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Sodium butyrate causes α-synuclein degradation by an Atg5-dependent and PI3K/Akt/mTOR-related autophagy pathway

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ABSTRACT

Aggregation of α-Synuclein is central to the pathogenesis of Parkinson's disease (PD). However, these α-Synuclein inclusions are not only present in brain, but also in gut. Enteroendocrine cells (EECs), which are directly exposed to the gut lumen, can express α-Synuclein and directly connect to α-Synuclein-containing nerves. Dysbiosis of gut microbiota and microbial metabolite short-chain fatty acids (SCFAs) has been implicated as a driver for PD. Butyrate is an SCFA produced by the gut microbiota. Our aim was to demonstrate how α-Synuclein expression in EECs responds to butyrate stimulation. Interestingly, we found that sodium butyrate (NaB) increases α-Synuclein mRNA expression, enhances Atg5-mediated autophagy (increased LC3B-II and decreased SQSTM1 (also known as p62) expression) in murine neuroendocrine STC-1 cells. Further, α-Synuclein mRNA was decreased by the inhibition of autophagy by using inhibitor bafilomycin A1 or by silencing Atg5 with siRNA. Moreover, the PI3K/Akt/mTOR pathway was significantly inhibited and cell apoptosis was activated by NaB. Conditioned media from NaB-stimulated STC-1 cells induced inflammation in SH-SY5Y cells. Collectively, NaB causes α-Synuclein degradation by an Atg5-dependent and PI3K/Akt/mTOR-related autophagy pathway.

1. Introduction

Recent studies suggest that brain function and behavior are influenced by microbial metabolite short-chain fatty acids (SCFAs) [1–3]. SCFAs, primarily acetate, propionate, and butyrate, are organic acids produced in the colon by bacterial fermentation of mainly undigested dietary carbohydrates [4]. Among SCFAs, butyrate has received particular attention for its beneficial effects on both intestinal and brain functions, such as colonic homeostasis and blood brain barrier permeability [5–7]. Growing evidence points to the impact of butyrate on the brain via the gut-brain axis. Our recent studies reported that fecal butyrate is increased in an acute and subacute Parkinson's disease (PD) mouse model induced by 1-methyl-4-phenyl-1,2,3,6-tetrathydropyridine (MPTP) compared to the control [8,9].

The pathological hallmark of PD is the presence of aggregated misfolded α-Synuclein (Lewy bodies) in the central nervous system (CNS) as well as enteric nervous system (ENS) [10]. Braak demonstrated that α-Synuclein appears in the ENS during the early-stage of PD, and this misfolded α-Synuclein then spreads cell to cell within the gut to reach the vagal projections, allowing pathological α-Synuclein to enter the CNS and result in neuron damage in the *substantia nigr*a as well as motor and non-motor symptoms [11–14]. In particular, EECs, which are part of the gut espithelium and are directly exposed to the gut lumen, could express α-Synuclein and directly connect to α-Synuclein-containing nerves. Therefore, it may be possible to form a neural circuit between the gut and nervous system in which environmental influences in the gut lumen could affect α-Synuclein folding in EECs, thereby enabling misfolded α-Synuclein to propagate from the gut epithelium to the brain [15]. How EECs in the gut respond to butyrate stimulation remains unknown. Here, we report that sodium butyrate (NaB) induces increases in α-Synuclein mRNA, but not α-Synuclein protein in the STC-1 EEC cell line.

The autophagy-lysosome pathway (ALP) or ubiquitin-proteasome system (UPS) has been suggested to contribute to α-Synuclein turnover [16]. Moreover, exosome/extracellular vesicles have also been found to be responsible for cell-to-cell transfer of α-Synuclein [17]. In particular,

Abbreviations: PD, Parkinson's disease; EECs, enteroendocrine cells; SCFAs, short-chain fatty acids; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrathydropyridine; CNS, central nervous system; ENS, enteric nervous system; mTOR, the kinase mammalian target of rapamycin; PI3K, phosphatidylinositol-3 kinase; Akt, serine/threonine kinase; BafA1, bafilomycin A1; FBS, fetal bovine serum; PBS, phosphate buffer saline

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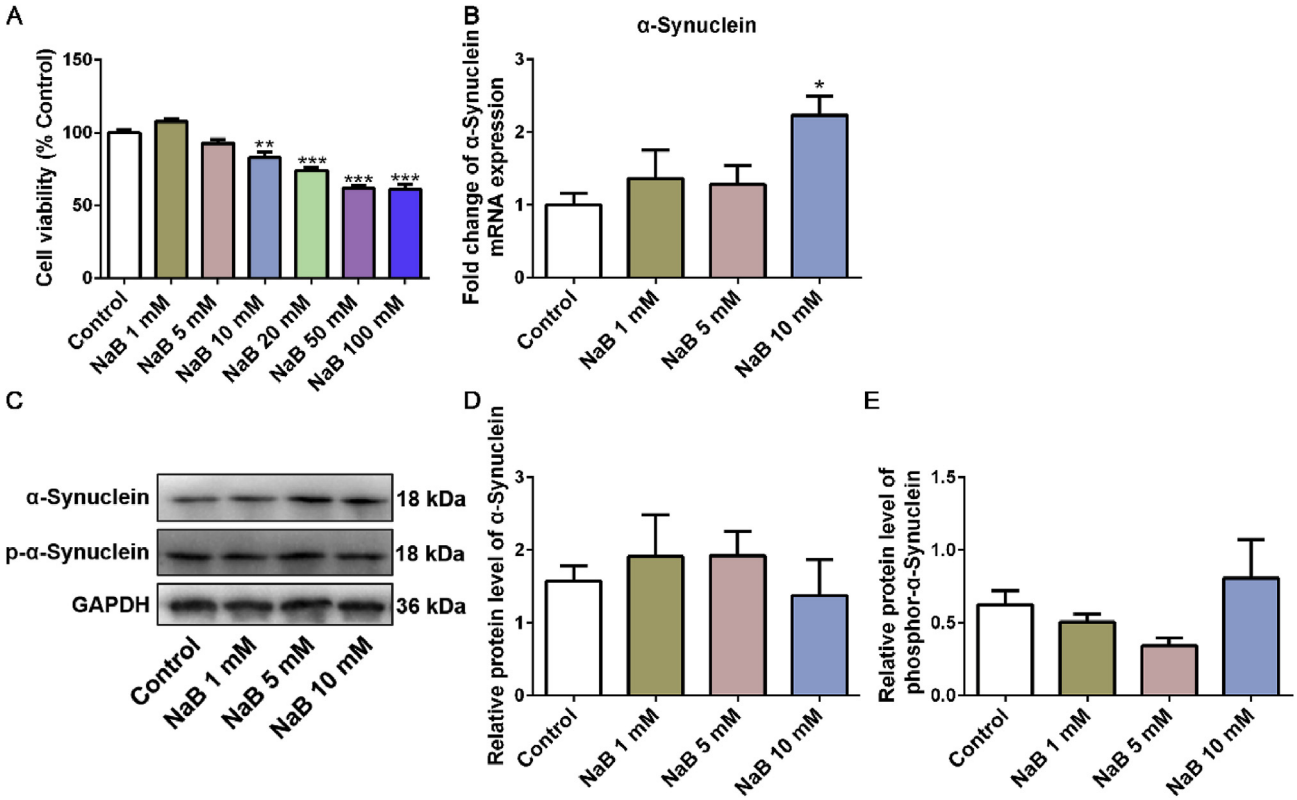

Fig. 1. A certain concentration of NaB induce α-Synuclein mRNA increased but not α-Synuclein protein in STC-1 cells. (A) MTT assay showed NaB-induced cytsssssssotoxicity is dose-dependent in STC-1 cells. STC-1 cells were treated with the indicated concentrations (1, 5, 10, 20, 50 and 100 mM) of NaB for 24 h n = 6. (B) qPCR analyzed α-Synuclein mRNA expression in STC-1 cells treated with different doses of NaB for 24 h n = 8. (C) Representative Western blot of total α-Synuclein and phospho-α-Synuclein expressions in STC-1 cells treated with different doses of NaB for 24 h. (D) Quantification of α-Synuclein expression was normalized to GAPDH. No significant difference was observed in the protein level of α-Synuclein. (E) Quantification of phospho-α-Synuclein expression was normalized to GAPDH. n = 6. No significant difference was observed on the protein level of phospho-α-Synuclein. Data represent the means ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$.

autophagy has been shown to be critically important for neuronal health and NaB has been demonstrated to induce autophagy and endoplasmic reticulum stress [18]. Autophagy is a highly conserved multistep process that is regulated by several autophagy-related (Atg) genes [19]. These genes (such as Atg3, Atg7, Atg10 and Atg5) have multiple functions in various physiological contexts [20]. Among these genes, Atg5 is a key autophagy protein required for conjugation of the ubiquitin-like protein LC3 to the phagophore and confirmed to be important for α-Synuclein degradation [21]. Within the autophagy network, the kinase mammalian target of rapamycin (mTOR) can be activated by phosphatidylinositol-3 kinase (PI3K) and serine/threonine kinase (Akt), resulting in the inhibition of autophagy [22,23]. Indeed, autophagy can often end with apoptosis and apoptosis may begin with autophagy. Studies have shown that caspases can be activated via recruitment to the autophagosomes, then leading to cell apoptosis [24]. The role of autophagy and α-Synuclein turnover in EECs is not completely understood.

Herein, we investigate whether NaB could induce α-Synuclein expression and release in the STC-1 EEC cell line, and regulate Atg5-dependent autophagy and PI3K/Akt/mTOR signaling, as well as cell apoptosis, and demonstrate that NaB activated Atg5-dependent autophagy pathway and suppressed PI3K/Akt/mTOR-related autophagy pathway, as well as enhanced caspase-3-mediated apoptosis in STC-1 cells. Moreover, we further demonstrate that supernatant from STC-1 cells following stimulation of NaB induced release of pro-inflammatory factors, such as TNF-α and IL-1β, from neuronal cells.

2. Material and methods

2.1. Cell culture and treatments

The murine STC-1 enteroendocrine cell line (ATCC®, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM, 11965092, Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The human neuroblastoma cell line SH-SY5Y (ATCC®, USA) was maintained in DMEM/F12 (C11330500BT, Gibco, USA) supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cells were grown in a 5% CO_2 humidified chamber at 37 °C, and split or harvested every 2–3 days.

NaB (B5887, Sigma Aldrich, USA) was prepared as a 1 M stock solution in sterile phosphate buffer saline (PBS) and was freshly diluted with culture medium to final treatment concentrations ranging from 1 to 100 mM. STC-1 cells were treated with NaB or PBS (control) for 24 h.

For autophagy inhibiton experiment, STC-1 cells were treated with 10 mM NaB for 24 h, then the culture medium was replaced with autophagy inhibitor bafilomycin A1 (BafA1, HY-100558, MedChemExpress, USA) used at 200 nM for 6 h.

For cell supernatant transfer experiments, STC-1 cells were treated with 10 mM NaB for 24 h. After incubation, the conditioned culture medium of STC-1 cells was collected and centrifuged at 13,000 rpm, 4 °C for 5 min to remove cell debris. Then, the new supernatant was collected and transferred to SH-SY5Y cells immediately for another 24 h.

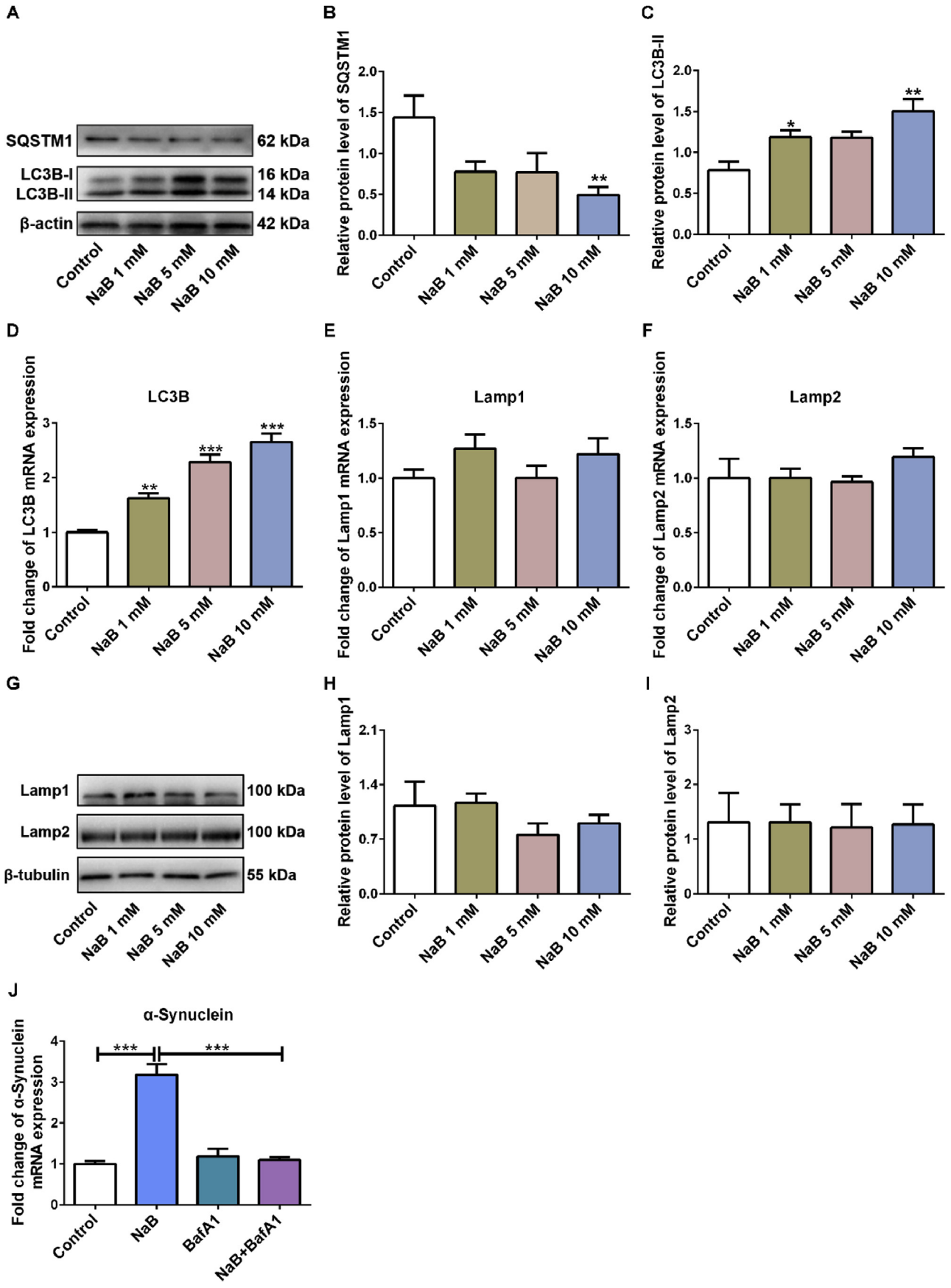

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Fig. 2. NaB induces autophagy and regulates α-Synuclein release at the mRNA level in STC-1 cells. (A) Representative Western blot of SQSTM1 and LC3B expressions in STC-1 cells which were treated with different doses of NaB for 24 h. (B) Quantification of SQSTM1 expression was normalized to β-actin. A decrease was observed on SQSTM1 protein level in NaB-treated STC-1 cells. (C) Quantification of LC3B expression was normalized to β-actin. Significant increases were observed on protein levels of LC3B following NaB treatment of STC-1 cells. n = 6. (D) qPCR analysis of LC3B mRNA, (E) Lamp1 mRNA and (F) Lamp2 mRNA expression in STC-1 cells, which were treated with different doses of NaB for 24 h n = 8 (G) Representative Western blot of Lamp1 and Lamp2 expressions in STC-1 cells, which were treated with different doses of NaB for 24 h. (H) Quantification of Lamp1 and (I) Lamp2 expression was normalized to β-tubulin. n = 6. No change was observed on Lamp1 or Lamp2 protein levels in NaB-treated STC-1 cells. (J) qPCR analysis of α-Synuclein mRNA expression in STC-1 cells treated with 10 mM NaB for 24 h or/and BafA1 for 6 h n = 8. Data represent the means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

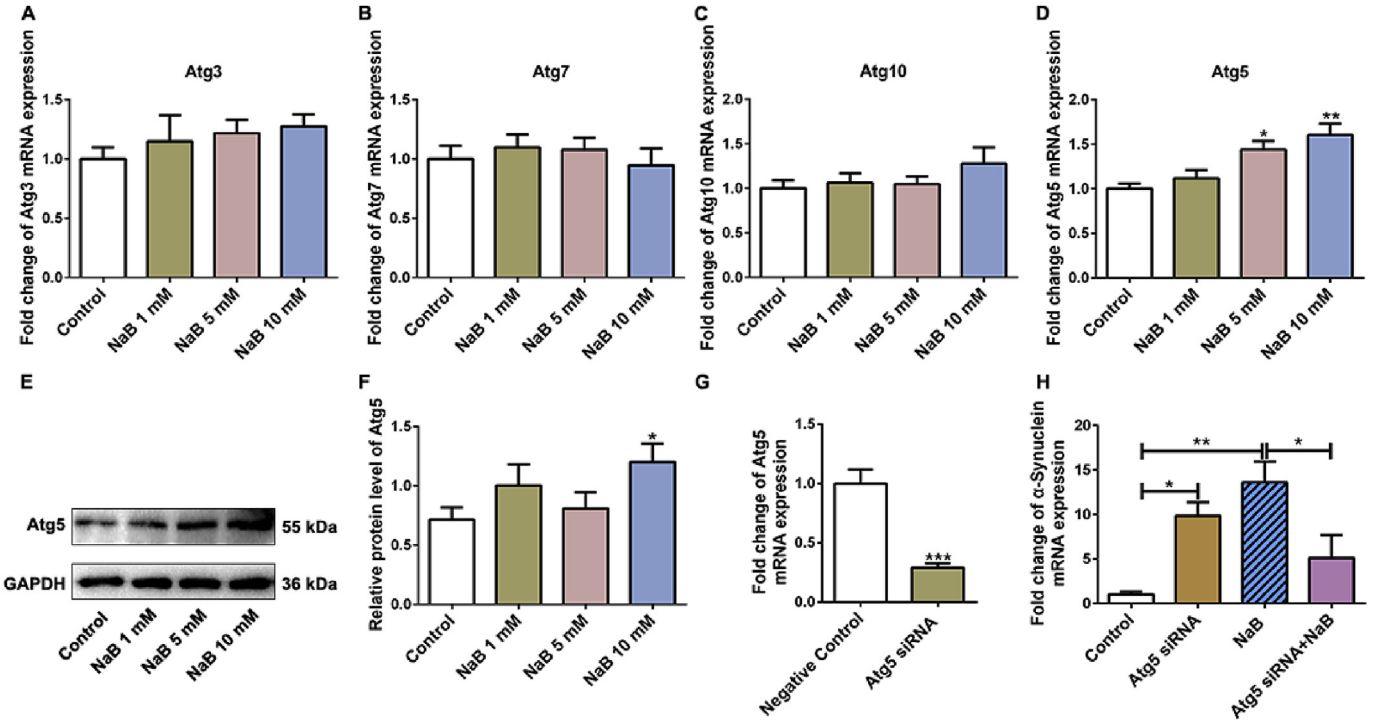


Fig. 3. NaB modulates α-Synuclein mRNA expression through Atg5-dependent autophagy pathway in STC-1 cells. qPCR analysis of (A) Atg3, (B) Atg7, (C) Atg10 and (D) Atg5 mRNA expression in STC-1 cells treated with different doses of NaB for 24 h n = 8. (E) Representative Western blot of Atg5 expression in STC-1 cells treated with different doses NaB for 24 h. (F) Quantification of Atg5 expression was normalized to GAPDH. Significant increases were observed on the protein level of Atg5 following treatment with 10 mM NaB. n = 6. (G) qPCR analysis of Atg5 mRNA expression in Atg5 siRNA-transfected STC-1 cells. (H) qPCR analysis of α-Synuclein mRNA expression in STC-1 cells treated with 10 mM NaB for 24 h or/and Atg5 siRNA for 24 h n = 8. Data represent the means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

2.2. Cell viability assay

Cell viability was estimated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 468941, J&K, China) assay. STC-1 cells were seeded in 96-well (5×10^5 cells/well) culture plates and treated with concentrations of 1, 5, 10, 20, 50 or 100 mM NaB for 24 h. After incubation, 20 μL MTT (5 mg mL^{-1}) was added to each well and incubated at 37 °C for 4 h. Subsequently, 150 μL of DMSO was added to each well and mixed thoroughly. Absorbance was determined by using an enzyme-linked immunosorbent assay reader at 490 nm, and viability was determined as the percentage absorbance of treated cultures compared with those of untreated control cultures.

2.3. Small-interfering RNA (siRNA) transfection

siRNA against Atg5 and a non-specific scrambled siRNA were purchased from GenePharma (Suzhou, China) and transfected into cells using Lipofectamine 2000 (11668019, Invitrogen, USA) according to the manufacturer's guidelines. STC-1 cells were cultured in 12-well plates with Opti-MEM™ containing control siRNA or Atg5 siRNA and Lipofectamine 2000 for 4 h. After 4 h incubation, the transfection mixtures were removed and were replaced with fresh DMEM containing 10% FBS with or without 10 mM NaB for 20 h. The siRNA sequences were as follows: negative control (NC) siRNA: 5′-UUCUCCGAACGUG UCACGUTT-3′ (sense), 5′-ACGUGACACGUUCGGAGAATT-3′ (antisense); Atg5 siRNA: 5′-GCUUUACUCUCUAUCAGGATT-3′ (sense), 3′-UCCUGAUAGAGAGUAAAGCTT-5′ (antisense).

2.4. RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was extracted from cells or mice tissue using TRIzol (15596018, Invitrogen, USA) reagent according to the manufacturer's instructions. The cDNA was synthesized using PrimeScript™ RT reagent kit (RR036A, TaKaRa, China), following the manufacturer's instructions. The following primers were used for STC-1 cells: α-Synuclein: 5′-GCAAGGGTGAGGAGGGGTA-3′ (forward) and 5′-CCTCTGAAGGCA TTTCATAAGCC-3′ (reverse); LC3B: 5′-TTATAGAGCGATACAAGGGG GAG-3′ (forward) and 5′-CGCCGTCTGATTATCTTGATGAG-3′ (reverse); TNF-α: 5′-CGTCAGCCGATTTGCTATCT-3′ (forward) and 5′-CGGACTC CGCAAAGTCTAAG-3′ (reverse); IL-1β: 5′-AAGCTCTCCACCTCAAT GGA-3′ (forward) and 5′-TGCTTGAGAGGTGCTGATGT-3′ (reverse); Atg5: 5′-TGTGCTTCGAGATGTGTGGTT-3′ (forward) and 5′-GTCAAAT AGCTGACTCTTGGCAA-3′ (reverse); Atg3: 5′- ACACGGTGAAGGGAAA GGC-3′ (forward) and 5′-TGGTGGACTAAGTGATCTCCAG-3′ (reverse); Atg7: 5′-GTTCGCCCCCTTTAATAGTGC-3′ (forward) and 5′-TGAACTC CAACGTCAAGCGG-3′ (reverse); Atg10: 5′-GTAGTTACCAAGTGCCGG

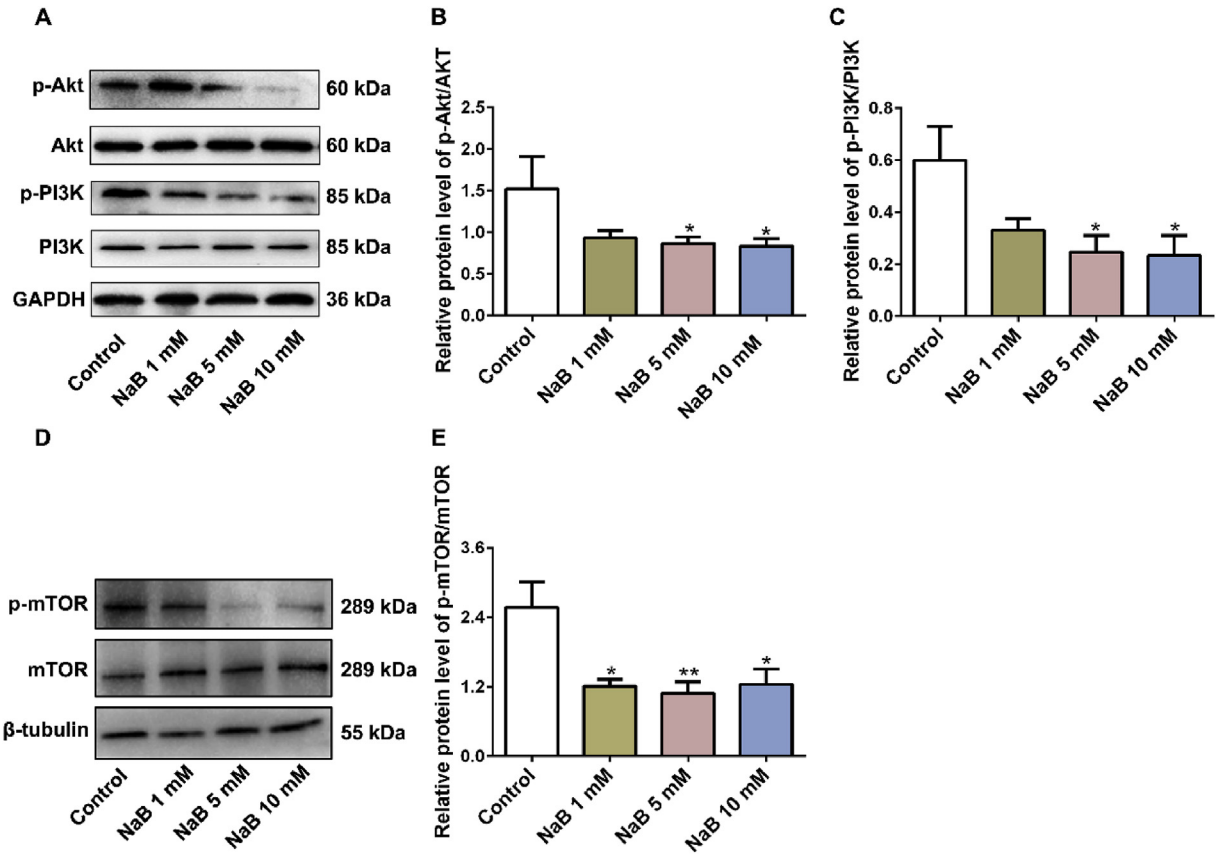


Fig. 4. NaB modulates autophagy via downregulation of mTOR/PI3K/Akt pathway in STC-1 cells. (A) Representative Western blot of *p*-Akt, total Akt, p-PI3K and total PI3K expressions in STC-1 cells treated with different concentrations of NaB for 24 h. (B) Quantification of the *p*-Akt/total Akt ratio. Significant decreases were observed at the protein level of the *p*-Akt/total Akt ratio in NaB-treated STC-1 cells. (C) Quantification of the p-PI3K/total PI3K ratio. Significant decreases were observed at the protein level of p-PI3K/total PI3K in NaB-treated STC-1 cells. (D) Representative Western blot of *p*-mTOR and total mTOR expressions in STC-1 cells treated with different concentrations of NaB for 24 h. (E) Quantification of the *p*-mTOR/total mTOR ratio. Significant decreases were observed in the *p*-mTOR/total mTOR ratio in NaB-treated STC-1 cells. n = 6. Data represent the means ± SEM; *P < 0.05, **P < 0.01.

TTC-3′ (forward) and 5′-AGCTAACGGTCTCCCATCTAAA-3′ (reverse); Lamp1: 5′-CAGCACTCTTTGAGGTGAAAAAC-3′ (forward) and 5′-ACG ATCTGAGAACCATTCGCA-3′ (reverse); Lamp2: 5′-TGTATTTGGCTAAT GGCTCAGC-3′ (forward) and 5′-TATGGGCACAAGGAAGTTGTC-3′ (reverse); GAPDH: 5′-AGGTCGGTGTGAACGGATTTG-3′ (forward) and 5′-TGTAGACCATGTAGTTGAGGTCA-3′ (reverse). The following primers were used for SH-SY5Y cells: α-Synuclein: 5′-AAGAGGGTGTTCT CTATGTAGGC-3′ (forward) and 5′-GCTCCTCCAACATTTGTCACTT-3′ (reverse); TNF-α: 5′-CCCTCACACTCAGATCATCTTCT-3′ (forward) and 5′-GCTACGACGTGGGCTACAG-3′ (reverse); IL-1β: 5′-ATGATGGCTTAT TACAGTGGCAA-3′ (forward) and 5′- GTCGGAGATTCGTAGCTGGA-3′ (reverse); GAPDH: 5′-GGAGCGAGATCCCTCCAAAAT-3′ (forward) and 5′-GGCTGTTGTCATACTTCTCATGG-3′ (reverse). Quantitative real-time PCR was carried out using SYBR® Premix Ex TaqTM II (RR820A, TaKaRa, China) according to the manufacturer's protocol. The mRNA quantification was estimated by the formula from the $2^{-\Delta\Delta Ct}$ method. Expression of mRNA was normalized to GAPDH mRNA, which served as the control gene in all samples.

2.5. Western blot analysis

Western blots were performed as previously described [25]. Briefly, cells were lysed with RIPA lysis buffer (P0013C, Beyotime, China) containing a commercial protease inhibitor (ST506, Beyotime, China) and phosphatase inhibitor (P1081, Beyotime, China). After centrifugation of the homogenate at 13,000 rpm, 4 °C for 5 min, the supernatant was collected and measured with a BCA protein assay kit (BL521A, Biosharp, China). Protein samples (30 μg) were subjected to 6%–12% SDS-PAGE, then transferred to PVDF membranes (ISEQ00010, Millipore, USA). After blocking with Tris-buffered saline/5% skim milk (36120ES76, Yeasen Biotech, China), membranes were incubated with the following primary antibodies: rabbit *anti*-α-Synuclein (#4179, Cell Signaling Technology, USA), rabbit *anti*-phospho-α-Synuclein (#23706, Cell Signaling Technology, USA), rabbit *anti*-LC3B (ab48394, Abcam, USA), mouse *anti*-SQSTM1/p62 (ab51416, Abcam, USA), rabbit anti-Lamp1 (ab24170, Abcam, USA), rat anti-Lamp2 (ab13524, Abcam, USA), rabbit *anti*-Atg5 (#12994, Cell Signaling Technology, China), rabbit *anti*-mTOR (#2983, Cell Signaling Technology, USA), rabbit *anti*-phospho-mTOR (#5536, Cell Signaling Technology, USA), rabbit anti-PI3K (#4257, Cell Signaling Technology, USA), rabbit *anti*-phospho-PI3K (#4228, Cell Signaling Technology, USA), rabbit *anti*-Akt (#4691, Cell Signaling Technology, USA), rabbit *anti*-phospho-Akt (#4060, Cell Signaling Technology, USA), rabbit *anti*-caspase-3 (#9665, Cell Signaling Technology, USA), rabbit anti-cleaved caspase-3 (#9664, Cell Signaling Technology, USA), rabbit *anti*-Bax (A0207, ABclonal, China), mouse *anti*-GAPDH (60004-1-Ig, Proteintech, USA), mouse *anti*-β-actin (60008-1-Ig, Proteintech, USA) and rabbit *anti*-β-tubulin (10094-1-AP, Proteintech, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (BA1054, BOSTER, China) or goat anti-mouse IgG (BA1050, BOSTER, China) was then applied as secondary antibody. After washing, the protein bands were visualized with a chemiluminescence detection kit (P90720, Millipore, USA) according to the manufacturer's instructions. Target protein signals were normalized to GAPDH, β-tubulin or β-actin as the loading control. Densitometry analysis was carried out using Image J software (National Institutes of Health, USA).

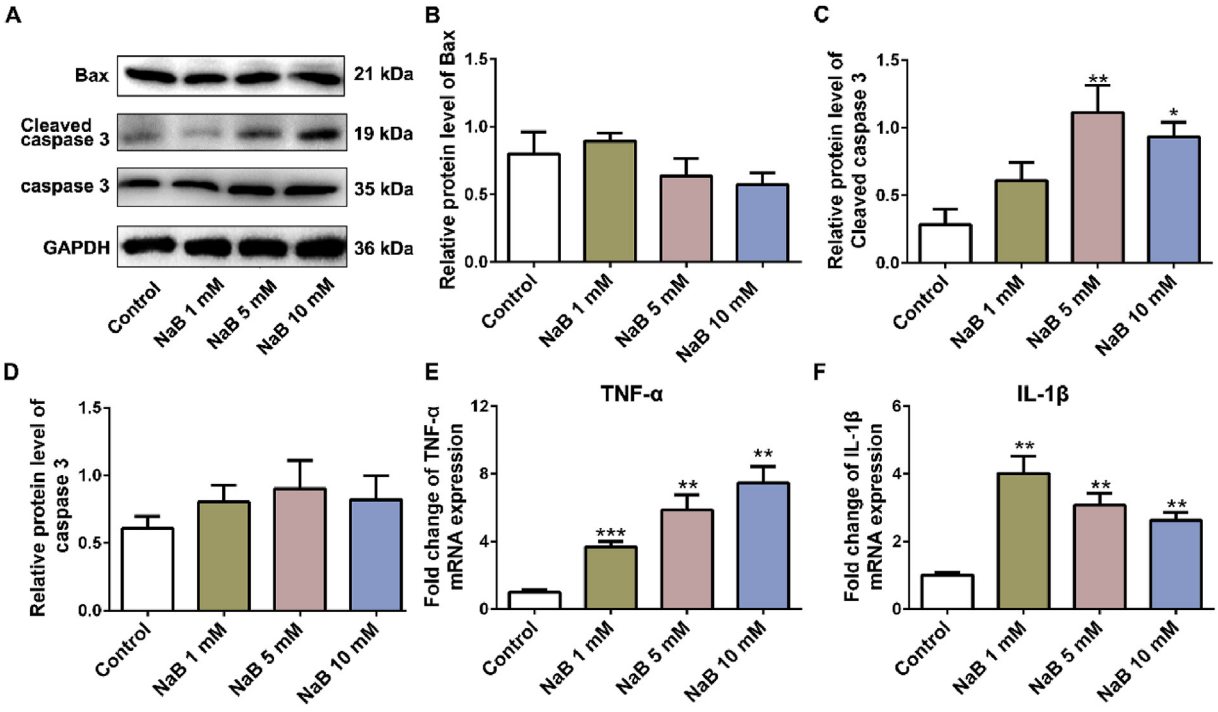


Fig. 5. NaB induces apoptosis and inflammation in STC-1 cells. (A) Representative Western blot of Bax, cleaved caspase-3 and caspase-3 expressions in STC-1 cells treated with different doses NaB for 24 h. (B) Quantification of Bax expression normalized to GAPDH. No significant difference was observed in protein levels of Bax in NaB-treated STC-1 cells. (C) Quantification of cleaved caspase-3 expression following normalization to GAPDH. Cleaved caspase-3 was increased in NaB-treated STC-1 cells. (D) Quantification of the caspase-3 expression was normalized to GAPDH. No significant difference was observed on the protein level of caspase-3 in NaB-treated STC-1 cells. n = 6. (E) qPCR analysis TNF-α mRNA expression in STC-1 cells treated with different doses of NaB for 24 h. (F) qPCR analysis of IL-1β mRNA expression in STC-1 cells treated with different doses of NaB for 24 h n = 8. Data represent the means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

2.6. Enzyme-linked immunosorbent assay (ELISA)

α-Synuclein concentration in culture medium was detected using a commercial ELISA kit (JL25187, Jianglai Biotech, China). The limit of detection is 0.75–24 ng/mL and the samples were diluted by five-fold. All experimental procedures were performed according to the manufacturer's instructions. α-Synuclein concentration was expressed as ng/mL protein.

2.7. Statistical analysis

Each experiment was performed independently in triplicates and SPSS 22.0 software (IBM SPSS Statistics, USA) was used for statistical analysis. Data were expressed as means ± SEM (standard error of the mean). A one way ANOVA with Turkey's post hoc *t*-test or Dunnett's T3 was used to determine statistically significant differences between treatments. We also used the independent-samples T test to detect the significance of differences between two groups. A $P < 0.05$ was required for results to be considered statistically significant.

3. Results

3.1. NaB induces α-synuclein mRNA but not α-synuclein protein in STC-1 cells

Initially, we performed a MTT assay to evaluate the growth-inhibitory effect of NaB on STC-1 cells. NaB (5, 10, 20, 50, and 100 mM) was found to be inversely proportional to cell viability and induces cell death in a dose-dependent manner after 24 h incubation. These observations indicate that NaB can inhibit STC-1 cell proliferation and cell survival at concentrations over 10 mM ($P < 0.001$) (Fig. 1A). To verify whether NaB induced increased release of α-Synuclein in STC-1 cells, we firstly analyzed α-Synuclein mRNA expression by qPCR. NaB treatment at doses of 1, 5 or 10 mM for 24 h, increased α-Synuclein mRNA at 10 mM ($P < 0.05$) in STC-1 cells compared with controls (Fig. 1B), indicating that NaB induced α-Synuclein mRNA in STC-1 cells. To further confirm whether NaB induces α-Synuclein expression at the protein level, we detected α-Synuclein protein and phospho-α-Synuclein protein expressions by Western blot. Interestingly, we found that neither total α-Synuclein nor phospho-α-Synuclein was increased in STC-1 cells under NaB treatment for 24 h compared with control (Fig. 1C–E), indicating that NaB failed to induce α-Synuclein at protein level.

3.2. NaB induces autophagy and regulates α-synuclein expression in STC-1 cells

Strikingly, differential expression of α-Synuclein between mRNA and protein levels was observed in NaB-treated STC-1 cells. Several studies using different cell culture models of synucleinopathies have shown that the autophagy pathway participates in α-Synuclein degradation and its alteration may support α-Synuclein mediated neurodegeneration [26–28]. Thus, to figure out whether newly synthesized α-Synuclein was degraded through the autophagy pathway or released extracellularly in response to NaB stimulation, we analyzed the expression of autophagy markers of free LC3B–I and lipid-bound LC3B-II as well as SQSTM1 by Western blot.

After NaB treatment for 24 h, the expression of the SQSTM1 protein was significantly decreased by 10 mM NaB treatment in STC-1 cells compared with the control ($P < 0.01$) (Fig. 2A and B). Conversion of LC3B–I to LC3B-II is an essential event for autophagosome-formation to induce autophagy. An increase in LC3B-II is, therefore, a marker of activated autophagy [29]. Accordingly, expression of the LC3B-II protein was significantly increased in STC-1 cells treated with NaB compared with the control ($P < 0.01$) (Fig. 2A and C), indicating that autophagy pathway plays an important role in degradation of α-

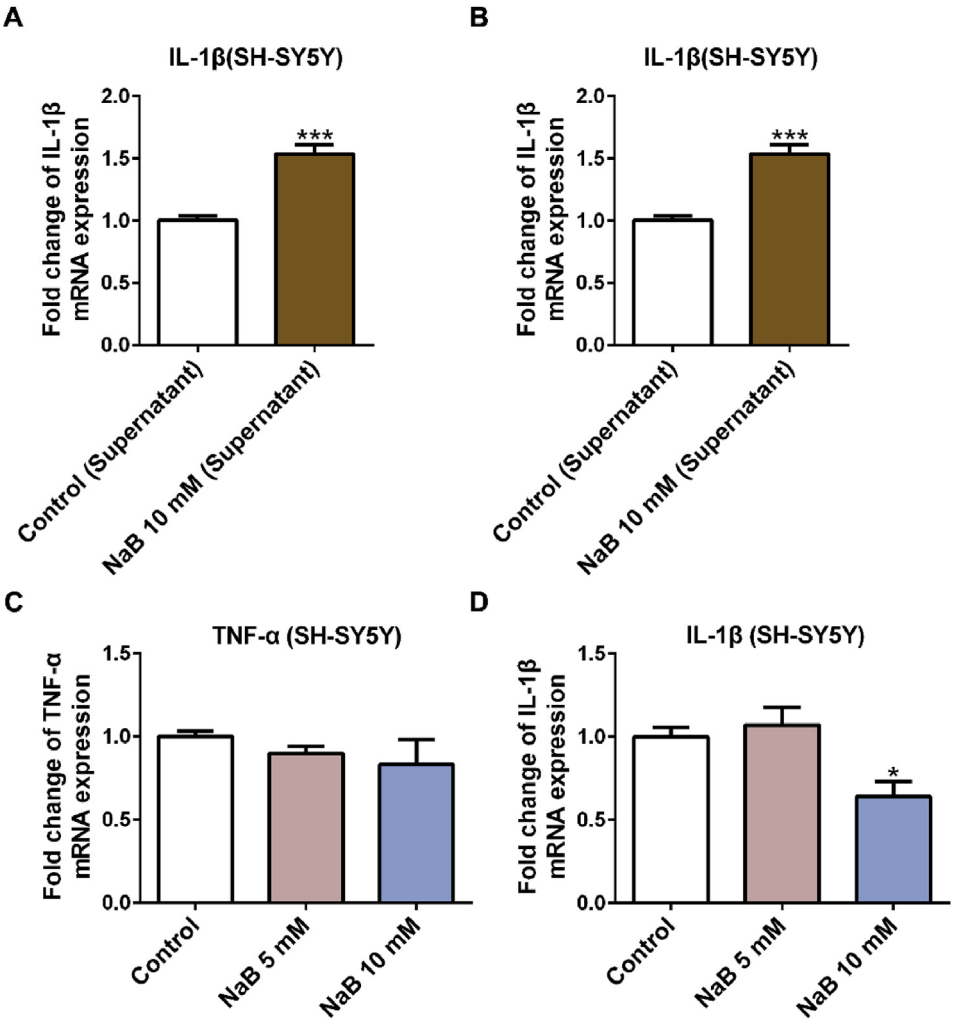


Fig. 6. Conditioned medium from NaB-stimulated STC-1 cells induces inflammation in SH-SY5Y cells. (A) qPCR analyzed TNF-α mRNA expression in SH-SY5Y cells which were treated with conditioned medium from 10 mM NaB-treated STC-1 cells for 24 h. (B) qPCR analysis of IL-1β mRNA expression in SH-SY5Y cells treated with conditioned medium from 10 mM NaB-treated STC-1 cells for 24 h. (C) qPCR analyzed TNF-α mRNA expression in SH-SY5Y cells which were treated with different doses of NaB for 24 h. (D) qPCR analysis IL-1β mRNA expression in SH-SY5Y cells treated with different doses of NaB for 24 h n = 8. Data represent the means ± SEM; *$P < 0.05$, ***$P < 0.001$.

Synuclein.

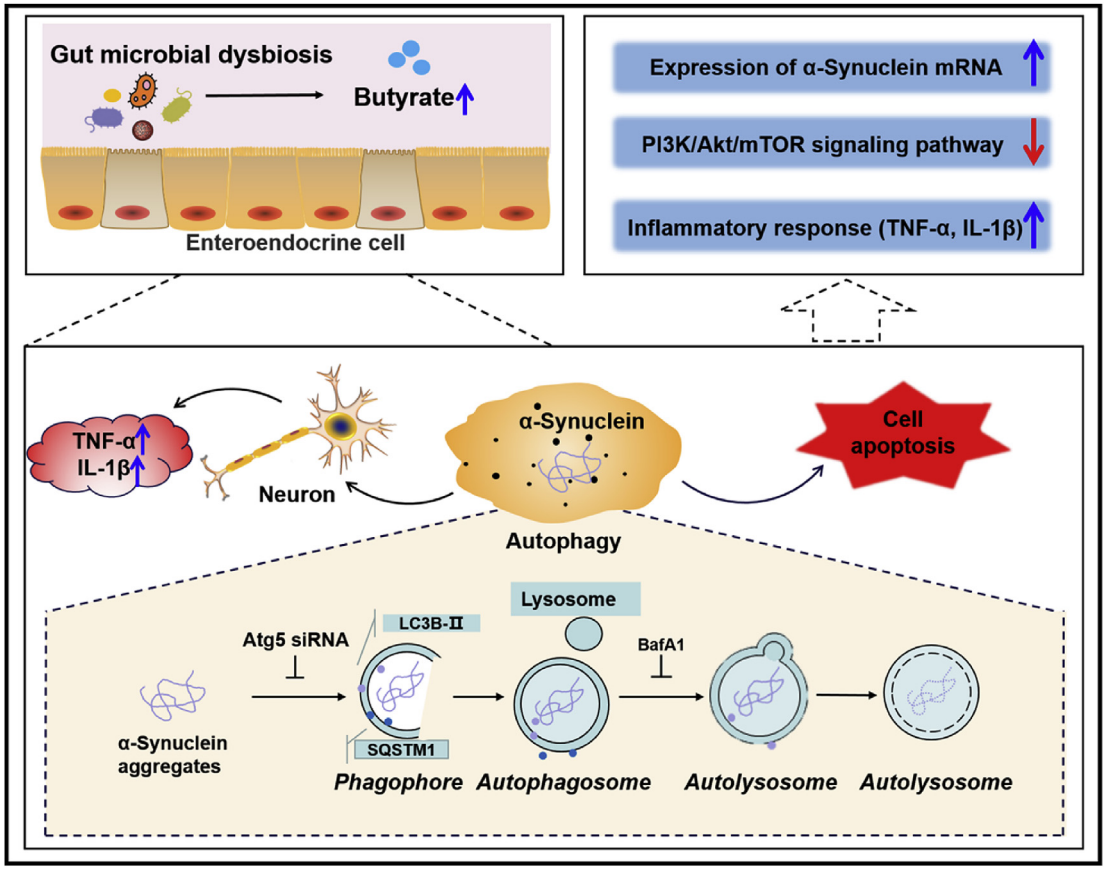
Autophagosomes form from a pre-autophagosomal structure, then mature to a phagophore that entraps cargo to a fully formed and sealed autophagosome prior to fusion with the endocytic compartment and termination at the lysosome [30]. To further confirm whether NaB influenced the formation of autophagosomes or lysosomes, we detected LC3B, SQSTM1 and lysosomal associated membrane protein 1 (Lamp1) and lysosomal associated membrane protein 2 (Lamp2) expressions in STC-1 cells. NaB significantly upregulated LC3B expression at the protein level and the mRNA level ($P < 0.01$) (Fig. 2C and D). However, there were no significant differences in Lamp1 and Lamp2 mRNA or protein expressions between NaB-treated groups and control, indicating that NaB selectively regulates autophagosomes, but not lysosomes, in STC-1 cells at the mRNA level or the protein level (Fig. 2E–I).

Considering that the discrepancy of α-Synuclein expression changes between mRNA and protein levels was regulated by autophagy pathway, we used the autophagy inhibitor BafA1 to verify whether NaB regulates α-Synuclein expression at the mRNA level by the autophagy pathway. An increase of α-Synuclein mRNA was observed at 10 mM NaB ($P < 0.001$) (Fig. 2J). After inhibition lysosomal fusion to autophagosomes by BafA1, α-Synuclein mRNA was statistically decreased in (NaB + BafA1)-treated cells compared with cells treated with NaB alone ($P < 0.001$) (Fig. 2J), indicating NaB could induce an autophagic response leading to decreased expression of α-Synuclein. Besides, BafA1 used alone had no effect on α-Synuclein mRNA expression in STC-1 cells (Fig. 2J).

3.3. NaB induces α-synuclein mRNA expression through an Atg5-dependent autophagy pathway

Studies in yeast have identified a series of autophagy-related genes forming the autophagy machinery [31]. To get an overall view of the changes of some important Atg genes induced by NaB in the process of autophagosome formation, we quantified Atg-related genes families by qPCR analysis. No significant change of Atg3, Atg7 or Atg10 mRNA was observed in NaB-treated groups compared with the control (Fig. 3A–C). Interestingly, Atg5 mRNA was significantly increased by 5 mM ($P < 0.05$) and 10 mM ($P < 0.01$) NaB compared with the control (Fig. 3D). Consistent with mRNA expression, Atg5 was increasingly expressed by 10 mM NaB compared with the control at the protein level ($P < 0.05$) (Fig. 3E–F), suggesting that Atg5 might play a key role in modulating autophagy by NaB.

Studies show that the Atg5 complex is necessary for formation of LC3-I and phosphatidylethanolamine conjugation to form LC3-II [32]. To characterize the mechanism through which NaB modulated expression of α-Synuclein mRNA by Atg5-dependent autophagy, we firstly knocked down Atg5 expression using siRNA in STC-1 cells. Compared with transfection with negative control siRNA, Atg5 mRNA expression was suppressed by 70.85% in Atg5 siRNA-transfected cells ($P < 0.001$), suggesting Atg5-specific siRNA effectively reduced the expression of Atg5 at the mRNA level compared with the control (Fig. 3G). Subsequently, we measured α-Synuclein mRNA expression in STC-1 cells by NaB stimulation and gene knockdown by Atg5 siRNA. Obviously, the expression of α-Synuclein mRNA in the NaB (10 mM)-



Scheme. 1. Schematic diagram of the effects of NaB on α-Synuclein degradation by Atg5-dependent and PI3K/Akt/mTOR-related autophagy pathways in STC-1 cells. NaB triggers α-Synuclein mRNA increases but does not induce α-Synuclein protein increase in STC-1 cells, followed by cell apoptosis and autophagy, which result in activation of Atg5 and inhibition of the PI3K/Akt/mTOR signaling pathway. In addition, expression of α-Synuclein mRNA is influenced by inhibition of autophagy when using Atg5 siRNA or BafA1. Moreover, NaB induces expression of pro-inflammatory factors TNF-α and IL-1β, as well as induces α-Synuclein mRNA, which may result in inflammation in neuronal cells.

treated group was 12-fold higher than that in the control ($P < 0.01$) (Fig. 3H). However, NaB failed to promote the expression of α-Synuclein mRNA when Atg5 was knocked down, suggesting that NaB induced α-Synuclein mRNA expression by Atg5-dependent autophagy. The increased expression of α-Synuclein mRNA in Atg5 siRNA group might due to the inhibition of autophagy.

3.4. NaB modulates autophagy via downregulation of PI3K/Akt/mTOR pathway in STC-1 cells

Previous studies have shown that the PI3K/Akt/mTOR pathway is one of the key pathways involved in regulating autophagy [33,34]. To evaluate whether the PI3K/Akt/mTOR pathway was affected by NaB stimulation, the expression levels of PI3K/Akt/mTOR were measured by Western blot. The *p*-Akt/total Akt ratio was reduced following treatment with 5 mM or 10 mM NaB compared with the control ($P < 0.05$) (Fig. 4A and B). Similarly, the ratio of p-PI3K/total PI3K was reduced by NaB treatment from 5 mM to 10 mM compared with the control ($P < 0.05$) (Fig. 4A and C). Additionally, the ratio of *p*-mTOR/total mTOR was significantly decreased by treatment with 1 mM–10 mM NaB compared with the control ($P < 0.05$) (Fig. 4D and E). These results suggest that PI3K/Akt/mTOR signaling was involved in the effect of NaB-induced autophagy.

3.5. NaB induces apoptosis and inflammation in STC-1 cells

To determine whether the autophagy was involved in the effect of NaB on cell apoptosis and inflammation, STC-1 cells were incubated with different doses of NaB for 24 h and apoptosis was measured. We examined expression of Bax, caspase-3 and cleaved caspase-3 protein by Western blot, as well as TNF-α and IL-1β production by qPCR analysis. NaB has no effect on Bax expression in STC-1 cells compared with the control (Fig. 5A and B). Similarly, there was no change of caspase-3 between NaB-treated STC-1 cells and the control (Fig. 5A and D). Interestingly, cleaved caspase-3 was increased by 2.9-fold in 5 mM NaB- and 2.3-fold in 10 mM NaB-treated groups compared with the control ($P < 0.05$) (Fig. 5A and C), indicating that NaB might induce apoptosis. Further, we measured pro-inflammatory cytokines TNF-α and IL-1β, which trigger the activation of complex signaling cascades. qPCR analysis demonstrated that TNF-α was increased by 3 to 6-fold in a dose-dependent manner by NaB stimulation ($P < 0.01$) (Fig. 5E). Interestingly, IL-1β was also significantly increased by different doses of NaB, but not in a dose-dependent manner ($P < 0.01$) (Fig. 5F).

3.6. Conditioned media from NaB-stimulated STC-1 cells induces inflammation in SH-SY5Y cells

It has been proposed that neuronal cell death is directly linked to the accumulation of α-Synuclein, and extracellular α-Synuclein aggregates are taken up by endocytosis [35,36]. Thus we measured α-Synuclein concentration in medium conditioned from NaB-treated STC-1 cells by ELISA, but the concentration was too low to be detected. Although microglia are considered to be the major player of neuroinflammation in brain, it has not been investigated yet whether neuronal cells can response to the stimulation of NaB on STC-1 cells. Thus, we cultured SH-SY5Y cells with medium conditioned by 10 mM NaB-

treated STC-1 cells for 24 h, then harvested SH-SY5Y cells for inflammation analysis. Interestingly, TNF-α was 1-fold elevated in NaB-treated SH-SY5Y cells compared with the control ($P < 0.001$) (Fig. 6A). Similarly, IL-1β was 0.5-fold increased in NaB-treated SH-SY5Y cells cultured with supernatant of STC-1 cells compared with the control ($P < 0.001$) (Fig. 6B). To further exclude the carryover effect of NaB in culture medium, we treated SH-SY5Y cells with 5 mM and 10 mM to confirm whether NaB treatment alone induced inflammation. As expected, there were no changes of TNF-α expression in SH-SY5Y cells between NaB-treated groups and the control without NaB treatment (Fig. 6C). In addition, there was no change in IL-1β expression in the 5 mM NaB-treated group, and was even decreased in the 10 mM NaB-treated group in SH-SY5Y cells compared with the control (Fig. 6D). These results indicate that some molecules or proteins secreted by stimulation of NaB from EECs stimulated pro-inflammatory factors expressions in neuronal cells.

4. Discussion

PD is a complex, chronic and progressive neurodegenerative disease, and α-Synuclein is a component central to the pathogenesis of the disease [37]. Interestingly, α-Synuclein pathology in PD is not limited to the brain, being also observed in the ENS. The major findings of our study provide evidence that α-Synuclein mRNA is markedly increased in EECs by administration of NaB (Fig. 1). Most studies have reported that α-Synuclein manifests in enteric neurons of the gut much earlier than its presence in dopaminergic neurons of the midbrain and the onset of PD symptoms [12,38]. Interestingly, recent research demonstrates that EECs containing α-Synuclein are more abundant in the proximal small intestine, where vagal neural innervation is more extensive [15]. In particular, EECs are chemosensory cells, which are distributed throughout the intestinal mucosa and oriented with their apical surface open to the intestinal lumen so that they can sense luminal contents, such as gut microbiota or SCFAs [39]. Our previous studies have demonstrated that gut microbial dysbiosis is involved in PD. In addition, microbial metabolite SCFAs, including butyrate, are elevated in PD mice, and may contribute to the over-activation of microglia and astrocytes in the *substantia nigra* [8,9]. On the one hand, our study demonstrates the SCFA NaB can induce high expression of pathologic α-Synuclein mRNA in STC-1 cells (Fig. 1). On the other hand, our study also demonstrates that some molecules or proteins results in pro-inflammatory factors, such as TNF-α and IL-1β, being expressed by neuronal cells (Fig. 6).

Accumulation and aggregation of intracellular α-Synuclein could result from disturbances in the proper function of autophagy-related mechanisms responsible for removal of unfolded and misfolded proteins [40]. Strikingly, NaB stimulation lead to changes of α-Synuclein mRNA expression but did not lead to changes in α-Synuclein protein expression in STC-1 cells (Fig. 1). To further investigate the different expression of α-Synuclein at mRNA and protein levels, we found that the autophagic pathway is mainly involved in the degradation of α-Synuclein protein by the autophagy marker LC3B assay combined with SQSTM1 assay. During autophagy, the cytoplasmic form of LC3-I is recruited to the autophagosome, where LC3-II is generated by site-specific proteolysis and lipidation near to the C-terminus [41]. On the other hand, SQSTM1 binds to LC3 and recruits proteins into autophagosomes for degradation [42]. Therefore, increased LC3-II and decreased SQSTM1 levels indicate autophagic activity. As expected, LC3B-II levels are significantly increased by NaB stimulation, while SQSTM1 is decreased in STC-1 cells (Fig. 2). SQSTM1 and LC3 interact with each other to participate in autophagosome formation, and the latter fuses with lysosomes to form autolysosomes, resulting in the degradation of the autophagic contents. We also confirmed NaB participates in the specific stage of autophagosome formation but lysosome, by detecting lysosome markers Lamp1 and Lamp2. In addition, NaB not only induced autophagy, but also influenced the expression of α-Synuclein at the mRNA level. The expression of α-Synuclein mRNA induced by NaB is partly influenced by the autophagy pathway. Interestingly, inhibition of autophagy by BafA1 followed by NaB treatment reduced α-Synuclein mRNA expression, indicating that NaB induced a strong autophagic response. (Fig. 2). Previous studies also demonstrate that NaB induces autophagy in cultured colorectal cells [18,43]. However, little is known about α-Synuclein expression induced by NaB until now.

Among autophagy-related genes, Atg5 protein is involved in the early stages of autophagosome formation [44]. The siRNA-mediated knockdown of Atg5 followed by NaB treatment decreased mRNA expression of α-Synuclein compared with the NaB-treated group alone, indicating that the Atg5 pathway was involved in NaB-induced autophagy and α-Synuclein induction (Fig. 3). As a negative regulator both in autophagy and apoptosis, the PI3K/Akt/mTOR signaling pathway plays vital roles in modulating the crosstalk between autophagy and apoptosis [45]. Given that NaB induced autophagic apoptosis by inhibiting AKT/mTOR signaling in nasopharyngeal carcinoma cells [46], we also verified that NaB inhibited PI3K/Akt/mTOR signaling in STC-1 cells (Fig. 4). In addition, prolonged autophagy has also been shown to promote cell apoptosis [47], we also demonstrated that cleavage of caspase3, as well as the expression of pro-inflammatory factors TNF-α and IL-1β, were elevated by NaB stimulation (Fig. 5).

Based on the role of α-Synuclein and butyrate in the pathology of PD, herein, we demonstrate that NaB elevates α-Synuclein mRNA expression by inducing Atg5-mediated autophagy and PI3K/Akt/mTOR signaling. In addition, NaB induces cell apoptosis and release of pro-inflammatory factors TNF-α and IL-1β, as well as promotes α-Synuclein release which may result in inflammation in SH-SY5Y cells (Scheme. 1). Collectively, these data suggest that the intestinal α-Synuclein and SCFAs, especially butyrate, may serve as a potential mechanism for PD pathogenesis.

Declaration of competing interest

The authors declare no conflicts of interest.

Author contributions

Chen-Meng Qiao: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Software, Supervision. Meng-Fei Sun: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Software, Supervision. Xue-Bing Jia: Conceptualization, Data curation, Investigation, Project administration. Yun Shi: Investigation. Bo-Ping Zhang: Investigation. Zhi-Lan Zhou: Software. Li-Ping Zhao: Software. Chun Cui: Formal analysis, Funding acquisition, Supervision. Yan-Qin Shen: Data curation, Funding acquisition, Project administration, Resources, Supervision.

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