then treated with LPS 20 μmol/l in the above transfected cells, respectively. After incubation for 24 h, and the cell was adjusted to concentration of 1×10^6/mL and then collected for further experiments.

2.3. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted using a Trizol reagent (Takara Bio, Inc.). RNA was reverse transcribed into cDNA using a RT kit. Using cDNA as template, the mRNA expression levels of lncRNA-NONMMUT020270.2 and ITPR2 were detected by qPCR using a SYBR Green kit (Takara Bio, Inc.), according to the manufacturer's instructions, on a qPCR instrument. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal reference for the real-time PCR products. Each sample was set with three duplicate holes and the experiment was repeated three times. The primer sequences used are listed in Table SI.

2.4. Enzyme linked immune sorbent assay (ELISA)

The cell culture was taken and centrifuged at 1000×*g* for 20 min. The supernatant is used for subsequent testing. The assay procedure is in accordance with the ELISA kit instructions (Mouse $A\beta_{1\text{-}42}$ ELISA Kit, ELK Biotechnology). The absorbance value at 450 nm were measured by using Multiskan SkyHigh enzyme labeling instrument (Multiskan SkyHigh, Thermo Scientific).

2.5. Western blot analysis

The cells from each group were collected and total proteins were extracted using a lysis buffer on ice for 1 h, followed by centrifugation at 4 °C for 30 min at 13,000 g. Subsequently, the supernatant was collected, and protein concentration was measured using a BCA assay. Equal amount of protein extracts was separated by SDS-PAGE and were then transferred onto PVDF membranes. Following blocking with 5 % skim milk, the membranes were incubated with primary antibodies against IP3R (dilution, 1:500), p-tau (dilution, 1:1000), tau (dilution, 1:5000) and caspase-1 (dilution, 1:1000) overnight at 4 °C. Following washing with PBS-Tween-20 for five times for 5 min each, the membranes were incubated with the corresponding secondary antibodies (dilution, 1:6000) for 1 h at room temperature. Then, the blots were developed and fixed. ImageJ software was used to scan membranes and calculate the grayscale value of each protein band. The relative protein expression level of p-tau was normalized to those of tau. The relative protein expression levels of IP3R and caspase-1 were normalized to those of GAPDH.

2.6. Cell viability assay

HT22 cells at a density of 10^4 cells/well in 100 μl culture medium were seeded into 96-well culture plates. Following incubation for 48 h, the culture medium was replaced, each well was supplemented with 10 μl CCK-8 solution (Dojin Laboratory) and cells were then incubated at 37 °C in 5 % CO_2 for an additional 2 h. Subsequently, the optical density (OD) at a wavelength of 450 nm was measured using a microplate reader (Varioskan LUX; Thermo Fisher Scientific, Inc.). The cell survival rate was measured using the following formula: Cell survival rate (%) = (OD value of the experimental group-blank group)/(OD value of the control group-blank group) x100 %. The experiment was repeated three times in each group. The optimal concentration of the drug was used for the follow-up experiments.

2.7. Hoechst staining

The methods of hoechst staining were previously reported by Farahzadi et al. [25]. HT22 cells at a density of 4.5×10^5 cells/well were cultured in 2 ml antibiotic-free medium. Subsequently, cells were treated with LPS for 24 h and each well was then supplemented with 0.5 ml fixative solution and incubated for 10 min. Subsequently, 0.5 ml Hoechst 33258 was added into each well and cells were incubated for an additional 5 min. Cells were observed under a fluorescence inverted microscope and analyzed by Image-Pro Plus software.

2.8. Flow cytometry

Flow cytometry by Annexin V-FITC/PI were elaborately explained in the research by Rasouliyan et al. [26] and Rafat et al. [27]. Cell suspensions were prepared, and cells were washed twice with PBS. Subsequently, the suspension was first supplemented with 300 μl binding buffer and then with 5 μl Annexin V-FITC and 5 μl PI (both from Beijing Solarbio Science & Technology Co., Ltd.), followed by incubation in the dark for 10 and 5 min, respectively. Flow cytometry was performed using the FACSCalibur system (Becton, Dickinson and Company).

2.9. Intracellular Ca^{2+} concentration

The method of Fluo-3/AM calcium were reported by Selvaraj et al. [28]. Cells in each group were prepared into a cell suspension and were then seeded into a confocal Petri dish. Following treatment with the indicated drugs, the medium was poured out and cells were washed thrice with HBSS solution, followed by staining with 1 μm Fluo-3/AM calcium (Fluo-3/AM; Beijing Solarbio Science & Technology Co., Ltd.) at 37 °C for 30 min. The fluorescence of intracellular calcium was observed under a laser confocal microscope and its intensity was analyzed by Image-Pro Plus software.

2.10. Statistical analysis

All data were analyzed using SPSS 22.0 and GraphPad Prism 5 software. The results are expressed as the mean ± standard deviation. The differences among multiple groups were compared with univariate ANOVA, while those between two groups with least significant difference (LSD)-t test. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. LPS downregulates lncRNA-NONMMUT020270.2 and ITPR2 in HT22 cells

The expression levels of lncRNA-NONMMUT020270.2, ITPR2 and IP3R were significantly lower in cells treated with LPS for 12 and 24 h compared with those in the 0 h LPS group ($p < 0.05$; Fig. 1A and B). In addition, p-tau and $A\beta_{1\text{-}42}$ was notably upregulated in the 12 and 24 h LPS groups compared with the 0 h LPS group ($p < 0.05$; Fig. 1C–E), thus indicating that the LPS-stimulated HT22 cells could reduce expressions of lncRNA-NONMMUT020270.2, ITPR2 and IP3R, as well as increase the expression levels of p-tau and $A\beta_{1\text{-}42}$, which were similar with the results observed in an LPS-induced AD mouse model [15].

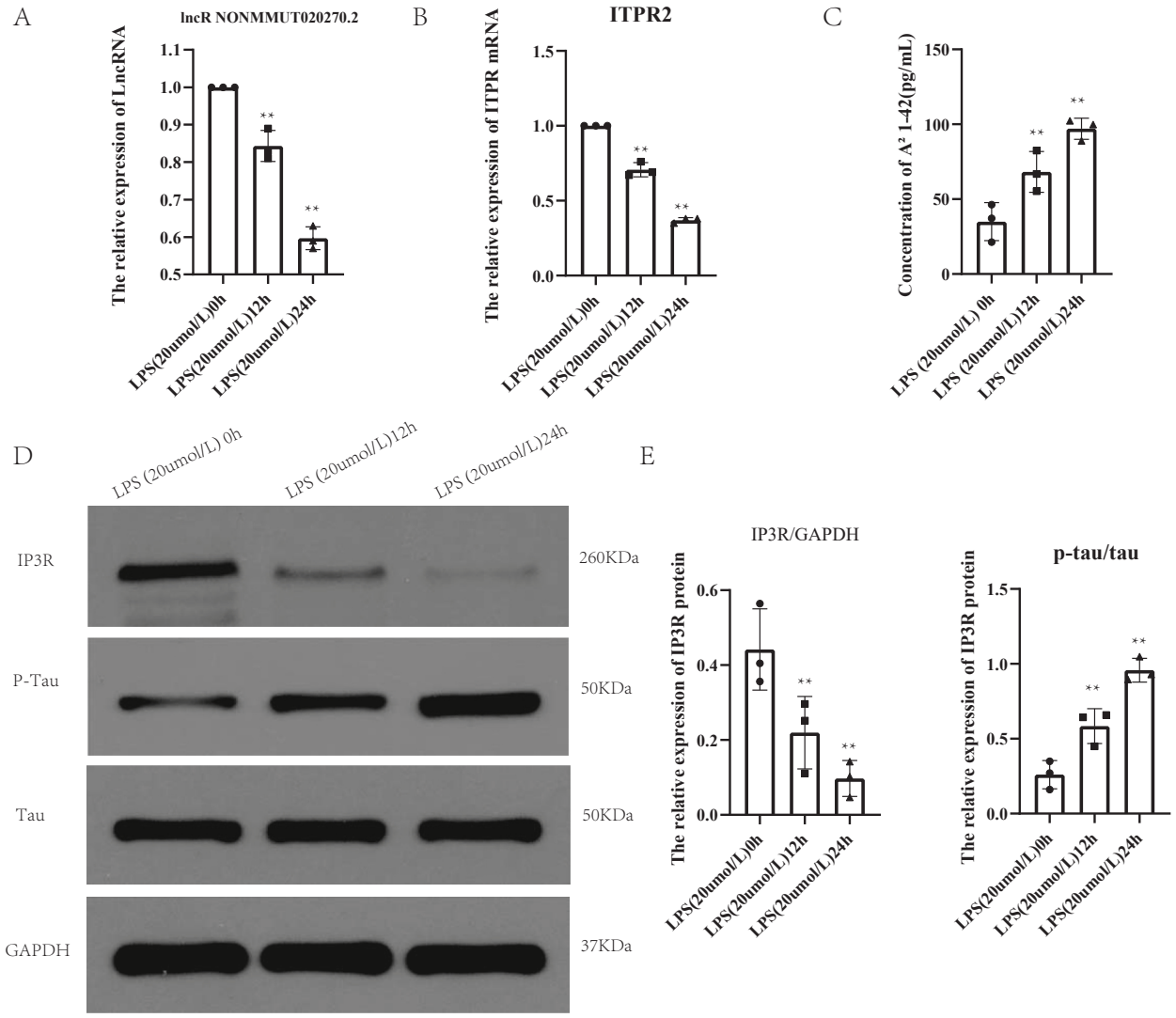

Fig. 1. The expression of NONMMUT020270.2, Itpr2 mRNAs and P-tau and IP3R proteins in HT22 cells treated with LPS. A-B. Relative expression of NONMMUT020270.2, Itpr2 mRNAs in HT22 cells treated with LPS. C. The expression of $A\beta_{1\text{-}42}$ in HT22 cells treated with LPS. D. The expression of P-tau and IP3R proteins in HT22 cells treated with LPS. E. The relative density of P-tau and IP3R proteins in HT22 cells treated with LPS. "**" indicate $p < 0.05$ versus LPS 0h. All experimental results were performed in triplicates.

3.2. ITPR2 expression is regulated by lncRNA-NONMMUT020270.2

To evaluate the regulatory association between lncRNA-NONMMUT020270.2 and ITPR2, HT22 cells were transfected with smart silencer- or pcDNA3.1(+)-lncRNA NONMMUT020270.2. After treated with LPS, the expression levels of NONMMUT020270.2 mRNA significantly decreased in the LPS treated groups compared with the LPS untreated groups ($p < 0.05$; Fig. 2A and H). Compared to the smart-silencer-NC group, the expression level of NONMMUT020270.2 mRNA significantly decreased in the smart-silencer-lncRNA NONMMUT020270.2 group ($p < 0.05$; Fig. 2A). Compared to the pcDNA3.1(+) group, the expression level of NONMMUT020270.2 mRNA was significantly increased in the pcDNA3.1(+)-lncRNA NONMMUT020270.2 group ($p < 0.05$; Fig. 2H).

In addition, the expression levels of ITPR2 mRNA and IP3R protein were significantly decreased in the LPS treated groups compared with the LPS untreated groups ($p < 0.05$; Fig. 2B-E, 2H-2L). The expression levels of ITPR2 mRNA and IP3R protein in the smart-silencer-lncRNA NONMMUT020270.2 group were significantly decreased compared with the smart-silencer-NC group ($p < 0.05$; Fig. 2B-E). After transfection with the pcDNA3.1(+)-lncRNA NONMMUT020270.2 group, the expression levels of ITPR2 mRNA and IP3R protein in the pcDNA3.1(+)-lncRNA NONMMUT020270.2 group were significantly higher than those in the pcDNA3.1(+) group ($p < 0.05$; Fig. 2H–L). After addition of LPS, ITPR2 mRNA and IP3R protein expressions in the pcDNA3.1(+)-lncRNA NONMMUT020270.2+LPS group were decreased ($p < 0.05$), but were still higher than those in the pcDNA3.1(+) + LPS group ($p < 0.05$; Fig. 2H–L). Therefore, we might speculate that LPS can down-regulate ITPR2 expression through lncRNA NONMMUT020270.2, and this down-regulation can be reversed by pcDNA3.1(+)-lncRNA NONMMUT020270.2. As for p-tau and $A\beta_{1\text{-}42}$, significant higher expression levels were detected in the LPS treated groups compared with the LPS untreated groups ($p < 0.05$). Compared with the smart silencer-NC group, the levels of p-tau and $A\beta_{1\text{-}42}$ were markedly enhanced in the smart silencer-lncRNA-NONMMUT020270.2 group ($p < 0.05$; Fig. 2F). And compared with the pcDNA3.1(+) group, the levels of p-tau and $A\beta_{1\text{-}42}$ were markedly decreased in the pcDNA3.1(+)-lncRNA NONMMUT020270.2 group ($p < 0.05$; Fig. 2M).

Furthermore, cell viability was inhibited in the LPS treated groups compared with the LPS untreated groups ($p < 0.05$). After transfection of smart silencer lncRNA NONMMUT020270.2, cell viability significantly reduced, compared with the smart silencer-NC group ($p < 0.05$; Fig. 2G). While, after transfection with pcDNA 3.1(+)-lncRNA NONMMUT020270.2 overexpression vector, the cell viability returned to normal ($p > 0.05$; Fig. 2N). The results indicated that pcDNA 3.1(+)-lncRNA NONMMUT020270.2 could inhibit the decrease cell viability induced by LPS, and smart silencer lncRNA-NONMMUT020270.2 group could promote the decrease cell viability induced by LPS.

3.3. LncRNA-NONMMUT020270.2 silencing accelerates cell apoptosis

To detect the effects of smart-silencer-lncRNA NONMMUT020270.2 and pcDNA3.1(+)-lncRNA NONMMUT020270.2 on the apoptosis rate, we transfected HT22 cells with both of them respectively, and then stained with Hoechst 33258 to observe the morphological changes of the nucleus. As shown in Fig. 3, nuclear condensation, chromatin condensation and deepening of staining

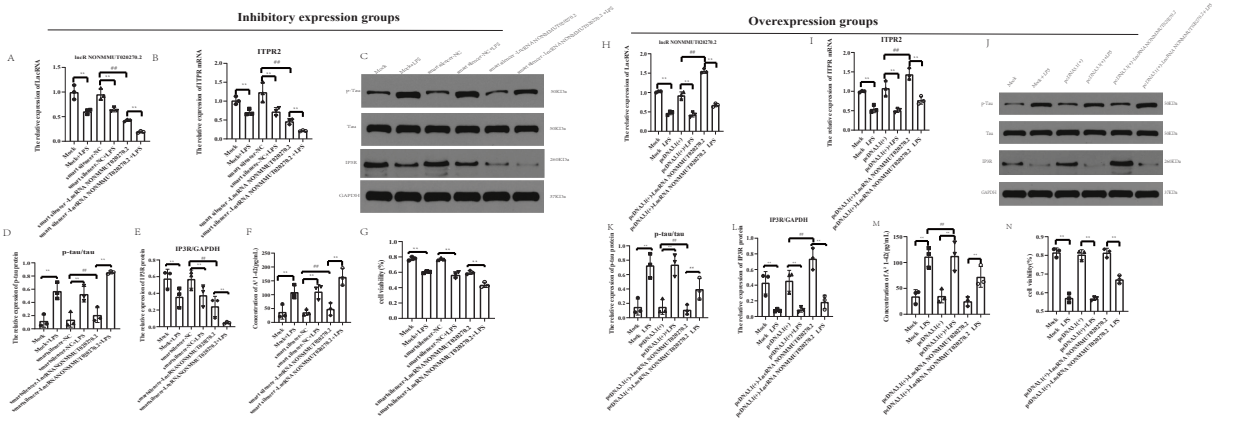

Fig. 2. The relative expression levels of LncRNA NONMMUT020270. and Itpr2 mRNAs, P-tau, $A\beta_{1\text{-}42}$, and IP3R proteins, as well as cell viability in HT22 cells after being transferred by smart-silencer-LncRNA NONMMUT020270.2 and pcDNA3.1(+)-LncRNA NONMMUT020270.2. A-B. The relative expression of LncRNA NONMMUT020270.2 and Itpr2 mRNAs in HT22 cells after being transferred by smart-silencer-LncRNA NONMMUT020270.2. C-E. The expression of P-tau and IP3R proteins in HT22 cells after being transferred by smart-silencer-LncRNA NONMMUT020270.2. F. The expression of $A\beta_{1\text{-}42}$ in HT22 cells after being transferred by smart-silencer-LncRNA NONMMUT020270.2. G. The cell viability in HT22 cells after being transferred by smart-silencer-NONMMUT020270.2., "**" compared to groups without treated LPS, $p < 0.05$. "##" compared to smart-silencer-NC group, $p < 0.05$. H–I. The relative expression of LncRNA NONMMUT020270.2 and Itpr2 mRNAs in HT22 cells after being transferred by pcDNA3.1(+)-LncRNA NONMMUT020270.2. J-L. The expression of P-tau and IP3R proteins in HT22 cells after being transferred by pcDNA3.1(+)-LncRNA NONMMUT020270.2. M. The expression of $A\beta_{1\text{-}42}$ in HT22 cells after being transferred by pcDNA3.1(+)-LncRNA NONMMUT020270.2. N. The cell viability in HT22 cells after being transferred by pcDNA3.1(+)-LncRNA NONMMUT020270.2. "**" compared to groups without treated LPS, $p < 0.05$. "##" compared to pcDNA3.1(+) group, $p < 0.05$. All experimental results were performed in triplicates.

were seen in the LPS treated groups. In the transfected cellgroups, the cells in the pcDNA3.1(+)-lncRNA NONMMUT020270.2 group restored normal nuclear morphology and homogeneous fluorescent staining compared with the pcDNA3.1(+) group. While the cells in the smart-silencer-lncRNA NONMMUT020270.2 group showed crescent-shaped nuclei, obvious aggregation of chromatin, and the appearance of tonoplast vesicles compared with the smart-silencer-NC group (Fig. 3A and F).

In addition, flow cytometry and western-blotting were used to further detect the apoptosis rate and the expression level of caspase-1 protein after transfection. Apoptosis rate and caspase-1 protein expression levels were significantly elevated in the LPS treated groups compared to the LPS untreated groups ($p < 0.05$). And the smart-silencer-lncRNA NONMMUT020270.2 group showed a significant higher apoptosis rate and expression level of caspase-1 compared to the smart-silencer-NC group ($p < 0.05$; Fig. 3B–E). Whereas the pcDNA3.1(+)-lncRNA NONMMUT020270.2 group showed a significantly lower apoptosis rate and expression level of caspase-1 protein than those in the pcDNA3.1(+) group ($p < 0.05$; Fig. 3G–J).

3.4. *LncRNA-NONMMUT020270.2 knockdown enhances the concentration of intracellular Ca^{2+}*

Intracellular Ca^{2+} concentrations in the LPS treated groups were significantly increased compared with LPS untreated groups ($p < 0.05$; Fig. 4A–D). In addition, the intracellular Ca^{2+} concentration was also notably enhanced in the smart silencer-lncRNA-NONMMUT020270.2 group compared with the smart silencer-NC group ($p < 0.05$; Fig. 4A and B). However, lncRNA-NONMMUT020270.2 overexpression exhibited the opposite effect ($p < 0.05$; Fig. 4C and D).

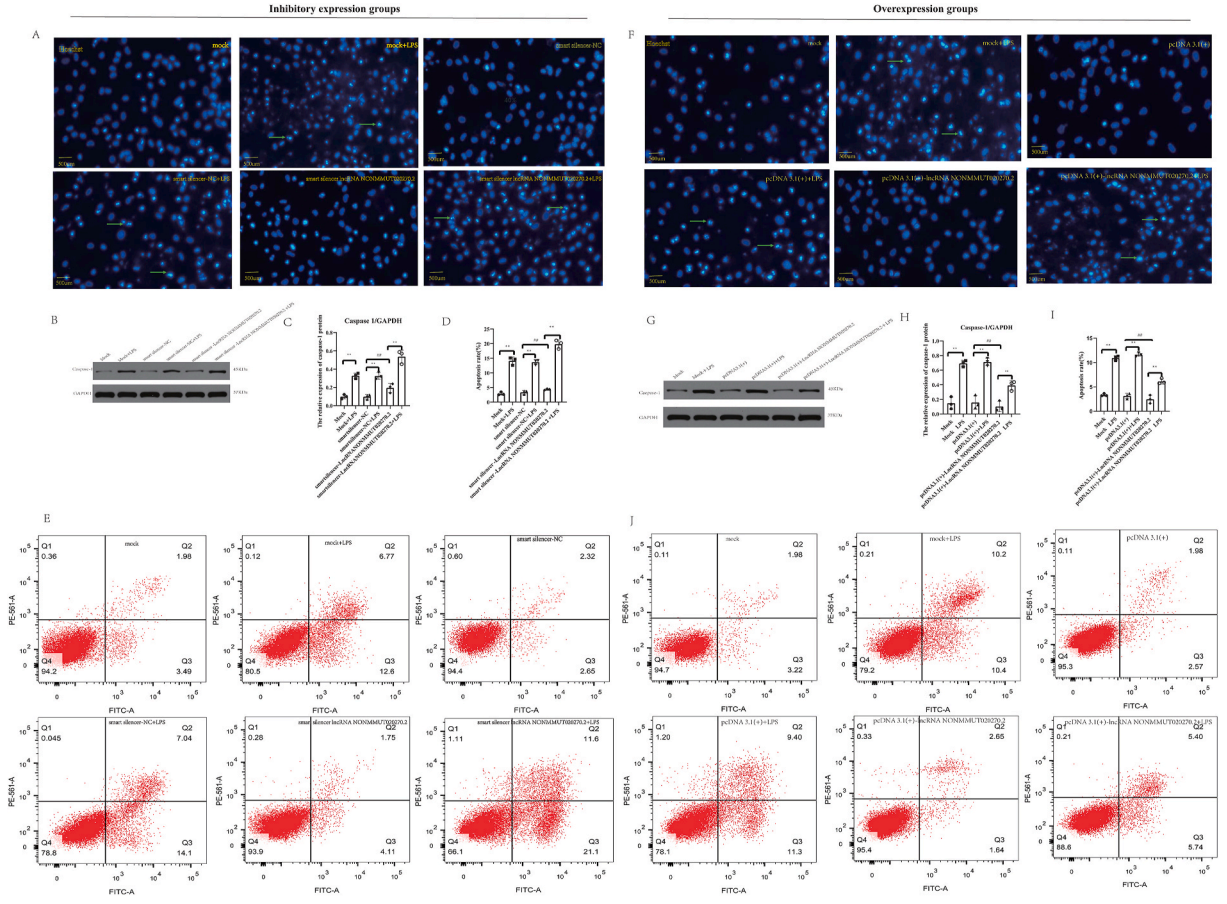

Fig. 3. The apoptosis in HT22 cells after being transferred by smart-silencer-NONMMUT020270.2 and pcDNA3.1(+)-LncRNA NONMMUT020270.2. A. Morphological changes of HT22 cell nuclei after being transferred by smart-silencer-NONMMUT020270.2 (Stained with Hoechst 33258 × 200). B–C. The relative expression level of caspase-1 in HT22 cells after being transferred by smart-silencer-NONMMUT020270.2. D-E. The apoptosis rate analysis and flow cytometry after being transferred by smart-silencer-NONMMUT020270.2. "**" compared to groups without treated LPS, $p < 0.05$. "##" compared to smart-silencer-NC group, $p < 0.05$. F. Morphological changes of HT22 cell nuclei after being transferred by pcDNA3.1(+)-LncRNA NONMMUT020270.2 (Stained with Hoechst 33258 × 200). G-H. The relative expression level of caspase-1 in HT22 cells after being transferred by pcDNA3.1(+)-LncRNA NONMMUT020270.2. I-J. The apoptosis rate analysis and flow cytometry after being transferred by pcDNA3.1(+)-LncRNA NONMMUT020270.2. "**" compared to groups without treated LPS, $p < 0.05$. "##" compared to pcDNA3.1 (+) group, $p < 0.05$. All experimental results were performed in triplicates.

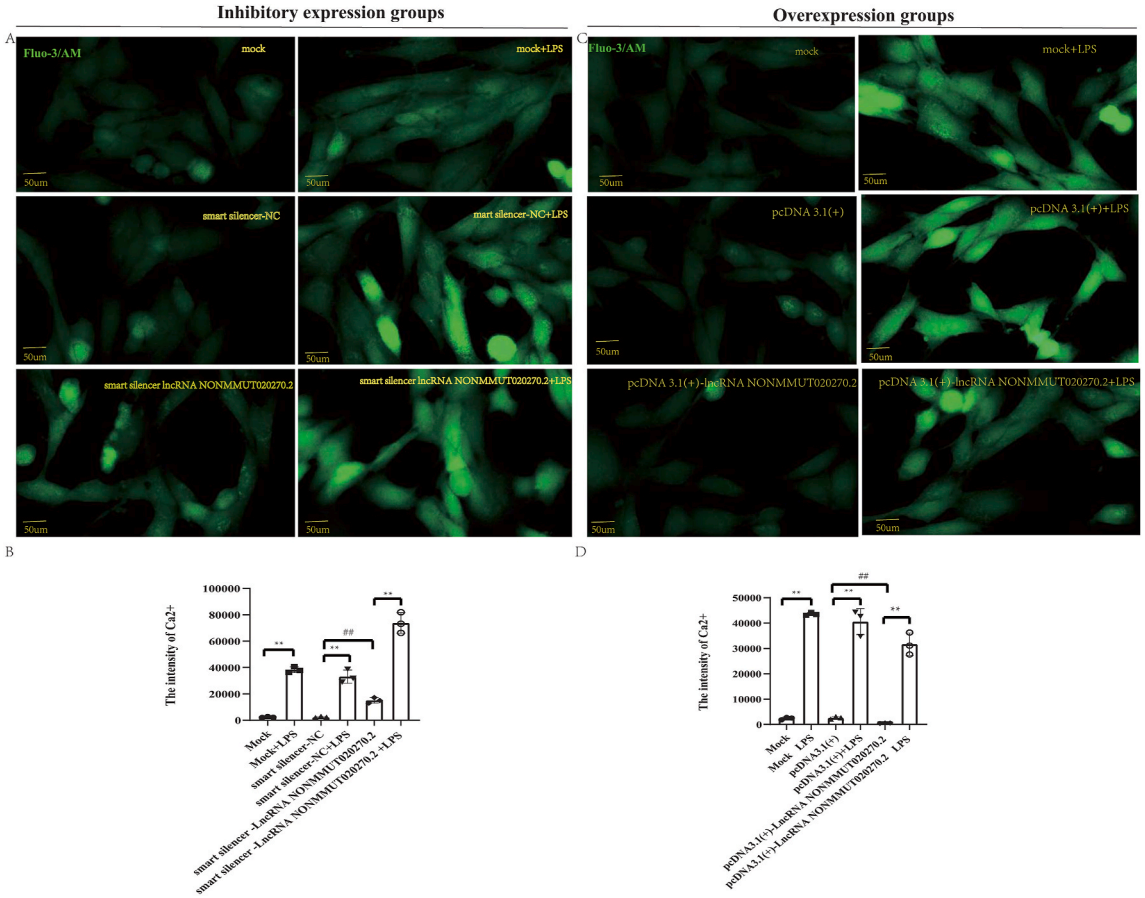

Fig. 4. The intensity of Ca^{2+} in HT22 cell after being transferred by smart-silencer-NONMMUT020270.2 and pcDNA3.1(+)-LncRNA NONMMUT020270.2. A-B. The intensity of Ca^{2+} in HT22 cell after being transferred by smart-silencer-NONMMUT020270.2. "**" compared to groups without treated LPS, $p < 0.05$. "##" compared to smart-silencer-NC group, $p < 0.05$ (200X). C-D. The intensity of Ca^{2+} in HT22 cell after being transferred by pcDNA3.1(+)-LncRNA NONMMUT020270.2. "**" compared to groups without treated LPS, $p < 0.05$. "##" compared to pcDNA3.1 (+) group, $p < 0.05$ (200X). All experimental results were performed in triplicates.

4. Discussion

Calcium homeostasis imbalance appears during the progression of AD disease or even early in the pathological changes of AD. It has been reported that calcium homeostasis imbalance could increase the production of Aβ, and increased Aβ could promote Ca^{2+} homeostasis imbalance [29,30]. High cytoplasmic Ca^{2+} can inhibit the activity of α-secretase, which in turn promotes the production of cytoplasmic $A\beta_{1\text{-}42}$ and accelerates the pathogenesis of AD [31]. Another study showed that the expression of Ca^{2+} channel proteins and the deposition of Aβ increased 3-fold when the cDNA of APP gene was transferred into HEK293 cells, indicating a positive correlation between cytoplasmic Ca^{2+} concentration and Aβ deposition [32]. It has also been shown that disturbed Ca^{2+} signaling pathways in PS1 transgenic mice may be caused by endoplasmic reticulum Ca^{2+} overload [33]. Aβ have toxic effects on a wide range of cells, including neurons, which can lead to neuronal loss in specific brain regions. Aβ can lead to neuronal loss through the apoptotic pathway in an in vitro cultured neuronal model [34]. Neuronal apoptosis was shown to be associated with Aβ-mediated intracellular Ca^{2+} homeostasis imbalance [34,35]. Previous studies revealed that Aβ could significantly increase the intracellular Ca^{2+} concentration and activate aspartate specific cysteine proteinase-3 (caspase-3), thus resulting in cell apoptosis [36,37]. Aβ-induced neuronal apoptosis has also been observed in the brain of human subjects, in transgenic mice and cultured nerve cells [38,39]. In addition, Aβ promoted the release of Ca^{2+} from the ER, thus leading to Ca^{2+} overload in the cytoplasm, decreased mitochondrial membrane potential and translocation of Bax to mitochondria, eventually resulting in oxidative stress-induced injury of mitochondria and the activation of apoptosis channels within mitochondria [40,41]. Intracellular calcium release channels include the IP3R and ryanodine receptor (Ryr) channels [42]. As the integrator of several signal transduction pathways, IP3R is present in the ER in the majority of cells and mediates the release of intracellular Ca^{2+}. The overactivation of this receptor can cause a rapid increase in the intracellular Ca^{2+} concentration, thus leading to apoptosis [43]. Another study revealed that Aβ could enhance the content of intracellular Ca^{2+}, activate the expression of apoptotic factors and induce apoptosis [44]. The toxic increase in intracellular Ca^{2+} could not be reversed by MK-801 or the voltage-dependent calcium channel antagonist, nimodipine, but only by the IP3R inhibitor, XeC, thus suggesting that IP3R/Ca^{2+}

could be a significant signaling pathway involved in the pathological process of AD [45].

On the other hand, Ca^{2+} can promote tau protein phosphorylation by activating multiple sites of Tau proteins such as Thr181, Thr231, and Ser396. In vitro cultured neurons increase cytoplasmic Ca^{2+} concentration by depolarization, which further - by activating protein kinase activities such as GSK3α/β and CDK5 and promoting tau protein phosphorylation [46]. Over-phosphorylated Tau proteins are prone to cause self-aggregation due to their inability to bind to microtubules, which in turn leads to the formation of double-stranded helical fibers, and ultimately NFTs, leading to neuronal death [47]. In summary, Ca^{2+} metabolic disorders can contribute to the pathogenesis of AD by modulating two major pathological features of AD. However, compared with studies related to Ca^{2+} regulation of Aβ deposition, there are relatively few studies on Ca^{2+} regulation of tau phosphorylation.

ER is the major intracellular organelle for Ca^{2+} storage, buffering and signal transduction. Its release is dependent on the binding of the second messenger IP3 to IP3R, which changes the conformation of the receptor complex and opens the ion channel [48,49]. The abnormal function of IP3R is closely associated with the pathogenesis of AD. A significant decrease in the amount of IP3R in the parietal lobe, hippocampus, upper frontal and temporal cortex, and cerebellum has been observed in patients with AD [50,51]. Street et al. [52] showed that IP3R deficiency could lead to the loss of the intracellular calcium pool in IP3R-knockout rats. In the present study, the expression levels of ITPR2 mRNA and IP3R protein were significantly reduced in LPS-treated cells. However, following cell transfection with smart silencer-lncRNA-NONMMUT020270.2, ITPR2 mRNA and IP3R protein were notably downregulated. By contrast, the levels of ITPR2 and IP3R were markedly enhanced in lncRNA-NONMMUT020270.2-overexpressing cells compared with the mock group. The above results suggested that the expression of ITPR2 and IP3R were notably altered following lncRNA-NONMMUT020270.2 overexpression or silencing, thus indicating that ITPR2/IP3R could be involved in the pathogenesis of AD.

The results of the current study demonstrated that promoting the expression of lncRNA-NONMMUT020270.2 could enhance the mRNA and protein expression levels of ITPR2, while lncRNA-NONMMUT020270.2 silencing in the LPS-induced AD cell model exhibited the opposite effect, thus suggesting that lncRNA-NONMMUT020270.2 could affect the occurrence and development of AD via regulating the expression of ITPR2. It has been reported that lncRNAs can act as competitive endogenous RNAs (ceRNAs) to interact with microRNAs, thereby participating in the regulation of the expression of their target genes and modulating several different physiological and pathological processes [53,54]. For instance, a study showed that BACE1-AS competed with microRNA (miR)-485-5p and miR-214-3p to bind their target genes, beta-secretase 1 and autophagy related 5, respectively, to inhibit the function of the corresponding miRNAs and positively regulate the expression of these genes [55,56]. In addition, IRT1-AS, a natural antisense lncRNA, could competitively bind miR-34a and increase the stability of sirtuin 1 (SIRT1) mRNA in C2C12 cells, thus being associated with muscle aging [57]. Furthermore, lncRNAs can also interact with transcription factors (TFs) and regulate the expression of target genes. The TFs CCAAT/enhancer-binding protein-alpha and yin yang 1 were separately reported to regulate the expression of lncRNAs involved in multiple biological processes [58,59]. Bioinformatics analysis in a previous study of our laboratory predicted the ceRNA network and TFs, including lncRNA-NONMMUT020270.2 and the ITPR2 gene [15]. The results implied that lncRNA-NONMMUT020270.2 could regulate the expression of ITPR2 via miRNAs, including miR-183-5p.2, miR-96-5p/1271-5p and miR-216a-5p. Furthermore, lncRNA-NONMMUT020270.2 was predicted to be regulated by several TFs, including forkhead box L1 and cell division cycle 5-like protein. To the best of our knowledge, the present study was the first to report that the expression of ITPR2 was regulated by lncRNA0-NONMMUT020270.2 in an LPS-induced cell. It was therefore hypothesized that lncRNA-NONMMUT020270.2 could also decrease the stability of ITPR2 mRNA by a mechanism like BACE1-AS or SIRT1-AS, thus downregulating ITPR2 in AD.

However, the current study has some limitations. Firstly, although the present study suggested that lncRNA-NONMMUT020270.2 could be involved in the development of AD, its function was not fully elucidated. Therefore, further studies are needed to verify the above hypothesis. Secondly, the pathway involved in the lncRNA-NONMMUT020270.2-mediated regulation of ITPR2 expression remains unclear and therefore further research is needed to excavate the specific underlying mechanisms. Thus, it is necessary to identify the results of the present study and investigate the in-depth regulating mechanism of lncRNA-NONMMUT020270.2 on ITPR2 in the future.

5. Conclusion

ITPR2 expression was positively correlated with lncRNA-NONMMUT020270.2 expression in LPS-induced cell. Downregulating the lncRNA-NONMMUT020270.2 and ITPR2 may promote cell apoptosis and increase intracellular Ca^{2+} concentration.

Data availability statement

Data included in article/supp. material/referenced in article.

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CRediT authorship contribution statement

Lan Liu: Writing – original draft, Methodology, Data curation. **Liang Tang:** Software, Formal analysis, Data curation. **Yan Wang:** Software, Formal analysis, Data curation. **Shanling Liu:** Writing – review & editing, Project administration, Funding acquisition. **Yongcang Zhang:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33491.

Abbreviations

LPS	lipopolysaccharide
AD	Alzheimer's disease
ITPR2	inositol 1,4,5-trisphosphate receptor type 2
IP3R	inositol trisphosphate receptor
ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
OD	optical density
TFs	transcription factors

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