AVE0991 Promotes Neuroprotection via Mas/ERK/mPTP Pathway in α -Synuclein A53T Transgenic Mice

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Research Article

Keywords: Angiotensin-(1-7), AVE0991, Mitochondrial permeability transition pore, Apoptosis, Parkinson's disease

Posted Date: November 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1067081/v1>

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Abstract

Parkinson's disease (PD) is the most prevalent neurodegenerative movement diseases featured by selective loss of dopaminergic (DA) neurons within the striatum and substantia nigra (SN). Accumulating evidence have indicated that angiotensin-(1-7) (Ang-(1-7)) prevents neuronal damage by binding to its specific receptor Mas in PD. To date, the underlying mechanisms is not known thus far. In the present study, by using α -synuclein A53T transgenic mice (A53T mice), we showed that the neuronal apoptosis in the SN of A53T mice may be attributed to a decrease in Ang-(1-7) levels. Additionally, we revealed that AVE0991, a recently found non-peptide analogue of Ang-(1-7), ameliorated neuronal apoptosis via Mas/ERK pathway in primary DA neurons. More importantly, we provided novel evidence that this beneficial impact was dependent on the suppression of mitochondrial permeability transition pore opening. In conclusion, these findings disclose the neuroprotective impact of Ang-(1-7) in the etiology of PD, and support the application of its nonpeptide analogue AVE0991 in the therapies of this neurodegenerative disease.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease affecting approximately 1% of the population over 60 years old worldwide [1]. One pathological hallmark of PD is the massive death of dopaminergic (DA) neurons within the striatum and substantia nigra (SN), which cause a dopamine deficiency in the striatum and subsequent appearance of cardinal features [2]. To date, there is no evidence to elucidate the precise mechanisms underlying the loss of DA neurons.

The renin-angiotensin system (RAS) is indispensable for modulating the homeostasis of sodium and water in the circulatory system. In addition to this classic RAS, accumulating evidence have revealed that a local RAS exists in the brain and participate in the pathogenesis of several neurological disorders including PD [3]. As a newly determined component of RAS, Angiotensin-(1-7) (Ang-(1-7)) exerts physiological function via binding to its specific receptor Mas and antagonizes the action of angiotensin-II (Ang II). Recent study from Rabie et al. indicated that Ang-(1-7) attenuated 6-hydroxydopamine (6-OHDA) induced neurotoxicity through activation of Mas receptor in hemiparkinsonian rats [4]. This result disclosed that the neuroprotective impact of Ang-(1-7)/Mas was closely related to anti-apoptotic property in DA neurons. However, the anti-apoptotic mechanism of Ang-(1-7) in DA neurons remain elusive thus far.

As an important molecule involved in nerve cell death, extracellular signal-regulated kinases (ERK) has sought immense attention and research. Previous study from Park and colleagues indicated that DA neurons viability regulated by D1 dopamine receptor agonist was dependent on activation of ERK-Bad-Bax pathway [5]. Meanwhile, recent research disclosed that activation of α 7-nAChRs protected DA neurons from MPTP induced apoptosis through ERK/p53 signaling pathway [6]. More importantly, mounting evidence have shown that the actions of Ang-(1-7) were closely associated with ERK activity in

vitro [7, 8]. These findings suggest that ERK may probably be involved in the protective effect of Ang-(1-7) on DA neurons.

Mitochondrial permeability transition pore (mPTP) was involved in the process of mitochondria-dependent apoptosis. Previous studies have revealed ERK acted as an upstream signaling molecule in the regulation of mPTP [9]. In addition, our previous study showed that the opening of mPTP contributed to Ang II-induced apoptosis in DA neurons [10]. On basis of the above-mentioned findings, we hypothesized that Ang-(1-7) may play a neuroprotective effect through Mas/ERK/mPTP signal pathways in DA neurons. Therefore, in the current study, by using α -synuclein A53T transgenic mice (A53T mice), an animal model of PD (α -synuclein A53T overexpression specifically in DA neurons), we indicated that the neuronal apoptosis in the SN may be attributed to the decline of Ang-(1-7) levels. Furthermore, we demonstrated that AVE0991, a recently found non-peptide analogue of Ang-(1-7), ameliorated neuronal apoptosis via Mas/ERK pathway in primary DA neurons. Finally, we provided novel evidence that this beneficial impact was dependent on the repression of mPTP opening.

Materials And Methods

Reagents and Chemicals

AVE0991 was provided by Medchem Express Inc. A-779 (a specific Mas inhibitor) was purchased from Abcam Inc. Atractyloside (Atr, a specific mPTP agonist), U-0126 (a specific ERK1/2 antagonist) were purchased from Sigma-Aldrich Inc. Antibodies against p-ERK1/2, ERK1/2 were ordered from Cell Signaling Technology. GAPDH were ordered from Santa Cruz Biotechnology.

Animals and Treatments

The A53T mice expressed mutant human A53T α -synuclein were provided by Model Animal Research Center of Nanjing University. Mice expressing A53T α -synuclein (line M83) is one of the early onset models of PD and mainly applied to the study of pathogenesis of PD. All mice were kept in an environmentally controlled room under a light/dark cycle of 12 h and supplied with water as well as standard mouse diet. Mice were employed to implement the experiment in according with the international guidelines for animal research. The experimental protocol was approved by the Biological Research Ethics Committee of Huai'an First People's Hospital.

To investigate the impact of AVE on anti-neuronal apoptosis, four-month old A53T mice were allocated to 5 groups randomly (6 mice per group): control group, vehicle (saline) group, AVE group (1 mg per kg per day), AVE group (3 mg per kg per day) and AVE group (10 mg per kg per day). Mice were given daily treatment with vehicle (saline, intraperitoneally) or AVE (1, 3 or 10 mg per kg per day, intraperitoneally) for 30 consecutive days. Afterwards, mice were deeply anesthetized with pentobarbital and sacrificed by cervical dislocation for the next experiment.

ELISA Experiments

Ang-(1-7) (R&D Systems, Inc.), Cleaved caspase-3 (R&D Systems, Inc.), Bcl-2 (Elabscience, Inc.) and Bax (Elabscience, Inc.) concentrations in the SN of mice were detected using mouse ELISA kits following the supplier's guidelines [11]. The absorbance was evaluated in each well solution at 450 nm with a spectrophotometer.

Primary Midbrain DA Neuron Culture

Ventral mesencephalic tissue was dissected from postnatal 0 day mouse according to previous protocol [12]. Then ventral mesencephalic tissue was trypsinized and dissociated. Afterwards, dissociated cell was plated on 0.5 mg/mL poly-D-lysine (Sigma)-coated glass bottom dishes (MatTek). The cells were cultured in DMEM/F12 medium containing 10 % fetal bovine serum, streptomycin (50 µg/ml), and penicillin (50 U/ml). Next day, B-27 Plus Neuronal Culture System (Thermo Fisher Scientific, A3653401) further replaced DMEM/F12 medium. After 7 days of culture, primary DA neurons were ready for administration and infected by adenovirus that expression either GFP or α -synuclein A53T.

Quantitative Real-Time PCR

Total RNA was extracted from the SN of mice with Trizol reagent (Invitrogen, USA) following the kit guidelines [13]. By utilizing the PrimeScriptTM RT Master Mix (Takara), equal quantity of total RNA was reverse transcribed under standard conditions. Afterwards, qRT-PCR reactions were employed to detect related mRNA expression using SYBR[®] Premix Ex TaqTM (Takara). Meanwhile, GAPDH was chosen as an internal control. Primers sequences: for Mas: forward 5'-CATCTAGGACTGGGCAGAGC-3', reverse 5'-AGTCAGGAGGTGGAGAGCAA-3', for GAPDH: forward 5'-CAACAGCAACTCCCACTCTTC-3', reverse 5'-GGTCCAGGGTTTCTTACTCCTT-3'.

Western Blot Analysis

Western blot assay was employed to measure the protein level as previously reported procedure [14]. Briefly, protein were extracted from primary DA neurons after receiving indicated administration. The BCA Protein Assay kit (Pierce, IL) was employed to determine the protein concentrations. Same amount of protein was subjected to electrophoresis on 15 % SDS-PAGE. Then transferred to a polyvinylidene difluoride (PVDF) membranes and further blocked with 5% skim milk. After that, PVDF membranes were stored overnight in a solution containing primary antibodies. After incubation with primary antibody, the membranes were further incubated with horseradish peroxidase-labeled secondary antibodies for 2 h. Finally, the visualized protein bands were exposed to X-ray film. Quantification of immunoreactivity using ImageJ software.

Double Immunofluorescence Assay

Immunofluorescence analysis was implemented following standard procedure [15]. In brief, primary DA neurons were subjected to appropriate treatment. The neurons were then fixed in 4 % paraformaldehyde (PFA) and permeabilized with 0.5 % Triton X-100. Afterwards, the neurons were stored in a primary

antibody (anti-caspase-3 antibody) solution with higher selectivity for caspase-3 (1:200, Cell Signaling Technology Inc.). After incubation with the primary antibody, neurons were further incubated with fluorescein isothiocyanate (FITC)-conjugated IgG (1:1000, bs-0295D-FITC; BIOSS Inc.). Moreover, the cell nuclei were counterstained with DAPI (Vector Laboratories). The fluorescence in DA neurons was observed with fluorescence microscope (Olympus BX53) at 488 nm. Images were acquired using Image-Pro Plus Software (Olympus).

Flow Cytometry Analysis

The apoptosis rate of primary DA neurons was evaluated using a flow cytometer with a FITC annexin V and PI double labeling apoptosis detection kit (BD Biosciences) as described elsewhere [16]. In brief, cultured DA neurons were trypsinized after indicated treatment, washed with 4 °C PBS and collected by centrifugation. After that, DA neurons were stained with FITC annexin V/PI binding buffer at 37 °C in the dark for 15 min. Finally, flow cytometry was utilized to quantitatively evaluate the apoptosis rate of DA neurons. The intracellular ROS production in primary DA neurons was detected utilizing flow cytometry with fluorescent DCFH-DA as probe (Enzo Life Sciences) [17]. The mPTP opening of primary DA neurons was evaluated with a flow cytometer after CoCl₂-calcein fluorescence quenching assay [18]. The data of aforementioned three assay were processed with CellQuest Pro software (BD Bioscience).

CoCl₂-Calcein Fluorescence Quenching Assay

CoCl₂-calcein fluorescence quenching assay was used to determine the opening of mPTP in primary DA neurons as previously described [19]. Calcein-acetomethoxy (AM) is permeable to intact cellular membranes but not to intact mitochondrial membranes under physiological conditions. Therefore, co-incubation of DA neurons with CoCl₂ quenches the fluorescent calcein in the cells except mitochondria. But the fluorescent calcein in the mitochondria is also quenched in the case of mPTP opening. The calcein fluorescence in DA neurons was observed with fluorescence microscope (Olympus Inc.).

Determination of Brain ACE2 Activity

ACE2 activity assay kit (SensoLyte 390, AnaSpec) with fluorescent Mc-Ala/Dnp was used to detect the ACE2 activity in the SN of mice following standard procedure [20]. The Mc-Ala fluorescence was taken at excitation and emission wavelengths of 330 nm and 390 nm, respectively.

Cell Viability Assay

MTT assay was employed to detect the viability of primary DA neurons following standard procedure [21]. In short, cultured DA neurons were subjected to indicated treatment, and the dissolved MTT was added to the media. Then the cultured media containing MTT were removed after incubation at 37 °C for 4 h. Finally, a microplate reader was used to measure the absorbance at 490 nm.

Immunohistochemical staining

DA neurons in the SN of mice were determined with monoclonal mouse anti-tyrosine hydroxylase (anti-TH) antibody [22]. Shortly, the brains were sliced into 30 μm thick sections using a cryostat. Then the slides of SN were blocked in 5 % bovine serum albumin and exposed to anti-TH (1:2000; Sigma Inc.) primary antibody overnight. Afterwards, the SN sections were stored in solution containing secondary antibody for 1 h. After secondary antibody incubation, sections were counterstained with Mayer's hematoxylin, fixed on slides following dehydration. The numbers of TH-positive cells (brown granules) were counted with microscope equipped with a CCD camera and then analyzed by three researchers not involved in the experimental treatments.

Statistical Analysis

Statistical analysis was performed by SPSS software (version 17.0, SPSS). Differences between groups were analyzed with one way ANOVA followed by Tukey's post hoc test. All data were presented as mean $\pm$ s.d. Value of $P<0.05$ was considered statistically significant.

Results

Neuronal Apoptosis was Increased in the Brain of A53T Mice during Aging

Firstly, we employed ELISA to measure the neuronal apoptosis in the SN of 2-, 4-, 8-month old A53T mice. As shown in Figure 1a-c, during the aging process, the protein levels of cleaved caspase-3 and Bax were obviously increased, but the protein levels of Bcl-2 were significantly decreased in the SN of A53T mice ($n=6$, $P<0.05$). In addition, we further detected the numbers of TH-positive neurons in the SN of 2-, 4-, 8-month old A53T mice. As shown in Figure 1d and 1e, the numbers of TH-positive neurons were markedly reduced in the SN of A53T mice during aging ($n=6$, $P<0.05$). These results revealed an elevated neuronal apoptosis in the brain of A53T mice.

Ang-(1-7) Levels were Reduced in the Brains of A53T Mice during Aging

Afterwards, we evaluated whether the brain ACE2/Ang-(1-7)/Mas axis was involved in this increased neuronal apoptosis. As seen in Figure 2b, evidently reduced Ang-(1-7) levels were noted in the SN of A53T mice during aging ($n=6$, $P<0.05$). However, as shown in Figure 2a and 2c, the ACE2 activity and Mas mRNA levels had no marked difference in the SN of A53T mice in the aging process. These results showed that the increase of neuronal apoptosis may be attributed to the decreased Ang-(1-7) levels in the brains of A53T mice.

AVE Ameliorated Neuronal Apoptosis in the Brains of A53T Mice

Since the half-life of Ang-(1-7) in vivo is very short, we used AVE in subsequent experiments, which is a non-peptide analog of Ang-(1-7) with a longer half-life. To validate the above-mentioned hypothesis, 4-month old A53T mice received daily treatment with vehicle or AVE (1, 3 or 10 mg per kg per day) for 30 consecutive days. As shown in Figure 3a and 3b, administration of AVE (3 or 10 mg per kg per day) markedly ameliorated cleaved caspase-3 and Bax protein levels of in the SN of A53T mice ($n=6$, $P<0.05$).

Meanwhile, as seen in Figure 3c, the protein levels of Bcl-2 was obviously increased after AVE (3 or 10 mg per kg per day) treatment ($n=6$, $P<0.05$). Moreover, AVE (3 or 10 mg per kg per day) can significantly increase the numbers of TH-positive neurons (Fig. 3d and 3e). These findings implied that AVE mitigated neuronal apoptosis in the brains of A53T mice.

AVE Protected Primary DA Neurons through a Mas Receptor Dependent Manner

We next explored the impact of AVE on neuronal apoptosis in vitro. To this end, primary DA neurons were isolated from the SN of 4-month old A53T mice. Next, DA neurons were treated with increasing concentrations of AVE (1×10^{-7} and 1×10^{-6} M) for 4 h. As shown in Figure 4a and 4b, AVE attenuated neuronal apoptosis in a concentration-dependent manner ($P<0.05$). Meanwhile, this correlation was further ascertained by immunofluorescence analysis utilizing the caspase-3 antibody with higher selectivity for caspase-3 (Fig. 4c). In addition, MTT assay was used to determine the cell viability. As shown in Figure 4d, low (1×10^{-7} M) and high dose (1×10^{-6} M) of AVE increased the viability of DA neurons by 16% ($P>0.05$) and 42% ($P<0.05$), respectively. Then flow cytometry was applied to assess the intracellular ROS levels of DA neurons. As seen in Figure 5a and 5b, when compared with control solution, low (1×10^{-7} M) and high dose (1×10^{-6} M) of AVE decreased intracellular ROS levels in DA neurons (For low dose: 23.8% vs. 19.3%; $P>0.05$; For high dose: 23.8% vs. 9.3%, $P<0.05$). Finally, as seen in Figure 5c and 5d, AVE also dose-dependently increased phosphorylation levels of ERK1/2 (p-ERK1/2) in DA neurons ($P<0.05$). It needed to be noted that there was no significant difference in the total protein levels of ERK1/2 among groups. These findings suggested that AVE dose-dependently protected DA neurons in vitro.

Then we attempted to clarify the underlying mechanisms by which AVE alleviated neuronal apoptosis. As shown in Figure 6b, DA neurons were co-incubated with AVE (1×10^{-6} M) and Mas inhibitor A-779 (1×10^{-6} M) for 4 h, and a higher intracellular ROS level was seen in AVE + A-779 group when compared to AVE group ($P<0.05$). Meanwhile, we found that the elevated protein levels of p-ERK1/2 caused by AVE were blocked by A-779 in DA neurons, but A-779 did not change the total protein levels of ERK1/2 (Fig. 6d-e). Furthermore, A-779 significantly increased the percentage of neuronal apoptosis (Fig. 6a) and decreased the viability of DA neurons (Fig. 6c). Our data showed that AVE protected DA neurons via binding to Mas receptor and might through downstream ERK.

AVE Protected Primary DA Neurons via inhibition of mPTP opening

Our previous study has shown that mitochondria was strongly associated with the pathogenesis of PD and mPTP may play a critical role in this regard. In light of this, we next tried to establish that whether mPTP contributed to the neuroprotective effect of AVE in DA neurons. DA neurons were co-incubated with AVE and mPTP agonist Atr (5 mmol/L) for 4 h. The mPTP opening in DA neurons following AVE administration (1×10^{-6} M) was detected by a CoCl_2 -calcein fluorescence quenching assay. As shown in Figure 7a and 7b, AVE evidently increased the signal of calcein fluorescence in DA neurons by 60.4% ($P<0.05$), suggesting a reduction in mPTP opening. Consistently, significant decline in mPTP opening

induced by AVE was observed using flow cytometry (Fig. 7c). However, Atr remarkably decreased the elevated signal of calcein fluorescence triggered by AVE ($P<0.05$). It should be noted that this result was further confirmed using flow cytometry (Fig. 7c). Furthermore, as seen in Figure 7d and 7e, the decrease of neuronal apoptosis rate and increase of cell viability triggered by AVE can be mostly reversed by Atr ($P<0.05$). These results demonstrated that AVE played a neuroprotective role by inhibiting mPTP opening in primary DA neurons.

Discussion

In this study, we first revealed that the neuronal apoptosis was evidently increased in the brain of A53T mice during the aging process, since the expression of caspase-3 and Bax were obviously increased in the SN of 4-, 8-month old A53T mice. These results were compatible with previous findings that neuronal death was significantly higher in the midbrain of A53T transgenic mouse model of PD when compared to non-transgenic group [23].

Ang-(1-7) is an essential bioactive peptide of RAS, which plays a role in signal transduction and biologic functions mainly by binding to the G protein-coupled receptor Mas. Mounting research have shown that Ang-(1-7) exerts neuroprotective role in various neurological diseases. For instance, Ang-(1-7) and its Mas receptor participated in the process of captopril protecting neuronal damage caused by focal cerebral ischemia [24]. Moreover, AVE could attenuate aging-related neuroinflammation via binding to MAS1 receptor [15]. In addition, recent study indicated that AVE ameliorated neuronal apoptosis via MAS/PKA/CREB/UCP-2 pathway after subarachnoid hemorrhage in vivo [25]. Consistent with these findings, our results indicated that Ang-(1-7) levels in the SN of A53T mice were remarkably decreased and AVE supplement can partially reverse neuronal apoptosis through a Mas receptor dependent manner.

ERK is an imperative signal pathway involved in neuronal survival and death. Emerging evidence have shown that ERK phosphorylation or activation contributes to neuroprotective effects in the mice model of PD. Recent findings from Shadfar and colleagues disclosed that antidepressants served as an important modulator in neuroprotection through up-regulation of ERK1/2 [26]. Furthermore, ERK activation participated in the attenuation of neurotoxicity and neuroinflammation induced by MPTP in vivo [27]. In our study, we also demonstrated that AVE attenuated neuronal apoptosis through phosphorylation of ERK in primary DA neurons. However, in contrast to our findings, Ma and colleagues reported that proanthocyanidins protected against rotenone-induced ROS generation and apoptosis in SH-SY5Y cells by inhibiting ERK signaling pathways [28]. This discrepancy needs to be investigated in future studies.

Accumulating scientific evidence showed that mPTP was a pivotal contributor to cell apoptosis [29]. Under pathological conditions, mPTP senses the stimulation of pro-apoptotic factors and facilitates the release of cytochrome c from the mitochondria into the cytoplasm, then initiating downstream apoptosis cascade [30]. Previous study from our group revealed that Ang II triggered mitochondrial-dependent apoptosis by promoting mPTP opening in DA neurons [10]. Meanwhile, the relationship between Ang-(1-7) and mitochondrial-mediated apoptotic pathway in DA neurons is less studied. Therefore, we attempted

to clarify whether mPTP was associated with the effect of Ang-(1-7) in primary DA neurons. As expected, by using mPTP agonist Atr, we demonstrated that AVE attenuated neuronal apoptosis by inhibiting mPTP opening in primary DA neurons, indicating that AVE protects DA neurons through a mPTP dependent manner.

To better clarify the potential neuroprotective mechanism of Ang-(1-7) in DA neurons, we further investigated the association between ERK and mPTP in our study. Several lines of evidence have shown that ERK acted as an upstream signaling molecule in regulating mPTP. For instance, previous study from Sun and colleagues revealed that ERK participated in the neuroprotective effect by regulating mPTP after ischemic stroke [31]. Recent study from Ping et al. disclosed that HSYA played an important role in the protection of cerebral ischemia/reperfusion damage by inhibiting the mPTP opening via MEK/ERK/CypD pathway [32]. Consistent with these findings, our study also showed ERK, as an upstream signaling molecule of mPTP, was involved in the neuroprotection of Ang-(1-7) in primary DA neurons.

Conclusion

In summary, in our study, we show that the neuronal apoptosis in the brain of A53T mice may be attributed to a decrease in Ang-(1-7) levels. In addition, we suggest that AVE ameliorates neuronal apoptosis via Mas/ERK pathway in primary DA neurons. More importantly, we also provide novel evidence that this beneficial impact is dependent on the inhibition of mPTP opening. Collectively, these findings disclose the neuroprotective effect of Ang-(1-7) in the etiology of PD, and raise the possibility of its non-peptide analogue AVE in the therapies of this neurodegenerative disease.

Declarations

Acknowledgments

We are grateful to Model Animal Research Center of Nanjing University for providing α -synuclein A53T transgenic mice.

Authors' contributions

ZO, LJX and FGX performed research and acquired the data. LJW analyzed the results. HYY, MW and XY developed methodology and discussed results. JLZ designed research. QT wrote the paper and supervised the study. All authors read and approved the final manuscript.

Funding

This study was funded by Double Innovation Doctoral Program in Jiangsu Province (SCBS2019 to Zhou Ou), National Natural Science Foundation of China (Grant no. 81600981 to Qiang Tong), Natural Science Foundation of Jiangsu Province (Grant no. BK20191212 to Qiang Tong) and Six Talent Peaks project in Jiangsu Province (CN) (WSN-282 to Qiang Tong).

Conflicts of interest

The authors declare no conflicts of interest.

Consent to Participate

Not applicable

Consent for Publication

Not applicable.

Availability of data and materials

All raw data are available upon reasonable request.

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Figures

Figure 1

Neuronal apoptosis was increased in the brain of A53T mice during the aging process. (a) The dynamic changes of cleaved caspase-3 protein levels in the SN of 2-, 4-, and 8-month-old A53T mice were assessed by ELISA. (b) The dynamic changes of Bax protein levels in the SN of 2-, 4-, and 8-month-old A53T mice were assessed by ELISA. (c) The dynamic changes of Bcl-2 protein levels in the SN of 2-, 4-, and 8-month-old A53T mice were assessed by ELISA. (d) TH-immunoreactive neurons in the right SN of each group. Scale bar: 500 μ m. (e) Quantitative analysis of TH-positive neurons in the right SN of each group. All data were analyzed by one-way ANOVA followed by Tukey's post hoc test. In panel e, data were expressed as a fold change relative to 2-month-old A53T mice. Columns represent mean $\pm$ SD ($n=6$ per group). [#] $P<0.05$ versus 2-month-old A53T mice.

Figure 2

Ang-(1-7) levels were decreased in the brains of A53T mice during the aging process. (a) The activity of ACE2 in the SN of 2-, 4-, and 8- month-old A53T mice was evaluated using a specific detection kit. (b) The Ang-(1-7) levels in the SN of 2-, 4-, and 8-month-old A53T mice were detected by ELISA. (c) The dynamic changes of Mas mRNA levels in the SN of 2-, 4-, and 8-month-old A53T mice were detected by qRT-PCR. GAPDH was used as an internal control. All data were analyzed by one-way ANOVA followed by Tukey's post hoc test and were expressed as a fold change relative to 2-month-old A53T mice. Columns represent mean $\pm$ SD (n=6 per group). #P<0.05 versus 2-month-old A53T mice.

Figure 3

AVE ameliorated neuronal apoptosis in the brains of A53T mice. Four-month-old A53T mice were injected intraperitoneally with vehicle or AVE (1, 3 or 10 mg/kg/day) for 30 days. Afterwards, mice were sacrificed for analysis. (a) The protein levels of cleaved caspase-3 in A53T mice were measured by ELISA. (b) The protein levels of Bax in A53T mice were measured by ELISA. (c) The protein levels of Bcl-2 in A53T mice were measured by ELISA. (d) TH-immunoreactive neurons in the right SN of each group. Scale bar: 500 μ m. (e) Quantitative analysis of TH-positive neurons in the right SN of each group. All data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Columns represent mean $\pm$ SD (n=6 per group). #P<0.05 versus vehicle-treated A53T mice.

Figure 4

AVE dose-dependently protected primary DA neurons in vitro. Primary DA neurons were treated with different doses of AVE (1×10^{-7} M and 1×10^{-6} M) for 4 h, and (a, b) Cell apoptosis was measured by flow cytometry using a commercial apoptosis detection kit. The apoptosis rate = $(\text{annexin V+PI+ cells} + \text{annexin V+PI- cells}) / \text{total cells} \times 100 \%$. (c) DA neurons were fixed and double-labeled with an anti-caspase-3 antibody with higher selectivity for caspase-3 (green) and DAPI (blue). Note that AVE decreased the caspase-3 immunoreactivity in DA neurons in a dose-dependent manner. Scale bar: 100 μm . (d) Cell viability was evaluated by MTT assay. All figures are representative of three independent experiments, performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Columns represent mean $\pm$ SD. [#] $P < 0.05$ versus control group.

Figure 5

AVE activated ERK in primary DA neurons. Primary DA neurons were treated with different doses of AVE (1×10^{-7} M and 1×10^{-6} M) for 4 h, and (a, b) Intracellular ROS levels was measured by flow cytometry with fluorescent DCFH-DA as probe (Enzo Life Sciences). Population P3 represents the ROS. (c) The protein levels of P-ERK1/2 and ERK1/2 were evaluated by Western blot. (d) Quantitative analysis of P-ERK1/2 protein levels. GAPDH was used as an internal control. All figures are representative of three independent experiments, performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Columns represent mean $\pm$ SD. # $P < 0.05$ versus control group.

Figure 6

AVE protected primary DA neurons through a Mas receptor dependent manner in vitro. Primary DA neurons were co-incubated with AVE (1×10^{-6} M) and Mas inhibitor A-779 (1×10^{-6} M) for 4 h, and (a) Cell apoptosis was measured by flow cytometry using a commercial apoptosis detection kit. (b) Intracellular ROS levels was measured by flow cytometry with fluorescent DCFH-DA as probe (Enzo Life Sciences). (c) Cell viability was evaluated by MTT assay. (d) The protein levels of P-ERK1/2 and ERK1/2 were evaluated by Western blot. (e) Quantitative analysis of P-ERK1/2 protein levels. GAPDH was used as an internal control. All figures are representative of three independent experiments, performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Columns represent mean $\pm$ SD. [#] $P < 0.05$ versus control group. ^{*} $P < 0.05$ versus AVE group.

Figure 7

AVE protected primary DA neurons through a mPTP dependent manner in vitro. Primary DA neurons were co-incubated with AVE (1×10^{-6} M) and mPTP agonist Atr (5 mmol/L) for 4 h, and (a, b) illustrate the CoCl₂-calcein fluorescence quenching assay which was employed to evaluate mPTP opening. Scale bar: 20 μ m. (c) The calcein fluorescence was further evaluated by flow cytometric analysis. (d) Cell apoptosis was measured by flow cytometry using a commercial apoptosis detection kit. (e) Cell viability was evaluated by MTT assay. All figures are representative of three independent experiments, performed in triplicate. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Columns represent mean $\pm$ standard deviation (SD). [#]P < 0.05 versus control group. ^{*}P < 0.05 versus AVE group.

Figure 8

AVE protected primary DA neurons via ERK/mPTP pathway in vitro. Primary DA neurons were co-treated with AVE (1×10^{-6} M) and ERK1/2 antagonist U-0126 (10 μ M) for 4 h, and (a) Cell apoptosis was measured by flow cytometry using a commercial apoptosis detection kit. (b) Intracellular ROS levels was measured by flow cytometry with fluorescent DCFH-DA as probe (Enzo Life Sciences). (c) Cell viability was evaluated by MTT assay. (d, e) illustrate the CoCl₂-calcein fluorescence quenching assay which was employed to evaluate mPTP opening. Scale bar: 20 μ m. (f) The calcein fluorescence was further evaluated by flow cytometric analysis. All figures are representative of three independent experiments, performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Columns represent mean $\pm$ SD. # $P < 0.05$ versus control group. * $P < 0.05$ versus AVE group.