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Nicotine and menthol independently exert neuroprotective effects against cisplatin- or amyloid- toxicity by upregulating Bcl-xl via JNK activation in SH-SY5Y cells

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Abstract: Nicotine and menthol, agonists of nicotinic acetylcholine receptor (nAChR) and transient receptor potential melastatin type 8 (TRPM8), serve important roles in the prevention of cell death-involved neurodegenerative diseases. However, the potential synergistic effects of nicotine and menthol on anti-apoptotic ability are still uncertain. In the present study, the potential synergistic effects of nicotine and menthol on cisplatin or amyloid $\beta_{1\text{-}42}$ induced cell model of the neurodegenerative diseases were explored by assessing cell viability, TNF-α expression, caspase-3 activation, and the collapse of mitochondrial membrane potential in human SH-SY5Y neuroblastoma cells. Statistical significance was tested using Student's *t*-test or one-way ANOVA with *post hoc* Newman-Keuls test. The results showed that: Firstly, SH-SY5Y cell viability was obviously increased by the treatments with nicotine and menthol. Secondly, nicotine and menthol independently alleviated cisplatin or amyloid $\beta_{1\text{-}42}$ induced TNF-α up-regulation. Thirdly, nicotine and menthol abrogated the effect of cisplatin and amyloid $\beta_{25\text{-}35}$ on caspase-3 activation. Interestingly, the effect of cisplatin and amyloid $\beta_{1\text{-}42}$ on the collapse of mitochondrial membrane potential was efficiently attenuated by nicotine and menthol treatments. Most importantly, the inhibition of c-jun kinase (JNK) activation abolished the effect of cisplatin, and amyloid $\beta_{1\text{-}42}$ stimulated Bcl-xl expression. All these findings indicate that nicotine and menthol independently exert neuroprotective effects by upregulating Bcl-xl *via* JNK activation. Nicotine and menthol augmented Bcl-xl expression and JNK phosphorylation, and thus they are potential therapeutic targets for altering the progress of neurodegenerative diseases.

Introduction

Apoptosis, which initiates either by the intrinsic pathway or the extrinsic pathway, is responsible for the physiological deletion of cells and appears to be intrinsically programmed (Pistritto *et al.*, 2016). While intrinsic cell stress is sensed by the intrinsic pathway, signals from other cells such as tumor necrosis factor-alpha (TNF-α) and Fas signals are sensed by the extrinsic pathway (van Opdenbosch and Lamkanfi, 2019). After the signals of cell death are sensed, cysteinyl aspartate specific proteinases (caspases) such as caspase-3 and caspase-6 are activated, and the degradations of the proteins and chromatin occur (van Opdenbosch and Lamkanfi, 2019).

Alzheimer's disease, which is characterized by memory loss and language deterioration, exhibits neurofibrillary tangle accumulation and β-amyloid protein-containing plaques (Rosenberg and Lambracht-Washington, 2013). β-amyloid induced neuron apoptosis, which is typically observed in the brains of the patients, features chromatin condensation, deoxyribonucleic acid (DNA) fragmentation, and caspase activation (Nalivaeva and Turner, 2013). Interestingly, there is evidence that nicotinic acetylcholine receptors (nAChR) provide neurotrophic support to neurons (Bono *et al.*, 2020). These findings indicate that nAChR might be involved in β-amyloid inducing neuronal loss, and it serves an important role in the development of the disease.

Nicotine, an agonist of nAChR (Islam *et al.*, 2013), prevents oxidative stress-induced hippocampal neuronal injury through α7-nAChR/Erk1/2 signaling pathway (Dong *et al.*, 2020). Although the treatment with menthol increases nicotine addiction (Nonnemaker *et al.*, 2019), menthol, an agonist of transient receptor potential melastatin Type 8

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(TRPM8), was documented to ameliorate the pathological abnormalities in hepatic and pancreatic islets (Muruganathan *et al.*, 2017; Rozza *et al.*, 2014). Hence, as the agonists of the nicotinic acetylcholine receptors, nicotine and menthol play important roles in activating c-Jun kinase (JNK) activation and altering the progress of neurodegenerative diseases (Go *et al.*, 2020; Lombardo and Maskos, 2015). In our previous studies, amyloid β and cisplatin were found to induce cell apoptosis in a concentration-dependent manner (Xue *et al.*, 2014; Yan *et al.*, 2014). By inhibiting JNK with SP600125, nicotine also exhibited an anti-apoptotic effect and facilitated the cross-presentation in dendritic cells (Gao and Gu, 2007; Xue *et al.*, 2014). But the potential synergistic effects of nicotine and menthol on anti-apoptotic ability are still uncertain.

In the present study, we aimed to investigate the potential synergistic effects of nAChR and TRPM8 agonist on cell viability by using human SH-SY5Y neuroblastoma cells as a cell model of neurodegeneration (Bono *et al.*, 2020). We demonstrated that as agonists of nAChR and TRPM8, nicotine and menthol exert neuroprotective abilities, which was supported by the following observations. Firstly, the treatments with nicotine and menthol efficiently increased cell viability. Secondly, cisplatin and amyloid $\beta_{1\text{-}42}$ augmented TNF-α up-regulation was abolished by the pretreatments with nicotine and menthol. Thirdly, the effects of cisplatin and amyloid $\beta_{25\text{-}35}$ on caspase 3 activation were abrogated by nicotine and menthol treatments. Interestingly, cisplatin and amyloid $\beta_{1\text{-}42}$ induced collapse of mitochondrial membrane potential was alleviated by nicotine and menthol pretreatments. Most importantly, the inhibition of JNK activation abrogated the effects of nicotine and menthol on Bcl-xl up-regulation. All these findings indicate that the upregulating Bcl-xl *via* JNK activation contributes to nicotine and menthol independently exert a neuroprotective effect. Bcl-xl and JNK phosphorylation are potential therapeutic targets for dealing with nAChR and TRPM8 associated with the progress of neurodegenerative diseases.

Materials and Methods

Reagents

Nicotine (-), amyloid $\beta_{1\text{-}42}$, amyloid $\beta_{25\text{-}35}$ and Rhodamine123 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Nicotine and menthol were provided by China Tobacco Guizhou Industrial Co., Ltd., Guiyang, China. Cisplatin was purchased from Calbiochem, Inc (Merck KGaA, Darmstadt, Germany). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc., Shanghai, China. SP600125 was from Cayman Chemical Company (Ann Arbor, MI, USA). Antibodies to β-actin (Mouse, #3700), myeloid cell leukemia-1 (Mcl-1) (Rabbit, #94296), Bcl-xl (Rabbit, #2764), phosphorylated JNK (Rabbit, #4668), TNF-α (Rabbit, #6945), cleaved caspase 3 (Rabbit, #9661) were purchased from Cell Signaling Technology, Inc., Beverly, MA, USA. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone, GE Healthcare Life Sciences (Logan, UT, USA). The stock solution of amyloid β and cisplatin was prepared by dissolving amyloid β and cisplatin in phosphate buffer saline (PBS) and dimethyl sulfoxide (DMSO), respectively.

Cell lines

Human SH-SY5Y neuro-blastoma cells were obtained from the Shanghai Cell Bank and authenticated by STR profiling using fluorescence-labeled amplification product length polymorphism analysis. Mycoplasma testing was performed for this cell line. Cells were cultured in DMEM with 10% FBS at 37°C in 5% CO_2. The cells were rinsed with 0.25% trypsin and passaged according to the subcultivation ratio of 1:20, and every 4 to 7 days, the medium was replaced. The concentrations of amyloid $\beta_{1\text{-}42}$, amyloid $\beta_{25\text{-}35}$, nicotine were based on previous studies (Xue *et al.*, 2014).

Plate scan and optical microscope observation

Optical microscope observation was performed according to a previous description (Jiang *et al.*, 2019). Briefly, a total of 5×10^4 SH-SY5Y cells were firstly seeded in 24-well plates. Then, synchronization was performed by serum starvation for at least 12 h to reduce the effect of cell proliferation. The cells were further pretreated with nicotine (1 μM), menthol (1 μM), nicotine and menthol used together (1 μM, respectively) for 16 h prior to 5 μg/mL cisplatin or 20 μM amyloid $\beta_{1\text{-}42}$ stimulation. In the end, the cells were cultured for a further 48 h–72 h, and the effects of nicotine, menthol, nicotine, and menthol used together on cell morphology and viability were assessed by plate scan with crystal violet staining, cell count using an optical microscope with 100× magnification factor, respectively.

Cell proliferation assay

Cell proliferation assay was determined by CCK-8 assay according to a previous description (Wang *et al.*, 2014). Based on the detection of the dehydrogenase activity in the viable cells, WST-8 products a water-soluble formazan dye upon reduction in the presence of an electron mediator. Briefly, a total of 3×10^4 SH-SY5Y cells were firstly inoculated in a 96-well plate in 100 μL/well medium. Then, the cells were subsequently treated with indicated amyloid $\beta_{1\text{-}42}$ and cisplatin. CCK-8 solution (10 μL per well) was added to each well. After 2 h incubation, the OD450 value was determined at the wavelength of 450 nm.

Mitochondrial membrane potential assay

Mitochondrial membrane potential was determined using flow cytometry according to a previous description (Ke *et al.*, 2013). Briefly, a total of 5×10^4 SH-SY5Y cells were seeded in 24-well plates. Then, the cells were pretreated with nicotine (1 μM), menthol (1 μM), nicotine and menthol used together (1 μM, respectively) for 16 h prior to 5 μg/mL cisplatin or 20 μM amyloid $\beta_{1\text{-}42}$ stimulation. After that, the cells were removed by trypsinization, rinsed with PBS. The cells were re-suspended in binding buffer containing Rhodamine123 (1 μM) for 20 min at room temperature. After a thorough wash, the mitochondrial membrane potential of the cell was determined using flow cytometric analyses on FACSCalibur. Data were analyzed using CellQuest software.

Western blot analyses

Western blot analyses were performed according to the methods previously described (Xue *et al.*, 2014). For TNF-α and cleaved caspase-3, SH-SY5Y cells were pretreated with DMSO/PBS, nicotine (1 μM), menthol (1 μM) or nicotine and menthol used together (1 μM, respectively) for 16 h prior to 5 μg/mL cisplatin or 20 μM amyloid $\beta_{25\text{-}35}$ 10 h stimulation. For Bcl-xl expression, the cells were pretreated with DMSO or SP600125 prior to the stimulations of nicotine, menthol, nicotine, and menthol used together. Left-handed nicotine, which has a powerful ability for cell proliferation (Wang *et al.*, 2014), was used as the positive control. The cellular proteins were extracted and loaded onto 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After 120 min electrophoresis with 80 V, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. Blocking was performed by incubation with 5% fat-free milk in TBST. The membrane was incubated with primary antibody at 4°C overnight with 1:1000 dilution. After washed six times with TBST (for 10 min each), the membrane was further incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature. The bound secondary antibody was visualized using enhanced chemiluminescence ECL. β-actin was used as a loading control. DMSO was employed as solvent control of cisplatin.

Statistical analysis

Each experiment was repeated at least 3 times to confirm data repeatability. Cell viabilities and Mean Fluorescent Index of Rhodamine123 in SH-SY5Y cells were expressed as the mean ± standard error. Statistical significance was tested using a Student's *t*-test or one-way ANOVA with a *post hoc* Newman-Keuls test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The treatments with nicotine and menthol augment cell viability in SH-SY5Y cells

In our previous studies, we have documented that amyloid β and cisplatin induce cell apoptosis in a concentration-dependent manner (Xue *et al.*, 2014; Yan *et al.*, 2014). Whereas as an agonist of nAChR, nicotine exhibits a positive effect in neurodegenerative disease and facilitates the cross-presentation in dendritic cells (Gao and Gu, 2007; Xue *et al.*, 2014), menthol, an agonist of TRPM8, was also demonstrated to promote cell proliferation (Muruganathan *et al.*, 2017; Rozza *et al.*, 2014). As cell viability is the comprehensive effects of cell apoptosis and cell proliferation, CCK-8 assay determined cell viability revealed that amyloid $\beta_{1\text{-}42}$ and cisplatin decrease SH-SY5Y cell viability in a concentration-dependent manner (Figs. 1A and 1B). Whereas the selection of the concentration of nicotine was documented (Xue *et al.*, 2014), the selected concentration of menthol achieved a similar effect on cell proliferation to 0.2 and 10 μM menthol (Fig. 1C). We further treated SH-SY5Y cells with nicotine, menthol, or nicotine and menthol used together and observed the potential synergistic effect on cell viability. The treatment with cisplatin increased 50% cell death (Fig. 1C), but pretreatments with nicotine, menthol, or nicotine and menthol used together efficiently increased cell viability in both plate scan and optical microscope observation assays (Figs. 2A and 2B). Meanwhile, similar phenomena can be observed in amyloid $\beta_{1\text{-}42}$ treated condition (Figs. 2C and 2D). Cell proliferation assay revealed that whereas the treatments with nicotine or menthol increased 89.6% and 91.4% cell viability, nicotine and menthol used together achieved about 88.2% increase in cell viability in cisplatin-treated condition (Fig. 2E). Similar results can also be derived in amyloid-$\beta_{1\text{-}42}$ treated conditions (Fig. 2F). Although the plate scan and microscopic images indicated the synergic effect of menthol and nicotine, the cell count test did not confirm any significant statistical differences among these treatments (Figs. 2E and 2F). All these findings indicate that as agonists of nAChR and TRPM8, nicotine and menthol exhibit an independent proliferation effect in SH-SY5Y cells.

The treatments with nicotine and menthol attenuate cisplatin and amyloid $\beta_{1\text{-}42}$ induced TNF-α expression in SH-SY5Y cells

Inflammations, especially indicated by the high level of TNF-α, play an important role in the progress of neurodegenerative diseases (Lombardo and Maskos, 2015). SH-SY5Y cells were incubated with nicotine, menthol, or nicotine and menthol used together prior to cisplatin and amyloid $\beta_{1\text{-}42}$

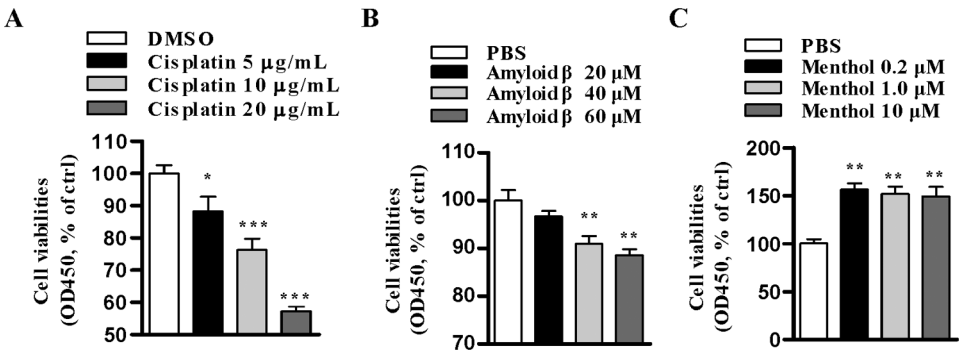

FIGURE 1. Cisplatin and amyloid β concentration-dependent decrease cell viability in SH-SY5Y cells.
1×10^4 SH-SY5Y cells were treated with cisplatin (A), amyloid $\beta_{1\text{-}42}$ (B), or menthol (C) at the indicated concentrations. Then, cell viability was determined by CCK-8 assay. Data are presented as mean ± SEM, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, compared with control group (one-way ANOVA with the post-Newman-Keuls test). Ni, nicotine; Me, menthol.

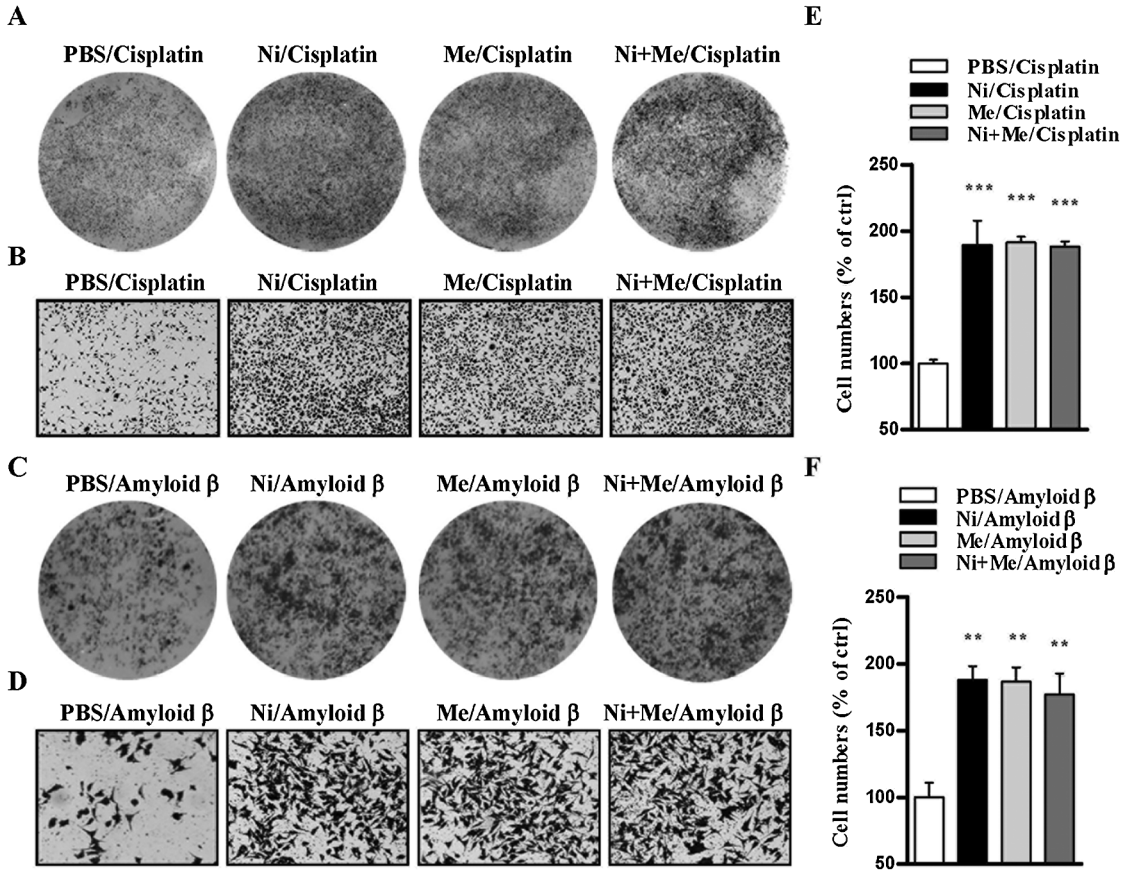

FIGURE 2. Nicotine and menthol augment cell viability in SH-SY5Y cells.
SH-SY5Y cells were pre-treated with nicotine (1 μM), menthol (1 μM) or nicotine and menthol used together (1 μM) for 16 h prior to 5 μg/mL cisplatin (A, B, E) or 20 μM amyloid $\beta_{1\text{-}42}$ (C, D, F) stimulation in complete DMEM medium. Cell morphology and viability were assessed using plate scan with crystal violet staining (A, C), optical microscope with 100× magnification factor (B, D), and cell count (E, F), respectively. Data are presented as the mean ± SEM, $^{**}P < 0.01$; $^{***}P < 0.001$. Data are representative of three independent experiments. Ni, nicotine; Me, menthol.

stimulation. The level of TNF-α in the cells was subsequently assessed by Western blot analyses. The treatments with cisplatin and amyloid $\beta_{1\text{-}42}$ significantly ($P < 0.05$) augmented TNF-α expression (Figs. 3A and 3B). However, pretreatments with nicotine, menthol, or nicotine and menthol used together achieved similar inhibitory effects of cisplatin and amyloid $\beta_{1\text{-}42}$ on TNF-α expression (Figs. 3C and 3D). These findings indicate that as agonists of nAChR and TRPM8, respectively, nicotine exhibited no synergistic effect with menthol on cisplatin and amyloid β-induced cell inflammation.

Nicotine and menthol alleviate cisplatin and amyloid $\beta_{25\text{-}35}$ induced caspase-3 activations in SH-SY5Y cells

Caspase-3 serves a vital role in the intrinsic and extrinsic pathways of apoptosis (Herr, 2018). SH-SY5Y cells were incubated with nicotine, menthol, or nicotine and menthol used together prior to cisplatin and amyloid $\beta_{25\text{-}35}$ stimulation. The activity of caspase 3 was assessed by Western blot analyses. Whereas the treatments with cisplatin and amyloid $\beta_{25\text{-}35}$ obviously increased the level of cleaved caspase 3 in a time-dependent manner (Figs. 4A and 4B), the pretreatments with nicotine, menthol, or nicotine and menthol used together efficiently abrogated the effects of cisplatin (Fig. 4C) and amyloid $\beta_{25\text{-}35}$ (Fig. 4D) on caspase 3 activation. These results indicate that as agonists of nAChR and TRPM8, nicotine exhibit no synergistic inhibitory effect with menthol on cisplatin and amyloid β induced caspase 3 activation.

The treatments with nicotine and menthol alleviate cisplatin and amyloid $\beta_{1\text{-}42}$ induced the collapse of mitochondrial membrane potential in SH-SY5Y cells

In the earlier stage of apoptosis, the outer membrane of the mitochondria disrupts, and intermembrane space proteins are released into the cytosol (Vringer and Tait, 2019). Hence, the collapse of mitochondrial membrane potential, which reflects mitochondrial function, serves as an earlier indicator of cell health (Sakamuru *et al.*, 2016). Whereas the treatment with cisplatin decreased 25.1% mitochondrial membrane potential (Fig. 5A), amyloid $\beta_{1\text{-}42}$ treatment attenuated 29.5% mitochondrial membrane potential (Fig. 5B), indicating that cisplatin and amyloid $\beta_{1\text{-}42}$ efficiently induced the collapse of the mitochondrial membrane potential. While the pretreatment with nicotine, menthol attenuated about 29.9%, 32.1% cisplatin-decreased mitochondrial membrane potential, respectively, nicotine and menthol used together alleviated 20.1% of the collapse of the mitochondrial membrane potential (Fig. 5C). Similar results can be derived in amyloid $\beta_{1\text{-}42}$ treated condition

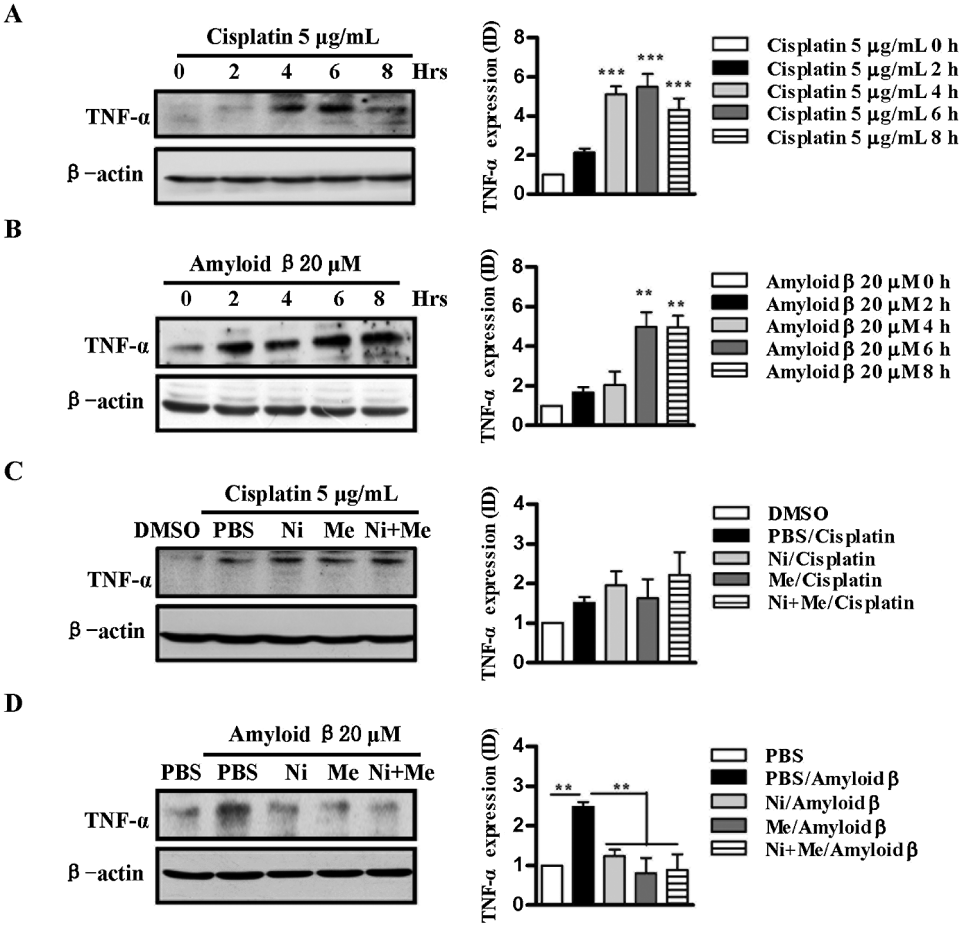

FIGURE 3. Nicotine and menthol abolish the effect of cisplatin and amyloid $\beta_{1\text{-}42}$ on TNF-α up-regulation in SH-SY5Y cells.
SH-SY5Y cells were pre-treated with (A) DMSO (B) PBS, or (C, D) nicotine (1 μM), menthol (1 μM) or nicotine and menthol used together (1 μM) for 16 h prior to (A, C) 5 μg/mL cisplatin or (B, D) 20 μM amyloid $\beta_{1\text{-}42}$ 10 h stimulation in complete DMEM medium. The whole cellular protein was extracted, and TNF-α expression was assessed using Western blot analysis. β-actin was used as an internal control. Data are representative of three independent experiments. TNF, tumor necrosis factor; Ni, nicotine; Me, menthol.

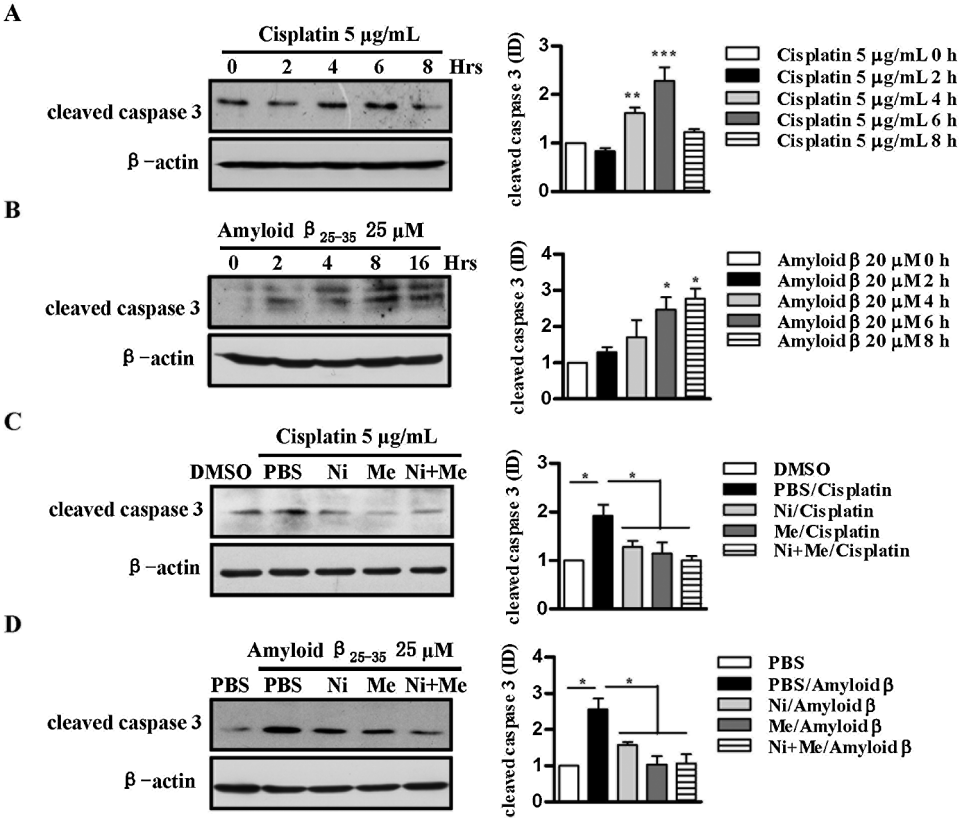

FIGURE 4. Nicotine and menthol abrogate cisplatin, and amyloid $\beta_{25\text{-}35}$ induces caspase 3 activation in SH-SY5Y cells.
SH-SY5Y cells were pre-treated with (A) DMSO (B) PBS, or (C, D) nicotine (1 μM), menthol (1 μM) or nicotine and menthol used together (1 μM) for 16 h prior to (A, C) 5 μg/mL cisplatin or (B, D) 20 μM amyloid $\beta_{25\text{-}35}$ stimulation in complete DMEM medium. The whole cellular protein was extracted, and cleaved caspase 3 was assessed using Western blot analysis. β-actin was used as an internal control. Data are representative of three independent experiments. Ni, nicotine; Me, menthol

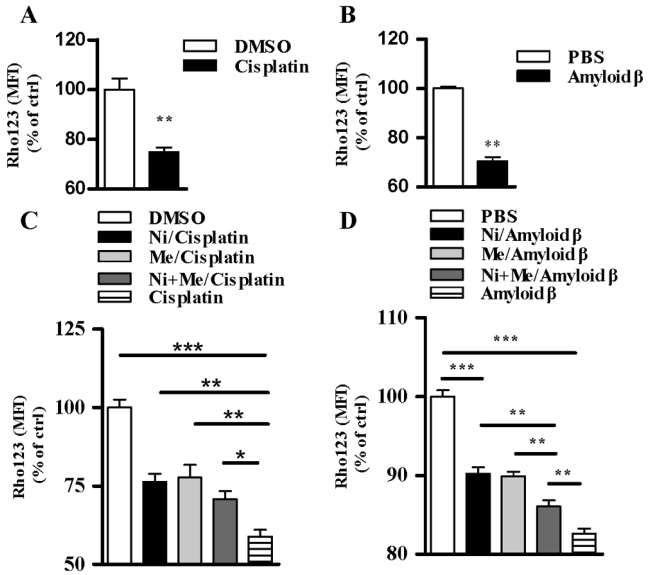

FIGURE 5. Nicotine and menthol alleviate cisplatin and amyloid $\beta_{1\text{-}42}$ induced the collapse of mitochondrial membrane potential in SH-SY5Y cells.
SH-SY5Y cells were pre-treated with (A) DMSO (B) PBS, or (C, D) nicotine (1 μM), menthol (1 μM) or nicotine and menthol used together (1 μM) for 16 h prior to (A, C) 5 μg/mL cisplatin or (B, D) 20 μM amyloid $\beta_{1\text{-}42}$ stimulation in complete DMEM medium. The mitochondrial membrane potential was determined using flow cytometric analyses with Rhodamine123 staining. Data are presented as the mean ± SEM, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Data are representative of three independent experiments. Ni, nicotine; Me, menthol.

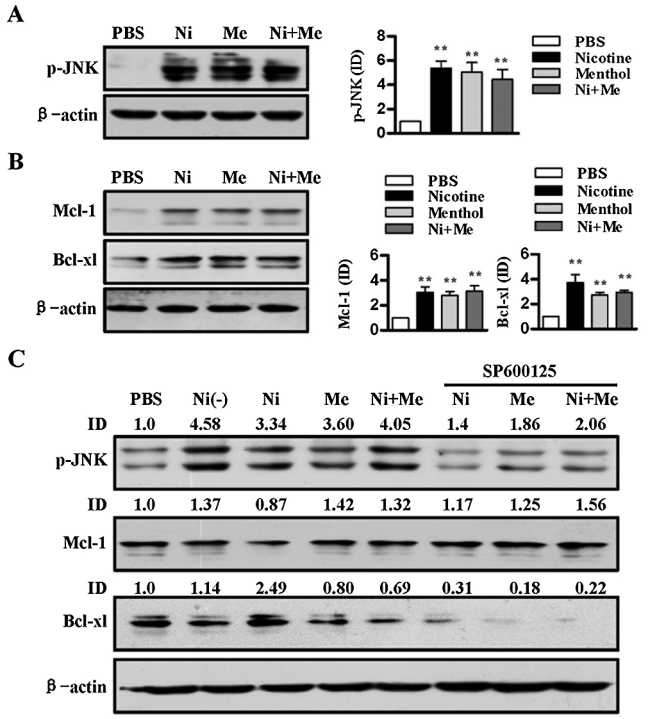

FIGURE 6. Nicotine and menthol augment Bcl-xl expression via JNK activation in SH-SY5Y cells. (A, B). SH-SY5Y cells were treated with PBS, nicotine (1 μM), menthol (1 μM), and nicotine and menthol used together (1 μM, respectively) for 30 min. (C) SH-SY5Y cells were pre-treated with DMSO or SP600125 (10 μM) prior to nicotine (1 μM), menthol (1 μM) or nicotine and menthol used together (1 μM, respectively) stimulation. The whole cellular protein was extracted, and the levels of phosphorylated JNK (A, C), Mcl-1 and Bcl-xl (B, C) were assessed using Western blot analysis. β-actin was used as an internal control. Data are representative of three independent experiments. Ni, nicotine; Me, menthol; Ni (−), left-handed nicotine.

(Fig. 5D). All these findings indicate that as agonists of nAChR and TRPM8, nicotine and menthol exhibited an anti-apoptotic effect by stabilizing the mitochondrial membrane potential.

Nicotine and menthol increase Bcl-xl expression via JNK activation in SH-SY5Y cells

JNK activation has a positive correlation with the nicotine-mediated anti-apoptotic ability (Xue *et al.*, 2014). Left-handed nicotine, which has a powerful ability for cell proliferation (Wang *et al.*, 2014), was used as a positive control. We incubated SH-SY5Y cells with nicotine, menthol, or nicotine and menthol used together and found that both the JNK phosphorylation (Fig. 6A) and the expressions of Mcl-1 and Bcl-xl (Fig. 6B) were efficiently increased by these treatments. The inhibition of JNK activation with SP600125 obviously abolished the effects of nicotine and menthol on Bcl-xl up-regulation (Fig. 6C). Despite the fact that SP600125 administration actually inhibited JNK activation, the inhibition of JNK phosphorylation had no effect on nicotine and menthol up-regulating Mcl-1 expression (Fig. 6C). All these results indicate that JNK activation is involved in an increase of Bcl-xl expression by nicotine and menthol.

Discussion

Neurodegenerative diseases, which are involved in the processes of inflammation and cell viability, exhibit increased prevalence in the developed community (Patel *et al.*, 2017). As agonists of nAChR and TRPM8 (Dong *et al.*, 2020; Lombardo and Maskos, 2015), nicotine and menthol have therapeutic benefits on neurodegenerative diseases (Pogocki *et al.* 2007), the synergistic effect of nicotine and menthol is definitely important for dealing with these neurodegenerative diseases. In the present study, we demonstrated that as agonists of nAChR and TRPM8, nicotine and menthol exerted neuroprotective effects by up-regulating Bcl-xl expression via JNK activation in SH-SY5Y cells. Interestingly, mitochondrial membrane potential, an indicator of the functional metabolic status of mitochondria, was found to underlie the mechanism of nicotine and menthol-enhanced cell viability. These findings indicate that Bcl-xl and JNK might be potential therapeutic target molecules in nAChR and TRPM8 associated diseases. Mitochondrial membrane potential might be useful for monitoring the progress of neurodegenerative diseases. Despite the fact that SH-SY5Y cell is a better cell model for Parkinson's disease (Krishna *et al.*, 2014; Xicoy *et al.*, 2017), a number of documents have conferred this cell line as a cell model of Alzheimer's disease (Prasad and Rao, 2015; Yang *et al.*, 2014). Whether nicotine exerts an anti-apoptotic effect and the potential synergistic effect with menthol in

Parkinson's disease still requires further investigations that therefore formed the basis of the present study.

The B cell lymphoma-2 (Bcl-2) family consists of a number of proteins containing Bcl-2 homology domains that regulate apoptosis by controlling mitochondrial membrane permeability and releasing cytochrome c (Kvansakul *et al.*, 2017). The Bcl-2 family can be separated into pro-survival proteins, pro-apoptotic proteins, and "BH3 only" proteins (Kvansakul *et al.*, 2017). Interactions between death-promoting and death-suppressing members lead to a rheostat model in which the ratio of pro-apoptotic and anti-apoptotic proteins controls the fate of acute myeloid leukemia (Ashkenazi *et al.*, 2017). Bcl-xl localizes in the outer mitochondrial membrane and protects cells against apoptotic stimuli (Kale *et al.*, 2012). Although nicotine increases Bcl-xl expression and facilitates multiple drug resistance of lung cancer cells (Ke *et al.*, 2013), the mechanisms by which nicotine up-regulates other anti-apoptotic proteins or increasing the interactions between death-promoting and death-suppressing members require further exploration. In the present study, the collapse of mitochondrial membrane potential was found to contribute to cisplatin- and amyloid β-induced apoptosis (Fig. 4). The treatments with nicotine and menthol increased Bcl-xl up-regulation (Fig. 5B), whereas the effects of cisplatin and amyloid β on the mitochondrial membrane potential were partly abolished by nicotine and menthol pretreatments (Fig. 4). As Bcl-xl and other Bcl-2 family proteins help preserve the mitochondrial potential by reducing mitochondrial membranes permeability (Suh *et al.*, 2013), the findings that nicotine up-regulating Bcl-xl expression indicate that mitochondrial membrane permeability contributes to nicotine-enhanced cell viability and the neuroprotective effect.

SP600125 abolishes the effect of nicotine on the anti-apoptotic ability by inhibiting JNK phosphorylation in Raw264.7 and El4 cells (Wang *et al.*, 2014). The treatment with amyloid β increases TNF-α expression *via* NF-κB pathway activation in the brain of TNF transgenic mice (Paouri *et al.*, 2017). In this study, we treated SH-SY5Y cells with nicotine and menthol and found that as agonists of nAChR and TRPM8, nicotine and menthol increased Bcl-xl expression *via* JNK signaling (Fig. 6). These findings indicate that JNK kinase might be an upstream molecule of the nuclear factor kappa-B (NF-κB) pathway activation. The precise roles of JNK phosphorylation in nicotine and menthol-inhibited TNF-α expression and in the protective effect on cell viability need to be clarified.

The treatment with menthol contributes to nicotine intake and addiction *via* its TRPM8 in C57BL/6 mice (Fait *et al.*, 2017; Fan *et al.*, 2016). Menthol administration enhances nicotine's effect by inducing neuron excitability and the expression of nicotinic acetylcholine receptors (Henderson *et al.*, 2017). Compared with nicotine exposure alone, chronic (±)-menthol plus nicotine exposure increases β2 nAChR levels in the hypothalamus (Mulcahy *et al.*, 2019). In the present study, despite the fact that the administrations of menthol and nicotine increased anti-apoptotic abilities by up-regulating Bcl-xl via JNK activation, the exact interactions between nAChR and TRPM8, as well as the up-regulation of β2 nAChR by menthol, require further studies.

Bhadania *et al.* (2012) documented that menthol administration (400 mg/kg, s.c.) successively for 10 days significantly improved spatial learning and memory ability of β-amyloid peptide-induced cognitive deficits by revealing that the higher dose of menthol showed improvement in both short-term and long-term memory. Also, when administered with nicotine, menthol showed psychoactive properties by affecting brain activity and behavior and facilitates dopamine-releasing effect in the mouse model (Thompson *et al.*, 2018; Zhang *et al.*, 2018). All these documents indicate that the effects of menthol and nicotine administration are attributable to the comprehensive function of the central nervous system. Aicher *et al.* (2003) found that 10^{-7} M nicotine has a better effect on surface molecules' expression than 10^{-5}–10^{-6} M nicotine does in dendritic cells. Our previous studies demonstrated that 10^{-6}–10^{-7} M nicotine has a similar effect on the cross-presentation in dendritic cells and the neuroprotective ability in SH-SY5Y cells (Gao and Gu, 2007; Xue *et al.*, 2014). In the present study, despite the short-term incubation of the SH-SY5Y cells with nicotine and menthol revealing an independent effect on cell viability, menthol's actions on nAchR increasing nicotine bioavailability in the body were also documented (Wickham, 2015). Hence. the exact interactions among different doses of nicotine and menthol in either the short-term exposure or the long-term exposure in the body need to be elucidated.

In the present study, despite the fact that nicotine and menthol independently decreased cisplatin and amyloid $\beta_{1\text{-}42}$ induced TNF-α expression and the collapse of mitochondrial membrane potential, the exact relationship between cytokine release and mitochondrial function still uncertain. A rapid increase of reactive oxygen species (ROS) occurs in acute ischemic stroke and overwhelms antioxidant defense, causing further tissue damage (Rodrigo *et al.*, 2013). The reduction of antioxidants was documented to produce an oxidation-reduction imbalance and lead to mitochondrial dysfunction (Poprac *et al.*, 2017). Whereas a lower concentration of ROS is essential for normal cellular signaling, a high concentration of ROS causes damage to cellular macromolecules and results in necrosis and apoptosis (Singh *et al.*, 2019). Hence, despite the fact we found that nicotine and menthol independently alleviate cisplatin and amyloid $\beta_{1\text{-}42}$-induced TNF-α expression and the collapse of mitochondrial membrane potential, further study should focus on the exact effects of nicotine and menthol on ROS production.

In conclusion, in the present study, as agonists of nAChR and TRPM8, nicotine and menthol exerted their neuroprotective effects by up-regulating anti-apoptotic protein Bcl-xl via JNK activation. These findings indicate that Bcl-xl and JNK might be potential therapeutic target molecules for the prevention and treatment of neurodegenerative diseases.

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Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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References

Aicher A, Heeschen C, Mohaupt M, Cooke JP, Zeiher AM, Dimmeler S (2003). Nicotine strongly activates dendritic cell-mediated adaptive immunity: Potential role for progression of atherosclerotic lesions. *Circulation* **107**: 604–611. DOI 10.1161/01.CIR.0000047279.42427.6D.

Ashkenazi A, Fairbrother WJ, Leverson JD, Souers AJ (2017). From basic apoptosis discoveries to advanced selective Bcl-2 family inhibitors. *Nature Reviews Drug Discovery* **16**: 273–284. DOI 10.1038/nrd.2016.253.

Bhadania M, Joshi H, Patel P, Kulkarni VH (2012). Protective effect of menthol on β-amyloid peptide induced cognitive deficits in mice. *European Journal of Pharmacology* **681**: 50–54. DOI 10.1016/j.ejphar.2012.01.035.

Bono F, Mutti V, Fiorentini C, Missale C (2020). Dopamine D3 receptor heteromerization: Implications for neuroplasticity and neuroprotection. *Biomolecules* **10**: 1016. DOI 10.3390/biom10071016.

Dong Y, Bi W, Zheng K, Zhu E, Wang S et al. (2020). Nicotine prevents oxidative stress-induced hippocampal neuronal injury through α7-nAChR/Erk1/2 signaling pathway. *Frontiers in Molecular Neuroscience* **13**: 961. DOI 10.3389/fnmol.2020.557647.

Fait BW, Thompson DC, Mose TN, Jatlow P, Jordt SE, et al. (2017). Menthol disrupts nicotine's psychostimulant properties in an age and sex-dependent manner in C57BL/6J mice. *Behavioural Brain Research* **334**: 72–77. DOI 10.1016/j.bbr.2017.07.027.

Fan L, Balakrishna S, Jabba SV, Bonner PE, Taylor SR et al. (2016). Menthol decreases oral nicotine aversion in C57BL/6 mice through a TRPM8-dependent mechanism. *Tobacco Control* **25**: ii50–ii54. DOI 10.1136/tobaccocontrol-2016-053209.

Gao FG, Gu JR (2007). *Ex vivo* nicotine stimulation augments the efficacy of therapeutic bone marrow-derived dendritic cell vaccination. *Clinical Cancer Research* **13**: 3706–3712. DOI 10.1158/1078-0432.CCR-07-0028.

Go YY, Mun JY, Chae SW, Chang J, Song JJ (2020). Comparison between in vitro toxicities of tobacco-and menthol-flavored electronic cigarette liquids on human middle ear epithelial cells. *Scientific Reports* **10**: 83. DOI 10.1038/s41598-020-59290-y.

Henderson BJ, Wall TR, Henley BM, Kim CH, Mckinney S, Lester HA (2017). Menthol enhances nicotine reward-related behavior by potentiating nicotine-induced changes in nAChR function, nAChR upregulation, and DA neuron excitability. *Neuropsychopharmacology* **42**: 2285–2291. DOI 10.1038/npp.2017.72.

Herr AB (2018). Evolution of an allosteric "off switch" in apoptotic caspases. *Journal of Biological Chemistry* **293**: 5462–5463. DOI 10.1074/jbc.H118.002379.

Islam MS, Ahmed MU, Sayeed MSB, Al Maruf A, Mostofa A et al. (2013). Lung cancer risk in relation to nicotinic acetylcholine receptor, CYP2A6 and CYP1A1 genotypes in the Bangladeshi population. *Clinica Chimica Acta* **416**: 11–19. DOI 10.1016/j.cca.2012.11.011.

Jiang YN, Ni XY, Yan HQ, Shi L, Lu NN et al. (2019). Interleukin 6-triggered ataxia-telangiectasia mutated kinase activation facilitates epithelial-to-mesenchymal transition in lung cancer by upregulating vimentin expression. *Experimental Cell Research* **381**: 165–171. DOI 10.1016/j.yexcr.2019.05.011.

Kale J, Liu Q, Leber B, Andrews DW (2012). Shedding light on apoptosis at subcellular membranes. *Cell* **151**: 1179–1184. DOI 10.1016/j.cell.2012.11.013.

Ke SZ, Ni XY, Zhang YH, Wang YN, Wu B, Gao FG (2013). Camptothecin and cisplatin upregulate ABCG2 and MRP2 expression by activating the ATM/NF-κB pathway in lung cancer cells. *International Journal of Oncology* **42**: 1289–1296. DOI 10.3892/ijo.2013.1805.

Krishna A, Biryukov M, Trefois C, Antony PM, Hussong R et al. (2014). Systems genomics evaluation of the SH-SY5Y neuroblastoma cell line as a model for Parkinson's disease. *BMC Genomics* **15**: 1154. DOI 10.1186/1471-2164-15-1154.

Kvansakul M, Caria S, Hinds MG (2017). The Bcl-2 family in host-virus interactions. *Viruses* **9**: 290. DOI 10.3390/v9100290.

Lombardo S, Maskos U (2015). Role of the nicotinic acetylcholine receptor in Alzheimer's disease pathology and treatment. *Neuropharmacology* **96**: 255–262. DOI 10.1016/j.neuropharm.2014.11.018.

Mulcahy MJ, Huard SM, Paulo JA, Wang JH, Mckinney S et al. (2019). Brain region-specific nAChR and associated protein abundance alterations following chronic nicotine and/or menthol exposure. *Journal of Proteome Research* **19**: 36–48. DOI 10.1021/acs.jproteome.9b00286.

Muruganathan U, Srinivasan S, Vinothkumar V (2017). Antidiabetogenic efficiency of menthol, improves glucose homeostasis and attenuates pancreatic β-cell apoptosis in streptozotocin-nicotinamide induced experimental rats through ameliorating glucose metabolic enzymes. *Biomedicine & Pharmacotherapy* **92**: 229–239. DOI 10.1016/j.biopha.2017.05.068.

Nalivaeva NN, Turner AJ (2013). The amyloid precursor protein: A biochemical enigma in brain development, function and disease. *FEBS Letters* **587**: 2046–2054. DOI 10.1016/j.febslet.2013.05.010.

Nonnemaker J, Feirman SP, Macmonegle A, Ambrose BK, Jackson KJ et al. (2019). Examining the role of menthol cigarettes in progression to established smoking among youth. *Addictive Behaviors* **98**: 106045. DOI 10.1016/j.addbeh.2019.106045.

Paouri E, Tzara O, Kartalou GI, Zenelak S, Georgopoulos S (2017). Peripheral tumor necrosis factor-alpha (TNF-α) modulates amyloid pathology by regulating blood-derived immune cells and glial response in the brain of AD/TNF transgenic

mice. *Journal of Neuroscience* **37**: 5155–5171. DOI 10.1523/JNEUROSCI.2484-16.2017.

Patel H, Mcintire J, Ryan S, Dunah A, Loring R (2017). Anti-inflammatory effects of astroglial α7 nicotinic acetylcholine receptors are mediated by inhibition of the NF-κB pathway and activation of the Nrf2 pathway. *Journal of Neuroinflammation* **14**: 399. DOI 10.1186/s12974-017-0967-6.

Pistritto G, Trisciuoglio D, Ceci C, Garufi A, D'Orazi G (2016). Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. *Aging (Albany NY)* **8**: 603–619. DOI 10.18632/aging.100934.

Pogocki D, Ruman T, Danilczuk M, Danilczuk M, Celuch M, Wałajtys-Rode E (2007). Application of nicotine enantiomers, derivatives and analogues in therapy of neurodegenerative disorders. *European Journal of Pharmacology* **563**: 18–39. DOI 10.1016/j.ejphar.2007.02.038.

Poprac P, Jomova K, Simunkova M, Kollar V, Rhodes CJ, Valko M (2017). Targeting free radicals in oxidative stress-related human diseases. *Trends in Pharmacological Sciences* **38**: 592–607. DOI 10.1016/j.tips.2017.04.005.

Prasad H, Rao R (2015). The Na^+/H^+ exchanger NHE6 modulates endosomal pH to control processing of amyloid precursor protein in a cell culture model of Alzheimer disease. *Journal of Biological Chemistry* **290**: 5311–5327. DOI 10.1074/jbc.M114.602219.

Rodrigo R, Fernández-Gajardo R, Gutiérrez R, Manuel Matamala J Carrasco R et al. (2013). Oxidative stress and pathophysiology of ischemic stroke: Novel therapeutic opportunities. CNS & Neurological Disorders Drug Targets **12:** 698–714.

Rosenberg RN, Lambracht-Washington D (2013). DNA Aβ42 vaccination as possible alternative immunotherapy for Alzheimer disease. *JAMA Neurology* **70**: 772–773. DOI 10.1001/jamaneurol.2013.1502.

Rozza AL, de Faria FM, Brito ARS, Pellizzon CH (2014). The gastroprotective effect of menthol: Involvement of anti-apoptotic, antioxidant and anti-inflammatory activities. *PLoS One* **9**: e86686. DOI 10.1371/journal.pone.0086686.

Sakamuru S, Attene-Ramos MS, Xia M (2016). Mitochondrial membrane potential assay. *High-Throughput Screening Assays in Toxicology*. New York, NY: Humana Press.

Singh A, Kukreti R, Saso L, Kukreti S (2019). Oxidative stress: A key modulator in neurodegenerative diseases. *Molecules* **24**: 1583. DOI 10.3390/molecules24081583.

Suh DH, Kim MK, Kim HS, Chung HH, Song YS (2013). Mitochondrial permeability transition pore as a selective target for anti-cancer therapy. *Frontiers in Oncology* **3**: 00041. DOI 10.3389/fonc.2013.00041.

Thompson MF, Poirier GL, Dávila-García MI, Huang W, Tam K et al. (2018). Menthol enhances nicotine-induced locomotor sensitization and *in vivo* functional connectivity in adolescence. *Journal of Psychopharmacology* **32**: 332–343. DOI 10.1177/0269881117719265.

van Opdenbosch N, Lamkanfi M (2019). Caspases in cell death, inflammation, and disease. *Immunity* **50**: 1352–1364. DOI 10.1016/j.immuni.2019.05.020.

Vringer E, Tait SW (2019). Mitochondria and inflammation: Cell death heats up. *Frontiers in Cell and Developmental Biology* **7**: 6627. DOI 10.3389/fcell.2019.00100.

Wang YY, Liu Y, Ni XY, Bai ZH, Chen QY et al. (2014). Nicotine promotes cell proliferation and induces resistance to cisplatin by α7 nicotinic acetylcholine receptor-mediated activation in Raw264.7 and El4 cells. *Oncology Reports* **31**: 1480–1488. DOI 10.3892/or.2013.2962.

Wickham R (2015). Focus: Addiction: How menthol alters tobacco-smoking behavior: A biological perspective. *Yale Journal of Biology and Medicine* **88**: 279.

Xicoy H, Wieringa B, Martens GJ (2017). The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Molecular Neurodegeneration* **12**: 384. DOI 10.1186/s13024-017-0149-0.

Xue MQ, Liu XX, Zhang YL, Gao FG (2014). Nicotine exerts neuroprotective effects against β-amyloid-induced neurotoxicity in SH-SY5Y cells through the Erk1/2-p38-JNK-dependent signaling pathway. *International Journal of Molecular Medicine* **33**: 925–933. DOI 10.3892/ijmm.2014.1632.

Yan HQ, Huang XB, Ke SZ, Jiang YN, Zhang YH et al. (2014). Interleukin 6 augments lung cancer chemotherapeutic resistance via ataxia-telangiectasia mutated/NF-kappaB pathway activation. *Cancer Science* **105**: 1220–1227. DOI 10.1111/cas.12478.

Yang W, Ma K, Chen X, Shi L, Bu G et al. (2014). Mitogen-activated protein kinase signaling pathways are involved in regulating α7 nicotinic acetylcholine receptor-mediated amyloid-β uptake in SH-SY5Y cells. *Neuroscience* **278**: 276–290. DOI 10.1016/j.neuroscience.2014.08.013.

Zhang M, Harrison E, Biswas L, Tran T, Liu X (2018). Menthol facilitates dopamine-releasing effect of nicotine in rat nucleus accumbens. *Pharmacology Biochemistry and Behavior* **175**: 47–52. DOI 10.1016/j.pbb.2018.09.004.