RESEARCH ARTICLE

Long-term adenosine A1 receptor activation-induced sortilin expression promotes α-synuclein upregulation in dopaminergic neurons

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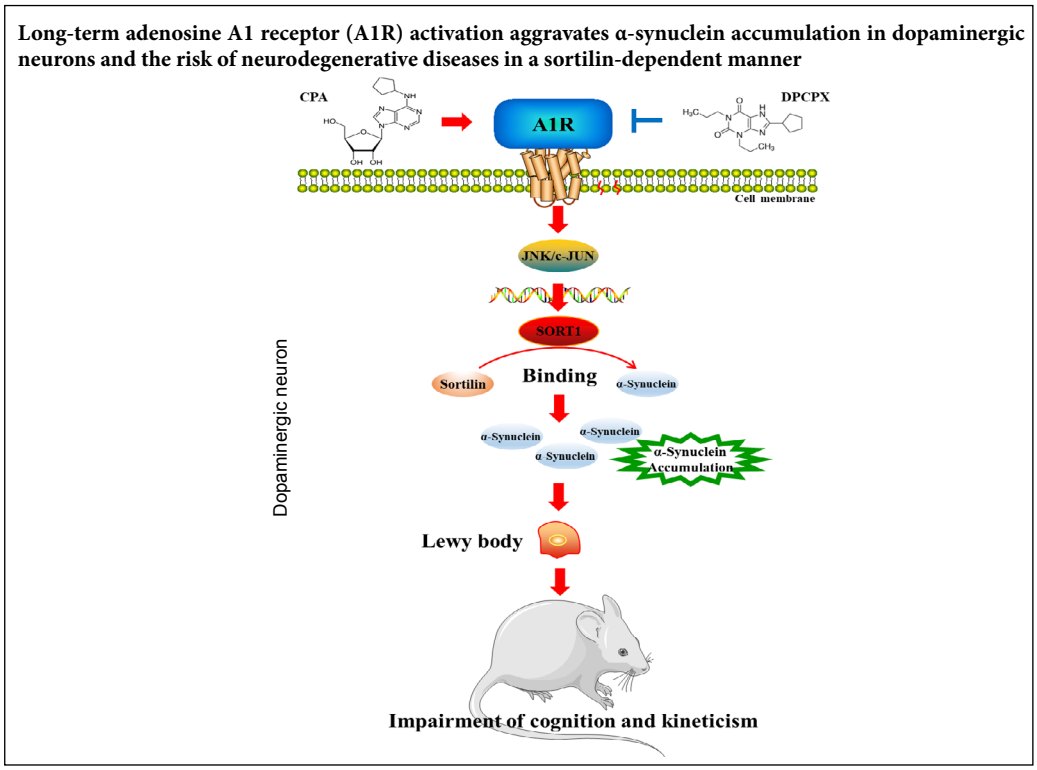
Graphical Abstract



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Abstract

Prolonged activation of adenosine A1 receptor likely leads to damage of dopaminergic neurons and subsequent development of neurodegenerative diseases. However, the pathogenesis underlying long-term adenosine A1 receptor activation-induced neurodegeneration remains unclear. In this study, rats were intraperitoneally injected with 5 mg/kg of the adenosine A1 receptor agonist N6-cyclopentyladenosine (CPA) for five weeks. The mobility of rats was evaluated by forced swimming test, while their cognitive capabilities were evaluated by Y-maze test. Expression of sortilin, α-synuclein, p-JUN, and c-JUN proteins in the substantia nigra were detected by western blot analysis. In addition, immunofluorescence staining of sortilin and α-synuclein was performed to detect expression in the substantia nigra. The results showed that, compared with adenosine A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (5 mg/kg) + CPA co-treated rats, motor and memory abilities were reduced, surface expression of sortin and α-synuclein in dopaminergic neurons was reduced, and total sortilin and total α-synuclein were increased in CPA-treated rats. MN9D cells were incubated with 500 nM CPA alone or in combination with 10 μM SP600125 (JNK inhibitor) for 48 hours. Quantitative real-time polymerase chain reaction analysis of sortilin and α-synuclein

mRNA levels in MN9D cells revealed upregulated sortilin expression in MN9D cells cultured with CPA alone, but the combination of CPA and SP600125 could inhibit this expression. Predictions made using Jasper, PROMO, and Alibaba online databases identified a highly conserved sequence in the sortilin promoter that was predicted to bind JUN in both humans and rodents. A luciferase reporter assay of sortilin promoter plasmid-transfected HEK293T cells confirmed this prediction. After sortilin expression was inhibited by sh-SORT1, expression of p-JUN and c-JUN was detected by western blot analysis. Long-term adenosine A1 receptor activation levels upregulated α-synuclein expression at the post-transcriptional level by affecting sortilin expression. The online tool Raptor-X-Binding and Discovery Studio 4.5 prediction software predicted that sortilin can bind to α-synuclein. Co-immunoprecipitation revealed an interaction between sortilin and α-synuclein in MN9D cells. Our findings indicate that suppression of prolonged adenosine A1 receptor activation potently inhibited sortilin expression and α-synuclein accumulation, and dramatically improved host cognition and kineticism. This study was approved by the University Committee of Animal Care and Supply at the University of Saskatchewan (approval No. AUP#20070090) in March 2007 and the Animals Ethics Committee of University of South China (approval No. LL0387-USC) in June 2017.

Key Words: *cognitive dysfunction; dopaminergic neuron; dyskinesia; JNK/c-JUN pathway; long-term adenosine A1 receptor activation; neural regeneration; neurodegenerative diseases; sortilin; α-synuclein*

Chinese Library Classification No. *R459.9; R741; Q421*

Introduction

The adenosine A1 receptor (A1R) has traditionally been described as a neuroprotective receptor because of its inhibitory effect in multiple neurodegenerative diseases (Gessi et al., 2011; Stockwell et al., 2017). Thus far, there has been substantial progress in expanding our understanding of the role of A1R activation in the brain (Gundlfinger et al., 2007; Gharibi et al., 2012; Gaudet et al., 2017), such as research indicating that A1R only effectively controls neuro-degeneration if activated in temporal proximity of the brain insult (Gessi et al., 2011). However, when A1R activation is prolonged under some pathogenic conditions, emerging evidence suggests that it will initiate neuronal damage and promote neurodegeneration through downstream signaling pathways such as the c-JUN N-terminal kinase (JNK)/c-JUN pathway (Stockwell et al., 2017). Furthermore, in our studies of the role of prolonged A1R activation in the brain, we found that treatment with the A1R agonist N6-cyclopentyladenosine (CPA) caused increased neuronal death in rat hippocampal slices (Stockwell et al., 2016, 2017).

Sortilin, which is encoded by the SORT1 gene and widely distributed throughout the central nervous system, has been increasingly implicated in multiple neurodegenerative diseases including Parkinson's disease (PD) (Wilson et al., 2014). As a multifunctional sorting receptor of the vacuolar protein sorting 10 protein domain receptor family, sortilin engages in intracellular sorting and trafficking of various proteinic substrates between the trans-Golgi network and plasma membrane compartments (Gao et al., 2017). Sortilin has been shown to direct the trafficking of substrates not only in biosynthetic pathways (anterograde traffic), but also during endocytic retrieval from the cell surface (retrograde traffic) (Kwon and Christian, 2011; Gao et al., 2017). Therefore, sortilin is a good candidate for the intracellular sorting receptor involved in the misprocessing and missorting of intracellular α-synuclein protein by these two trafficking pathways.

Encoded by the SNCA gene, α-synuclein is an extremely heat-resistant, small acidic protein (14 kDa, 140 amino acid residues) that is predominantly expressed in the brain, particularly the hippocampus, striatum, and substantia nigra (Kalia and Kalia, 2015). α-Synuclein is a soluble, natively unfolded protein with an extended structure primarily composed of random coils, but may acquire secondary structural elements upon interaction with a variety of ligands and proteins that alter its native state conformation, thus leading to a misfolded state (Recchia et al., 2004; Hewitt and Whitworth, 2017). Subsequently, α-synuclein becomes insoluble and aggregates into oligomers that form intracellular inclusions within cell bodies (Lewy bodies) and the processes of neurons (Lewy neurites), which initiates progressive neurodegeneration of dopaminergic neurons and contributes to the pathogenesis of PD (Recchia et al., 2004; Kalia and Lang, 2015). Thus, it is crucial to avoid the accumulation of α-synuclein protein in dopaminergic neurons to decrease the likelihood of PD development.

In the present study, we established that prolonged A1R activation activates the downstream JNK/c-JUN pathway to increase sortilin expression in dopaminergic neurons, which causes upregulation of α-synuclein protein and altera-tions of PD-like behavior.

Materials and Methods

Model establishment and drug intervention

Fifty male Sprague-Dawley rats (specific pathogen-free) were provided by the Experimental Animal Center of University of South China (License No. SYXK-2015-0001). Rats were maintained on a 12-hour light/dark cycle with unlimited access to food and water until they were seven weeks old. All rats were intraperitoneally injected with 5 mg/kg CPA alone (ab120398, Abcam, Cambridge, UK; CPA group, n = 20) or CPA with an equal dose of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, A1R antagonist; ab120396, Abcam; CPA + DPCPX group, n = 10), with the same volume of solvent (dimethyl sulfoxide) injected into the control group (n = 20) for five weeks. Behavioral testing was performed for each group. Subsequently, five rats from each group were anaesthetized with halothane (3441, ChemSpider, London, UK) and rapidly decapitated according to protocols approved by the University Committee of Animal Care and Supply at the University of Saskatchewan (approval No. AUP#20070090) in March 2007 and Animals Ethics Committee of University of South China (approval No. LL0387-USC) in June 2017. Brains were extracted and placed into a high-sucrose,

high-Mg^{2+} dissection medium and cut with a slicer (Vibram VT1200S, Leica, Wetzlar, Germany) into 400-μm thick slices. Substantia nigra slices were collected and maintained for one hour in artificial cerebrospinal fluid prior to biochemical experiments. The remaining rats were perfused with 0.9% saline and 4% paraformaldehyde for 30 minutes, then decapitated for harvesting of brains. Brain samples were fixed with 4% paraformaldehyde for one week, subsequently submerged into a 30% sucrose solution for one week, and then cut with a freezing microtome (CM2000, Leica) into 40-μm thick slices. All sections were collected and maintained in 6-well plates filled with Millonig's buffer for subsequent immunohistochemistry tests.

Forced swimming test

The forced swimming test (FST) (SANS, Nanjing, China) is one of the most commonly used methods to assess motor capabilities (Detke et al., 1995). After treatment with CPA alone or together with DPCPX, rats were individually placed into a glass cylinder (40-cm height, 18-cm diameter) containing 30 cm of water at 23°C. After adapting for ten minutes, rats were returned to a 30°C drying environment (pre-test). Twenty-four hours later, rats were placed into the cylinder for ten minutes (test), and the entire experimental process was recorded with a video camera. Video recordings were observed, and scores for success and vigor were assessed for every thirty-second period by an experimenter blinded to animal treatments. Criteria for success scores were as follows: 3, continuous movement of all four limbs; 2.5, occasional floating; 2, floating more than swimming; 1.5, occasional swimming using all four limbs; 1, occasional swimming using only hind limbs; 0, no use of limbs. Criteria for vigor scores were as follows: 3, entire head above water; 2.5, ears but not eyes are usually below water; 2, eyes but not nose are usually below water; 1.5, entire head below water for three seconds; 1, entire head below water for periods ≥ six seconds; 0, animal on the bottom of the tank for periods of ten seconds or longer. A sum of scores for the last three minutes of the test was used to evaluate the immobility of rats undergoing different treatments.

Y-maze test

The Y-maze (SANS) consisted of three arms that were 50 cm long, 18 cm wide, 35 cm high, and positioned at equal angles (Kim et al., 2013). The test consisted of two trials (sample and test) that were separated by a 90-minute interval. In the sample trial, one arm (the new arm) of the Y-maze was closed with a separator. Rats were placed in one of the two other arms with their head oriented in the direction opposite the center of the maze (start arm), with the remaining arm was defined as the old arm. Rats were allowed to visit these two arms freely for 15 minutes. Test trials were performed in the absence of the separator. Rats were allowed to explore the maze for five minutes. Time spent in each arm was recorded with a video camera. To judge the cognitive capacity of animals, percentages were calculated as follows: time spent in each arm × 100%/sum of time spent in the three arms.

Cell culture

The dopaminergic cell line MN9D and human embryonic kidney 293T cell line (HEK293T) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MN9D cells, which were generated by the fusion of rostral mesencephalic neurons from embryonic C57BL/6J (embryonic day 14) mice with N18TG2 neuroblastoma cells, endogenously express α-synuclein and tyrosine hydroxylase (Blume et al., 2015). HEK293T cells were used for transient transfection of luciferase reporter plasmids. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. MN9D cells were cultured in DMEM/F12 supplemented with 10% neonatal calf serum (Gaithersburg, MD, USA), and HEK293T cells were cultured in DMEM/F12 with 2% neonatal calf serum. Cells were treated with biochemical reagents as indicated during experiments.

Biotinylation assay

Substantia nigra slices (400-μm thick) were prepared and equilibrated in ice-cold oxygenated artificial cerebrospinal fluid bubbled constantly with 95%/5% (v/v) O_2/CO_2. Subsequently, substantia nigra slices were incubated with 0.5 mg/mL sulfo-NHS-biotin (Thermo Fisher Scientific, Waltham, MA, USA) dissolved in ice-cold artificial cerebrospinal fluid on ice for one hour. The biotin reaction was quenched by rinsing twice with quenching buffer at 4°C. Biotinylated slices were transferred into homogenizing tubes, ground in 500 μL of 1% lysis buffer for 30 minutes, and then centrifuged at 15,000 × *g* for five minutes. The supernatant was collected into separate tubes. After the protein concentration of the supernatant was determined by Bradford assay, 500 μg of protein sample from each rat was immunoprecipitated overnight using 50 μL of streptavidin-agarose beads (Invitrogen, Carlsbad, CA, USA) and topped off with 1% lysis buffer to a 500-μL total volume. Agarose beads were washed with 0.1% ice-cold lysis buffer, added to 50 μL of sample buffer, boiled for five minutes, and then evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot assay.

Western blot analysis

After behavioral tests, proteins (20 μg per lane) from the freshly harvested substantia nigra were loaded on 7.5% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed for 90 minutes at 120 V in gel running buffer, and trans-ferred to polyvinylidene fluoride membranes. After blocking in 5% (w/v) fat-free milk dissolved in Tris-buffered saline with Tween 20 (TBST), membranes were incubated with primary rabbit polyclonal antibody against sortilin (1:1000; ab16640, Abcam) and c-JUN (1:500; ab31419, Abcam), rabbit monoclonal antibody against phosphorylated-c-JUN (phosphor-serine 63, p-JUN; 1:1000; ab32385, Abcam), or mouse monoclonal antibody against α-synuclein (1:1000; ab1903, Abcam), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:2000; MAB374, Millipore, Billerica, MA, USA) overnight at 4°C. After a series of rinses, membranes were further incubated with a peroxidase-conjugated secondary goat anti-rabbit IgG (H+L) (1:1000; A0208, Beyotime,

Shanghai, China) or goat anti-mouse IgG (H+L) (1:1000; A0216, Beyotime) antibody in TBST-dissolved milk for one hour at room temperature. Finally, proteins were visualized using a chemiluminescence method (Enhanced Chemiluminescence Plus Western Blotting Detection System; Amersham Biosciences, Foster City, CA, USA), and protein levels were evaluated semiquantitatively using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

Substantia nigra slices were washed three times with 0.1 M phosphate buffer and then blocked for 1 hour at room temperature with phosphate-buffered saline containing 5% horse serum, 94.75% 0.1 M phosphate buffer, and 0.25% Triton X-100 (blocking buffer). Sortilin and α-synuclein proteins in slices were labeled by overnight incubation at 4°C with rabbit anti-sortilin (Abcam) and mouse or chicken anti-α-synuclein (Abcam) antibodies diluted at 1:200 in blocking buffer. Following three washes (10 minutes each), slices were incubated with Alexa Fluor 488-conjugated donkey anti-rabbit (for labeling sortilin; Invitrogen) and Alexa Fluor 594-conjugated goat anti-mouse or Alexa Fluor 546-conjugated goat anti-chicken secondary antibodies (for labeling α-synuclein; Invitrogen) at 1:500 for 1 hour at room temperature. Dopaminergic neurons within the substantia nigra were further labeled with mouse anti-rat tyrosine hydroxylase (1:500, Abcam) and Alexa Fluor 594-conjugated goat anti-mouse secondary antibodies, or labeled with rabbit anti-rat tyrosine hydroxylase antibody (1:500, Abcam) and Alexa Fluor 488-conjugated donkey anti-rabbit or Alexa Fluor 647-conjugated chicken anti-rabbit secondary antibody at 1:500. After rinsing, two drops of 4′,6-diamidino-2-phenylindole were added to each well to stain nuclei for five minutes. Slices were mounted on gelatin-coated microscope slides using Prolong Gold anti-fade reagent (Invitrogen) and then observed with a 63× Apochromat (NA = 1.40) oil objective and LSM 700 laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Images were exported as 8-bit .tiff files and analyzed with ImageJ software.

Quantitative real-time polymerase chain reaction

Sortilin and α-synuclein mRNA levels in MN9D cells were quantified by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated using TRIzol reagent (Omega Bio-Tek, Norcross, GA, USA). RNA purity was determined by A260/A280 absorption ratio. Complementary DNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Takara, Beijing, China), followed by 40-cycle two-step PCR with sequence-specific primer pairs in an iCycler IQ real-time detection system (Bio-Rad, Shanghai, China). Primers were purchased from Biology Engineering Corporation (Shanghai, China). Human primers were as follows: sortilin, forward 5′-GGG GAC CAA ACA ACA TCA TC-3′ and reverse 5′-AAG GGC TCA TGA CCA CAG TC-3′; α-synuclein, forward 5′-ATA AGA ATG CGG CCG CAT GGA TGT ATT CAT GAA AG-3′ and reverse 5′-CCG CTC GAG GCT TCA GGT TCG TAG TCT TGA-3′; GAPDH, forward 5′-GAG CTG AAC GGG AAA CTC AC-3′ and reverse 5′-GGT CTG GGA TGG AAA CTG TG-3′. PCR analysis was performed in strict accordance with the instructions of the SYBR Green Fluorescence Quantitation Kit (Thermo Fisher Scientific) as follows: pre-denaturation at 94°C for five minutes; 40 cycles of denaturation at 94°C for 45 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds. All PCR products were assessed by melt-curve analyses, in which DNA duplexes were identified. Measurements were quantified using the ΔΔCt method, and GAPDH expression was used as an internal control.

Promotor analysis and protein binding prediction

SORT1 promoter sequences were obtained from the National Center for Biotechnology Information (NCBI) sequence database. Transcription factors and their binding sites within the SORT1 promoter were predicted by JASPAR (http://jaspar.genereg.net/), Alibaba 2.1 (http://gene-regulation.com/pub/programs/alibaba2/index.html), and PROMO (http://alggen.lsi.upc.es/). For the prediction of protein binding between sortilin and α-synuclein, the online tool Raptor-X-Binding (http://raptorx.uchicago.edu/BindingSite/) and Discovery Studio 4.5 software (Biovia, Boston, MA, USA) were jointly used to generate a three-dimensional binding structure model and calculate the main binding sites within the amino acid sequence of the sortilin protein.

Plasmid constructs and luciferase reporter assays

psiCHECK™-2 vector was purchased from Promega (Madison, WI, USA). Briefly, sortilin cDNA was amplified from MN9D cells, sequence confirmed (Gene ID: 6272), and then cloned upstream of the firefly luciferase open reading frame of the psiCHECK™-2 vector (psiCHECK™-2-WT-sortilin), which contains a constitutively expressed Renilla luciferase gene as an internal control for normalization. Mutant sortilin promotor was generated by site-directed mutagenesis (259–265) and inserted into the psiCHECK™-2 vector (psiCHECK™-2-MUT-sortilin). Next, 0.5 μg/well of empty or reconstructed vector were transfected into HEK293T cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Cells were simultaneously co-incubated with CPA alone or with 10 μM SP600125 (JNK inhibitor; ab120065, Abcam) for 12 hours. A Dual-Luciferase Assay Kit (Promega) was used to assess luciferase activities, which were normalized to the corresponding Renilla luciferase activity and plotted as a percentage of internal control.

Chromatin immunoprecipitation assay

MN9D cell lysates were prepared and centrifuged at 16,000 × *g* for ten minutes at 4°C after treatment with CPA alone or combined with the JNK inhibitor SP600125. The supernatant containing sheared chromatin was subjected to immunoprecipitation using a chromatin immunoprecipitation assay kit (Abcam) and antibody against c-JUN (Abcam) or non-immune human IgG control. DNA was eluted and collected as a template for PCR amplification of a specific sortilin

promoter sequence. Sortilin primers (forward 5′-TGG TTT TAC CTG TGC TAC GTG T-3′ and reverse 5′-ACT CTG CAG TGA GAG CTT CG-3′) were designed to target a 163-bp fragment of the sortilin promoter. PCR amplification of a 189-bp fragment of GAPDH was used as a negative control (GAPDH primers: forward 5′-AGG TGA GAC ATT CTT GCT GGG-3′ and reverse 5′-ATA GCC TAG GAC TGG AGC GA-3′).

Coimmunoprecipitation

MN9D cells were homogenized and lysed in immunoprecipitation lysis buffer. Lysates were incubated with sortilin antibodies overnight, followed by one hour incubation with Protein G Plus Agarose (Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C. After 12 hours of end-over-end shaking, 20 μL of Protein G agarose beads were washed five times with immunoprecipitation lysis buffer. After 2 hours, samples were centrifuged, and the supernatant from each sample was removed. The pellet containing the beads was washed three times with lysis buffer, and bead-bound proteins were eluted with 40 μL of 1× sodium lauryl sulfate sample buffer (Beyotime). Each sample (20 μL) was subjected to western blot using a primary antibody against α-synuclein.

Statistical analysis

All statistical analyses were performed using SPSS 22.0 software (IBM SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant (one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test).

Results

Long-term A1R activation upregulates sortilin expression

To explore the detrimental effects of long-term A1R activation on dopaminergic neurons within the substantia nigra, we first performed a series of behavioral tests to examine alterations in cognition and kineticism of rats treated with CPA for five weeks. Compared with control rats, CPA-treated rats exhibited a remarkable decrease in both success and vigor scores in the FST, indicating motor lesion generation in response to long-term A1R activation ($P < 0.05$; **Figure 1A** & **B**). In addition, the percentage of time spent in the new arm of the Y-maze was obviously reduced in CPA-treated rats compared with control rats, suggesting that long-term A1R activation produces cognitive impairment ($P < 0.05$; **Figure 1C**). Next, we examined sortilin and α-synuclein expression in dopaminergic neurons of the substantia nigra under long-term A1R activation. Surface sortilin protein was reduced, whereas total sortilin was increased in dopaminergic neurons of rats chronically injected with CPA as detected by western blot assay (**Figure 1D** & **F**); analysis of α-synuclein protein expression yielded similar results ($P < 0.05$; **Figure 1E** & **G**). Furthermore, when labeled with the dopaminergic neuronal marker tyrosine hydroxylase, confocal images revealed significantly increased expression of sortilin and α-synuclein proteins in dopaminergic neurons within the substantia nigra of CPA-treated rats (**Figure 1H** & **I**). These results indicate that long-term A1R activation severely impaired the cognition of rats and promoted the expression of sortilin and α-synuclein proteins in dopaminergic neurons of the substantia nigra.

Long-term A1R activation upregulates sortilin expression through the JNK/c-JUN pathway

JNK/c-JUN is a downstream pathway of A1R activation (Schulte et al., 2004). To determine whether the JNK/c-JUN pathway was involved in long-term A1R activation-induced sortilin expression, dopaminergic MN9D cells were treated with CPA alone or with the c-JUN inhibitor SP600125. CPA incubation evidently increased p-JUN levels, while co-treatment with SP600125 abolished the pro-phosphorylation role of CPA in MN9D cells ($P < 0.05$; **Figure 2A** & **B**). However, total c-JUN (t-JUN) levels were not significantly altered when cells were incubated with CPA alone or together with SP600125. Further research found that sortilin mRNA was upregulated when MN9D cells were incubated with CPA alone ($P < 0.05$; **Figure 2C**). In contrast, coincubation with CPA and SP600125 downregulated sortilin mRNA levels in MN9D cells. These results suggest that CPA induced sortilin expression at the transcriptional level via the JNK/c-JUN pathway.

To determine if the JNK/c-JUN pathway promotes sortilin expression through the binding of p-JUN to its promoter, online prediction databases JASPAR, PROMO, and Alibaba 2.1 were employed. As expected, a highly conserved sequence was found in both human and rodent sortilin promoters that can bind to JUN (**Table 1**). We further confirmed this prediction in HEK293T cells transfected with luciferase reporter plasmids containing a wild-type (WT) or mutant (MUT) sortilin promoter (**Figure 2D**). When HEK293T cells were transfected with psiCHECK^{TM}-2-WT-sortilin, luciferase activity was clearly enhanced in HEK293T cells incubated with CPA alone, but was inhibited by co-incubation with SP600125 ($P < 0.05$). Even if activation by CPA occurred, luciferase activity was not significantly altered in HEK293T cells transfected with psiCHECK^{TM}-2-MUT-sortilin ($P > 0.05$). Chromatin immunoprecipitation assays were used to test whether CPA stimulates the binding of p-JUN to its putative binding site within the sortilin promoter ($P < 0.001$; **Figure 2E**). CPA indeed promoted the binding of c-JUN to the sortilin promoter, while SP600125 cancelled this action of CPA. Collectively, these results indicate that long-term A1R activation promoted the binding of p-JUN to the sortilin promoter to enhance sortilin expression at the transcriptional level.

Table 1 Transcription factor c-JUN and its binding sites within the SORT1 promoter were predicted by online websites (Jasper, PROMO, and Alibaba) in human or rodent species

Group	Model name	Score	Start	End	Strand	Predicted site sequence
Homo	c-JUN	6.065	259	265	1	tgactga
Rodent	c-JUN	8.680	1389	1395	1	tgactga

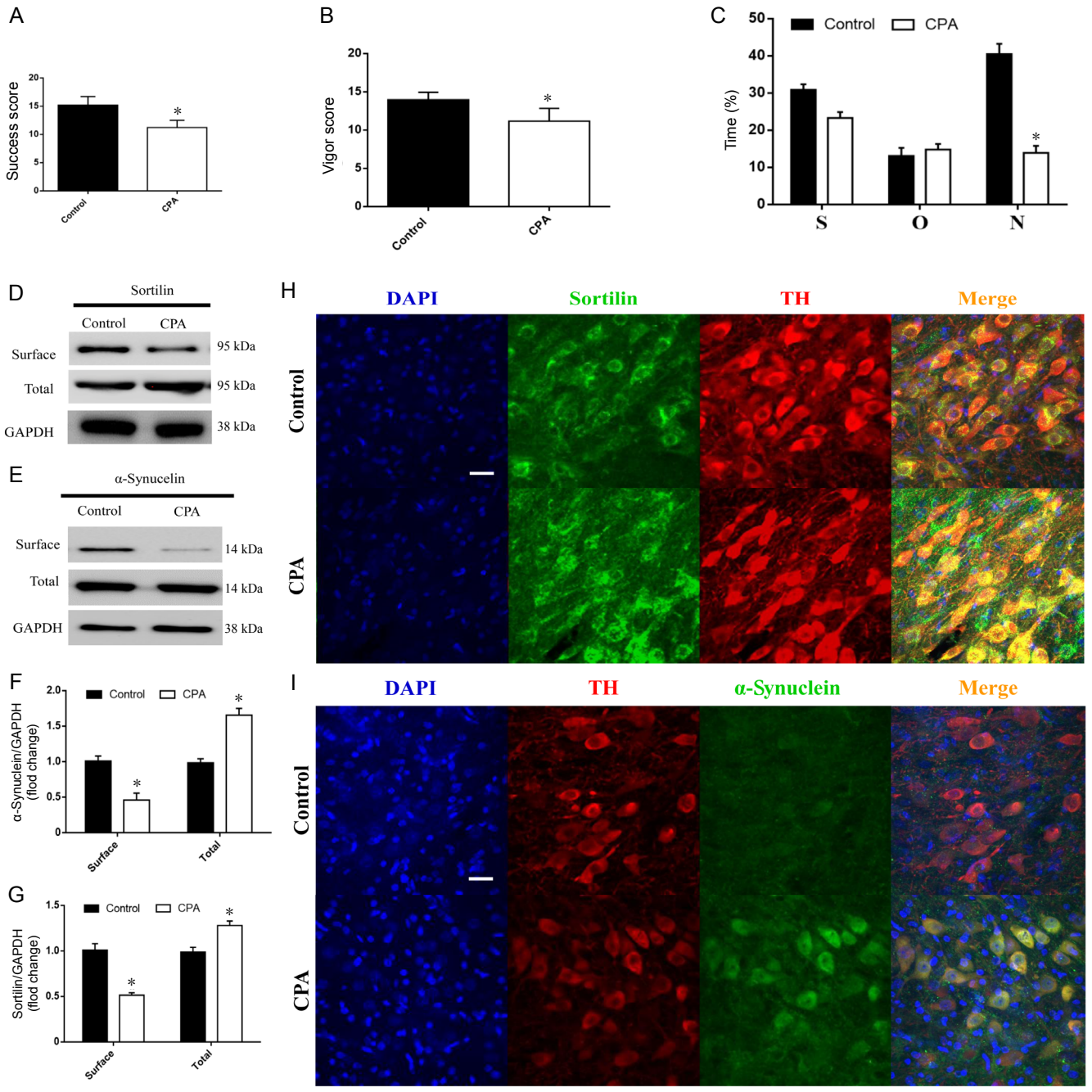

Figure 1 Chronic treatment with the A1R agonist CPA impaired cognition and kinesis in Sprague-Dawley rats, and upregulated sortilin and α-synuclein expression in dopaminergic neurons within the substantia nigra.
(A–C) Success (A) and vigor (B) scores in the forced swimming test, and percentages of time spent in the Y-maze test (C) of Sprague-Dawley rats chronically injected with 5 mg/kg CPA for five weeks. (D, E) Surface and total levels of sortilin (D) and α-synuclein (E) were analyzed by western blot in the substantia nigra of rats chronically injected with 5 mg/kg CPA for five weeks. (F, G) Quantification of surface and total protein levels of sortilin (F) and α-synuclein (G) in the substantia nigra. (H) Immunodetection of sortilin (green, stained by Alexa Fluor 488) expression in dopaminergic neurons labeled with TH (red, stained by Alexa Fluor 594) within the substantia nigra (right side). DAPI (blue) was used to stain nuclei. (I) Immunodetection of α-synuclein (red, stained by Alexa Fluor 594) expression in dopaminergic neurons labeled with TH (green, stained by Alexa Fluor 488) within the substantia nigra (right side). DAPI (blue) was used to stain nuclei. Scale bars: 25 μm. All data are presented as mean ± SD (n = 10 rats per group) from three independent experiments, each performed in triplicate. *P < 0.05, *vs.* control group (one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test). A1R: Adenosine A1 receptor; CPA: N6-cyclopentyladenosine; DAPI: 4′,6-diamidino-2-phenylindole; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; N: new arm; O: old arm; S: starting arm; TH: tyrosine hydroxylase.

Sortilin inhibition abolishes the effect of long-term A1R activation on α-synuclein

Long-term A1R activation causes α-synuclein upregulation in dopaminergic neurons. Thus, we next examined whether sortilin is involved in CPA-induced upregulation of α-synuclein protein. Sortilin expression was knocked down with the small hairpin RNA shSORT1 in MN9D cells (P < 0.001; **Figure 3A**). Compared with the control group, CPA treat-

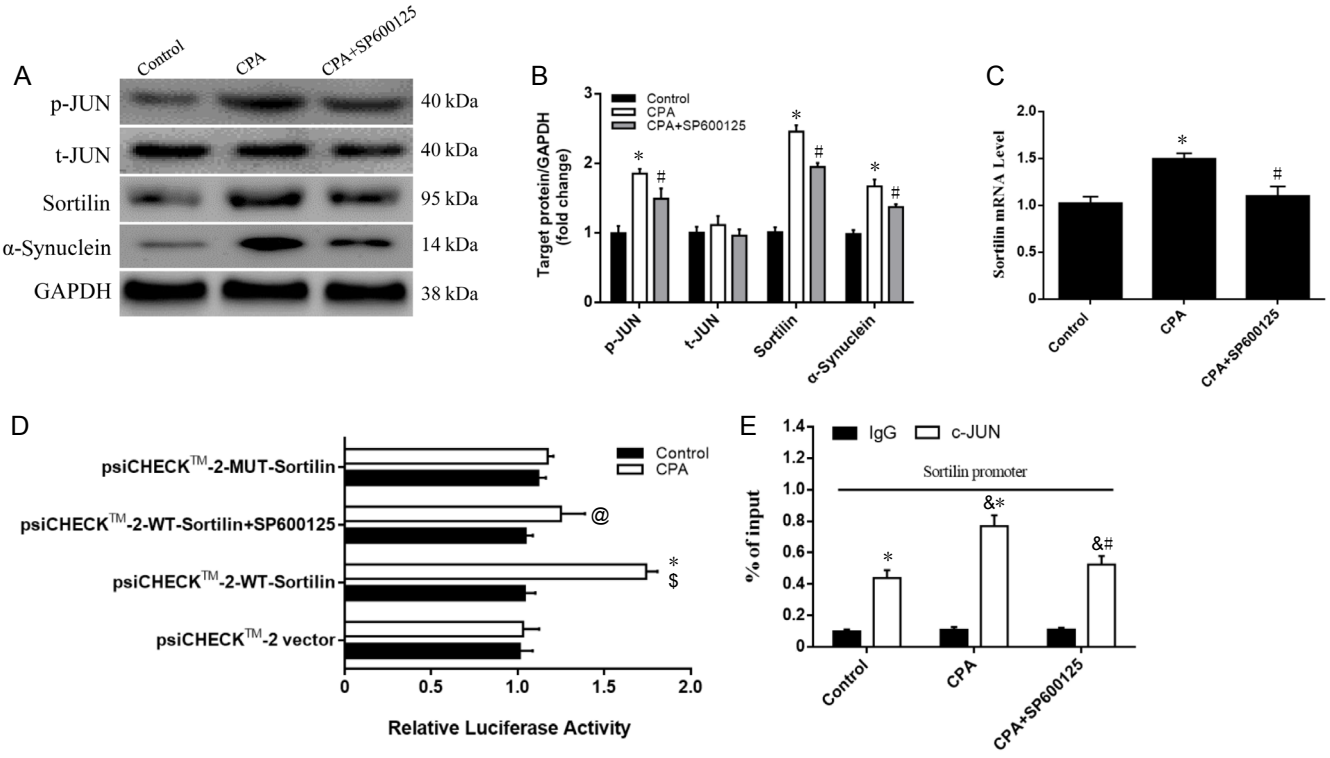

Figure 2 The A1R agonist CPA enhanced sortilin expression through the JNK/c-JUN pathway in MN9D cells.
(A) Protein levels of p-JUN, t-JUN, sortilin, and α-synuclein were analyzed by western blot assay in MN9D cells treated with 500 nM CPA alone or CPA supplemented with 10 μM SP600125 (JNK inhibitor) for 48 hours. (B) Quantification of p-JUN, t-JUN, sortilin, and α-synuclein as analyzed by western blot assay. (C) Sortilin mRNA levels were analyzed by quantitative real-time polymerase chain reaction in MN9D cells treated with 500 nM CPA alone or CPA supplemented with 10 μM SP600125 for 48 hours. (D) Luciferase activities in HEK293T cells transfected with control vector, WT-sortilin vector, or MUT-sortilin vector for 48 hours and co-incubated with CPA alone or CPA supplemented with 10 μM SP600125. (E) Chromatin immunoprecipitation assays of p-JUN bound to the sortilin promoter in MN9D cells treated with 500 nM CPA alone or supplemented with 10 μM SP600125 for 48 hours. Unprecipitated chromatin was used as input. All data are presented as mean ± SD from three independent experiments, each performed in triplicate. $*P < 0.05$, *vs.* control group; $\#P < 0.05$, *vs.* CPA group; $\$P < 0.05$, *vs.* psiCHENK™-2-vector group; $@P < 0.05$, *vs.* psiCHENK™-2-WT-Sortilin group; $\&P < 0.05$, *vs.* IgG group (one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test). A1R: Adenosine A1 receptor; CPA: N6-cyclopentyladenosine; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HEK293T cells: human embryonic kidney 293T cells; JNK: c-JUN N-terminal kinase; MUT: mutant; p-JUN: phosphorylated-c-JUN; t-JUN: total c-JUN; WT: wild type.

ment potently augmented protein levels of p-JUN, sortilin, and α-synuclein, while treatment with CPA together with sh-SORT1 significantly decreased sortilin and α-synuclein protein levels ($P < 0.05$), but did not change p-JUN levels ($P > 0.05$; **Figure 3B** & **C**). Furthermore, there was a nonsignificant alteration in α-synuclein mRNA levels in rats treated with CPA alone or together with shSORT1 ($P > 0.05$; **Figure 3D**). These results indicate that long-term A1R activation upregulated α-synuclein protein through sortilin at posttranscriptional levels.

Sortilin binds diverse substrate proteins and transports them between the trans-Golgi network and endosomes, thereby post-transcriptionally regulating cellular levels of cargo proteins (Gao et al., 2017). To predict whether sortilin can bind to α-synuclein, the online tool Raptor-X-Binding and Discovery Studio 4.5 prediction software were employed (**Figure 3E**). Prediction analyses indicated the capability of sortilin to bind α-synuclein protein, with multiple sites within the amino acid sequence of the sortilin protein identified as likely responsible for this binding. Moreover, amino acids 129–138 (AKNFVQTDLP) were calculated to be the main binding site within the sortilin protein sequence (**Figure 3F**). As CPA promotes sortilin and α-synuclein protein expression, the interaction between sortilin and α-synuclein was examined by coimmunoprecipitation assay of MN9D cells incubated with CPA (**Figure 3G**). We found that CPA significantly enhanced sortilin binding to α-synuclein. Furthermore, CPA-treated rats exhibited increased immunofluorescence co-localization of sortilin and α-synuclein in dopaminergic neurons of substantia nigra sections compared with the control group (**Figure 4E**). These results demonstrate that long-term A1R activation upregulated α-synuclein protein by enhancing binding of sortilin to α-synuclein.

A1R inhibition abolishes the effect of long-term A1R activation on neurodegeneration

The above experiments demonstrated that long-term A1R activation increased protein expression of sortilin and α-synuclein, eliciting a detrimental effect to dopaminergic neurons within the substantia nigra. Therefore, we examined the effect of CPA on dopaminergic neurons in the context of blocked A1R activation using intraperitoneally injected

DPCPX prior to CPA administration. Western blot assay results revealed that DPCPX relieved CPA-induced sortilin expression in dopaminergic neurons within the substantia nigra, and similarly altered the protein level of α-synuclein ($P < 0.05$; **Figure 4A** & **B**). We also found that the body weight of rats treated with CPA alone or co-treated with DPCPX was not significantly changed ($P > 0.05$; **Additional Table 1**). Confocal imaging also revealed that protein levels of both sortilin and α-synuclein were reduced in substantia nigra sections of rats co-treated with DPCPX and CPA (**Figure 4C** & **D**). Immunofluorescence co-localization was also decreased in substantia nigra sections under co-treatment with DPCPX and CPA, thus highlighting sortilin downregulation as a DPCPX-protective action for dopaminergic neurons *in vivo* (**Figure 4E**). Compared with CPA-treated rats, DPCPX-co-treated rats effectively recovered success and vigor scores in the FST ($P < 0.05$; **Figure 4F** & **G**). DPCPX co-treatment also significantly increased the percentage of time spent in the new arm in the Y-maze test compared with times observed for CPA-treated rats ($P < 0.05$; **Figure 4H**). Overall, these results demonstrate that A1R inhibition alleviated the detrimental effects of long-term A1R activation on dopaminergic neurons, as well as cognition and motor capabilities.

Discussion

Recent studies have indicated that prolonged A1R activation contributes to neural damage in the brain and the initiation of neurodegenerative diseases through downstream signaling pathways (Stockwell et al., 2016, 2017). In our research, we found that prolonged A1R activation by CPA dramatically increased sortilin expression via the JNK/c-JUN pathway, which caused upregulation of α-synuclein protein levels in dopaminergic neurons, ultimately resulting in impaired cognition and kineticism yielding PD-like behavior in Sprague-Dawley rats. Elevated levels of sortilin bound to α-synuclein protein and aggravated its accumulation in dopaminergic neurons. In contrast, blocking long-term A1R activation with DPCPX or its downstream signaling pathway with JNK inhibitor reduced sortilin ex-pression and α-synuclein levels in the substantia nigra, significantly improving host cognition and kineticism (**Figure 5**). Our findings support a destructive rather than protective role of A1R in dopaminergic neurons under long-term A1R activation, likely accelerating the development of neurodegenerative diseases such as PD.

A1R has generally only been described as a neuroprotective G protein-coupled adenosine receptor that functions as a brake to prevent lavish neuronal excitement in the brain (Trincavelli et al., 2010). Indeed, temporary A1R activation exhibits a protective inhibitory action in neurons, including inhibited glutamate release in presynaptic neurons and descended neuronal excitability in postsynaptic neurons, by affecting downstream transporters and receptors such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, N-methyl-D-aspartate receptors, and adenosine triphosphate-sensitive K^+ channels (Chen et al., 2016; Murley and Nunnari, 2016). However, scientists recently realized that long-term A1R activation elicits not only protective, but also destructive effects to neurons. Through intracellular kinases and phosphatases involved in its downstream signaling pathway, prolonged A1R activation is likely implicated in the neurodegeneration of dopaminergic neurons by upregulating expression of related target genes (Brust et al., 2006; Chen et al., 2014; Stockwell et al., 2016). In the present study, we found that prolonged A1R activation elevated sortilin expression at the transcriptional level through the JNK/c-JUN pathway in dopaminergic neurons, as sortilin upregulation was abolished by treatment with a JNK inhibitor. In contrast, long-term A1R activation only increased α-synuclein protein levels, but not mRNA levels, suggesting an indirect mechanism by which prolonged A1R activation enhances neuronal accumulation of α-synuclein protein in the substantia nigra.

Sortilin, a human homologue of the yeast vacuolar protein sorting 10 protein domain receptor family, is intimately involved in the pathogenesis of neurodegenerative diseases such as PD (Lane et al., 2012; Wilson et al., 2014). As a sorting receptor, sortilin affects intracellular trafficking and lysosomal degradation of substrate proteins to regulate the expression and function of multiple target genes (Cole et al., 2002; Carlo, 2013). Previous studies reported that sortilin can bind to various protein substrates, including lipoprotein lipase, low-density lipoprotein, apolipoprotein E, apolipoprotein A–V, and especially apolipoprotein B100. Sortilin transports these proteins from the trans-Golgi network to the lysosome for degradation or to the plasma membrane for exocytosis by endosomal trafficking. In addition, sortilin can bind clusterin to promote its lysosomal degradation in neurons, leading to the progression of Alzheimer's disease (Wang et al., 2017). Here, we demonstrated that α-synuclein protein is a novel substrate of sortilin in dopa-minergic neurons. Unexpectedly, sortilin did not decrease, but rather elevated the protein level of α-synuclein in the substantia nigra. The reasons for this phenomenon are probably as follows: first, sortilin directly bound α-synuclein protein and reduced its surface distribution for secretion, suggesting that sortilin exacerbates intracellular accumulation of α-synuclein in dopaminergic neurons, likely as a result of disturbed anterograde trafficking; second, cellular accumulation of α-synuclein suffocated sortilin-mediated lysosomal degradation under the circumstance of long-term A1R activation, causing blockade of α-synuclein protein removal. These underlying mechanisms may be involved in the accumulation of α-synuclein protein and require further investigation in future studies.

Growing evidence highlights the action of α-synuclein in neuronal vesicular transport processes and neurodegeneration progression (Miraglia et al., 2018; Pinheiro and Ventura, 2019). Normally, α-synuclein is involved in neuronal vesicle trafficking and regulates synaptic activity. Abeliovich and Gitler (Abeliovich and Gitler, 2016) verified that the α-synuclein protein mainly localizes to presynaptic terminals and regulates synaptic vesicle release to control synaptic activity in the rodent brain. Moreover, release of vesicular transmitter was potentiated in α-synuclein knockout mice.

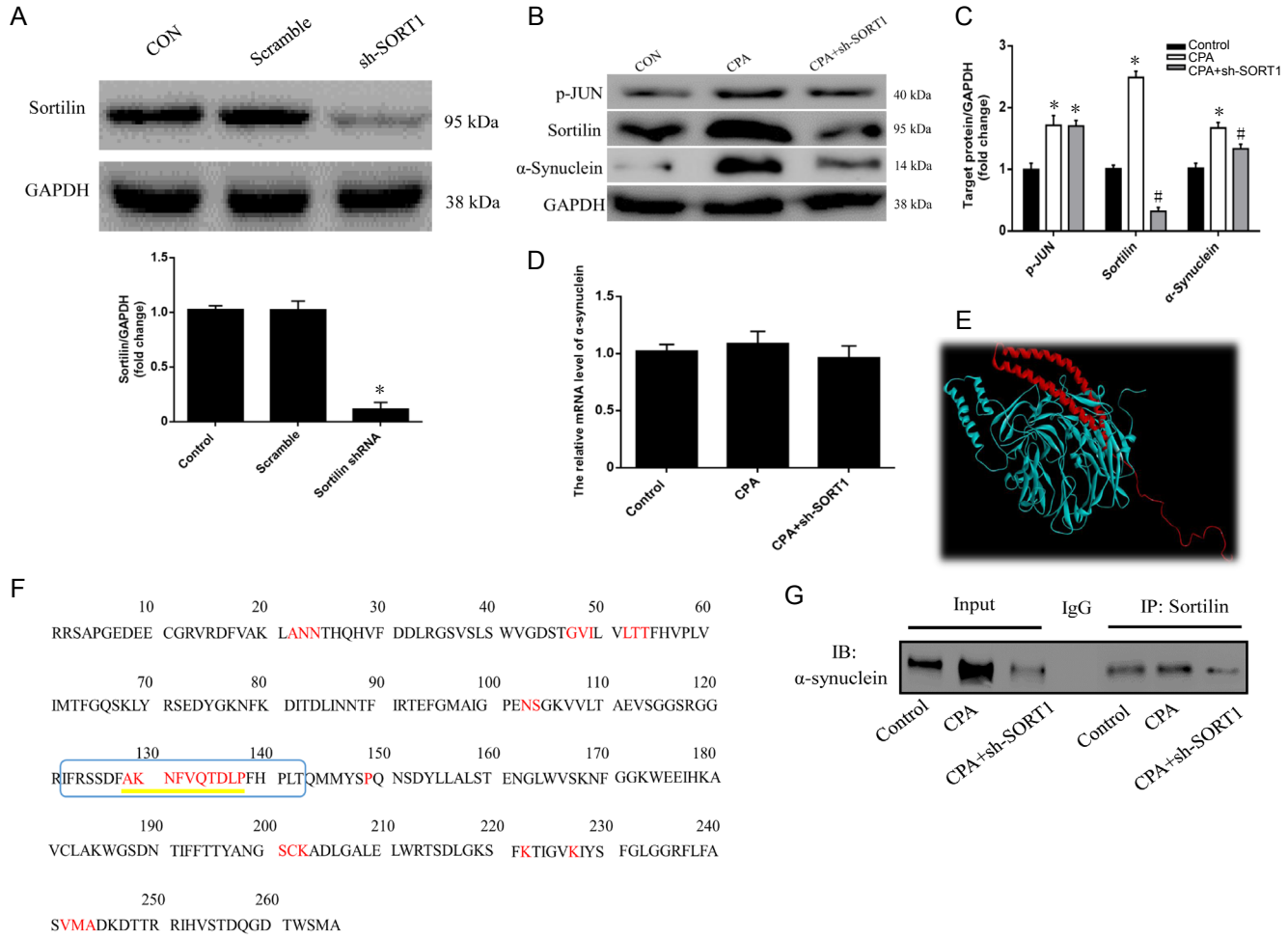

Figure 3 Sortilin is required for induction of increased α-synuclein levels by the A1R agonist CPA in MN9D cells.
(A) Protein levels of sortilin were analyzed by western blot in MN9D cells treated with 50 nM shSORT1 for 48 hours. (B) Protein levels of p-JUN, sortilin, and α-synuclein were analyzed by western blot in MN9D cells treated with 500 nM CPA alone or CPA together with sh-SORT1. (C) Quantification of p-JUN, sortilin, and α-synuclein protein levels analyzed by western blot. (D) α-Synuclein mRNA levels were analyzed by quantitative real-time polymerase chain reaction in MN9D cells treated with 500 nM CPA alone or CPA together with shSORT1. All data are presented as the mean ± SD from three independent experiments, each performed in triplicate. $*P < 0.05$, *vs.* control group; $\#P < 0.05$, *vs.* CPA group (one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test). (E) A three-dimensional binding structure model was predicted between sortilin and α-synuclein using the online tool Raptor-X-Binding and Discovery Studio 4.5 software. The structure in green represents sortilin, while red represents α-synuclein. (F) Binding sites within the sortilin protein sequence likely to bind to α-synuclein protein. Amino acids possibly responsible for binding are shown in red letters. Amino acids (AKNFVQTDLP) from 129 to 138 underlined with a yellow line were predicted to be the main binding site. (G) The interaction of sortilin and α-synuclein in total cell homogenates was analyzed by co-immunoprecipitation (IP) in MN9D cells treated with 500 nM CPA alone or CPA together with shSORT1. IgG is indicated as a negative control. A1R: Adenosine A1 receptor; CPA: N6-cyclopentyladenosine; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; p-JUN: phosphorylated-c-JUN.

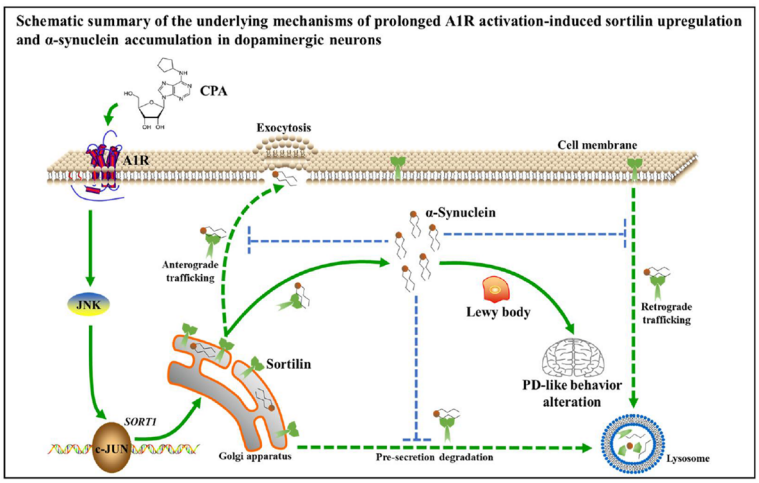

Figure 5 Schematic summary of mechanisms underlying prolonged A1R activation-induced sortilin upregulation and α-synuclein accumulation in dopaminergic neurons.
Prolonged A1R activation induced by CPA administration dramatically increased sortilin expression via the JNK/c-JUN pathway. Subse-quently, sortilin bound to α-synuclein protein to aggravate its accumulation in dopaminergic neurons. Substantial accumulation of α-synuclein disturbs its transport by sortilin to the lysosome for degradation or to the plasma membrane for exocytosis by vesicular traf-ficking, which in turn accelerates cellular accumulation of α-synuclein protein. Consequently, long-term A1R activation facilitated sortilin upregulation and α-synuclein accumulation to damage dopaminergic neurons, ultimately leading to host PD-like behavior. A1R: Adenosine A1 receptor; CPA: N6-cyclopentyladenosine; JNK: c-JUN N-terminal kinase; PD: Parkinson's disease.

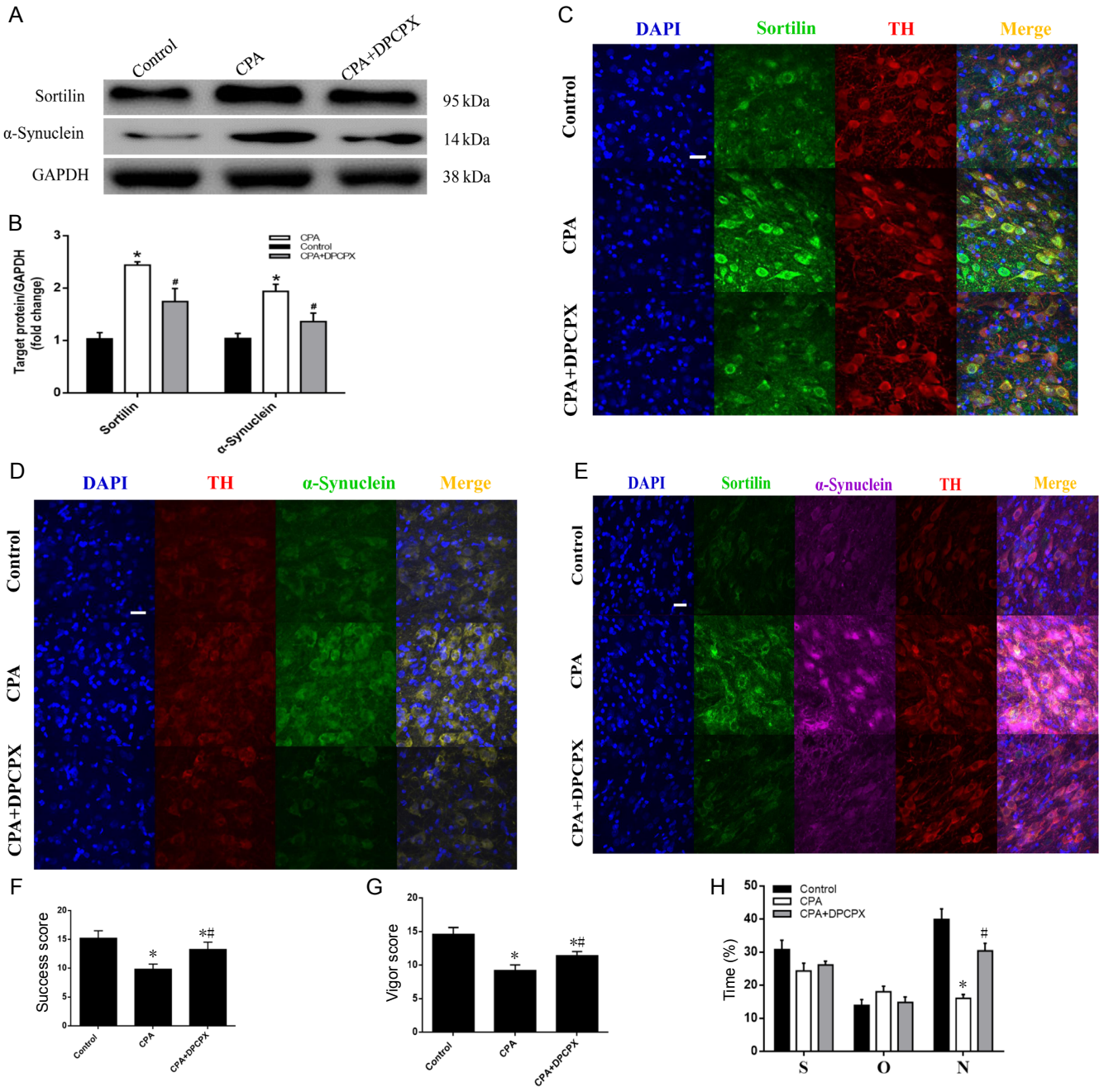

Figure 4 The A1R antagonist DPCPX opposed CPA-induced upregulation of sortilin and α-synuclein in dopaminergic neurons within the substantia nigra, and impaired cognition and kinesis in Sprague-Dawley rats.

(A) Protein levels of sortilin and α-synuclein were analyzed by western blot in the substantia nigra of rats treated with 5 mg/kg CPA alone or CPA with an equal dose of DPCPX for 5 weeks. (B) Quantification of sortilin and α-synuclein protein levels in the substantia nigra analyzed by western blot assay. (C) Immunodetection of sortilin (green, stained by Alexa Fluor 488) in dopaminergic neurons labeled with TH (red, stained by Alexa Fluor 594) within the substantia nigra (right side). DAPI (blue) was used to stain nuclei. (D) Immunodetection of α-synuclein (red, stained by Alexa Fluor 594) in dopaminergic neurons labeled with TH (green, stained by Alexa Fluor 488) within the substantia nigra (right side). DAPI (blue) was used to stain nuclei. (E) Co-localization (white) of sortilin (green, stained by Alexa Fluor 488) and α-synuclein (purple, stained by Alexa Fluor 546) in dopaminergic neurons labeled with TH (red, stained by Alexa Fluor 647) within the substantia nigra (right side). DAPI (blue) was used to stain nuclei. Scale bars: 25 μm. (F–H) Success (F) and Vigor (G) scores in the forced swimming test, and percentages of time spent in the Y-maze test (H) of rats treated with 5 mg/kg CPA alone or CPA with an equal dose of DPCPX for five weeks. All data are presented as mean ± SD from three independent experiments, each performed in triplicate (n = 10 rats per group in B). *P < 0.05, *vs.* control group; #P < 0.05, *vs.* CPA group (one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test). A1R: Adenosine A1 receptor; CPA: N6-cyclopentyladenosine; DAPI: 4′,6-diamidino-2-phenylindole; DPCPX: 8-cyclopentyl-1,3-dipropylxanthine.; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; TH: tyrosine hydroxylase.

α-Synuclein was established to be a molecular chaperone that binds to phospholipids of the plasma membrane and synaptobrevin-2, which affects synaptic vesicle fusion and activity (Petrov et al., 2016). α-Synuclein has also been implicated in activity of the neuronal Golgi appa-ratus and intracellular trafficking of substrate proteins to the lysosome (Cooper et al., 2006). However, accumulation of α-synuclein protein results from an impaired ubiquitin proteasome system and oxidative stress, which contributes to the formation of Lewy bodies in dopaminergic neurons and neurodegenerative processes within the substantia nigra (Kalia and Kalia, 2015; Mazzulli et al., 2016). Accumulated α-synuclein, a key pathological feature in PD, induces neurodegeneration and PD-like behavior. Recent studies ranging from yeast cells to transgenic mice have shown that elevated levels of α-synuclein disrupt intracellular vesicular trafficking and lysosomal degradation. With regard to neuronal vesicular trafficking, accumulated α-synuclein led to excessive intracellular vesicle fusion and inhibition of other steps, such as sortilin-dependent vesicular trafficking. For lysosomal degradation, α-synuclein accumulation was shown to impair lysosomal function by disrupting the trafficking of lysosomal hydrolase at the early secretory pathway (Abeliovich and Gitler, 2016; Mazzulli et al., 2016; Gao et al., 2017). Our research unexpectedly found that sortilin promotes α-synuclein accumulation in dopaminergic neurons under long-term A1R activation, likely as a result of the interfering role of accumulated α-synuclein in sortilin trafficking behavior. Sortilin binds α-synuclein and transports it to the cell membrane for secretion in an anterograde trafficking pathway, while it retrieves it from the cell surface and transports it to the lysosome for degradation in a retrograde trafficking pathway. However, accumulated α-synuclein possibly disrupts sortilin-dependent vesicular trafficking functions including both anterograde trafficking for exocytosis and retrograde trafficking for degradation. Therefore, accumulated α-synuclein likely disturbs sortilin-mediated vesicular trafficking, resulting in decreased surface distribution and in-creased intracellular amounts of α-synuclein protein in dopaminergic neurons under prolonged A1R activation. One plausible explanation for our unexpected results is that this mechanism interferes with sortilin-dependent vesicular trafficking and promotes an inexorable aggregation of α-synuclein in dopaminergic neurons, thus accelerating neuro-degeneration in the substantia nigra and impairment of cognition and kinesis in animals.

As the effect of overexpressed sortilin was preliminarily demonstrated in the context of α-synuclein accumulation in dopaminergic neurons under long-term A1R activation, the potential mechanisms involved remain speculative and need confirmation in ongoing studies. Ser63 and Ser73, the major phosphorylation sites of c-JUN protein, are normally associated with its transcriptional activity. However, whether other sites are also phosphorylated under long-term A1R activation (with the exception of Ser63) needs confirmation. In addition, why c-Jun expression, which is supposedly increased by high-dose CPA, remains unchanged needs further investigation. Furthermore, A1R intervention has been demonstrated to alter the activity of extracellular signal-regulated protein kinases 1 and 2, which functionally substitute JNK to activate c-JUN phosphorylation on Ser63 (Kunduri et al., 2013). Thus, it is necessary to exclude the supplemental role of extracellular signal-regulated protein kinases 1 and 2 in c-JUN activation under JNK inhibition in the context of prolonged A1R activation. The mechanistic link between sortilin trafficking and α-synuclein accumulation should also be illuminated; for instance, whether sortilin overexpression triggers α-synuclein accumulation in dopaminergic neurons or accumulated α-synuclein initiates disrupted sortilin trafficking first. We also need to know how to build a balance between the level of α-synuclein and sortilin-dependent vesicular trafficking, which aids the secretion or lysosomal degradation of intracellular α-synuclein protein. DPCPX administration prevented long-term A1R activation, elicited a pronounced protective effect to dopaminergic neurons, and improved host cognition and movement, indicating that durable and stable suppression of A1R activation is a valuable strategy to control neurodegeneration and PD progression under pathological conditions. As such, it is worth exploring a feasible method to intervene in prolonged A1R activation in patents with neurodegenerative diseases.

In conclusion, our study verified that long-term A1R activation increases sortilin expression via the JNK/c-JUN pathway. Upregulated sortilin binds with and arrests α-synuclein protein within dopaminergic neurons, resulting in α-synuclein accumulation in the substantia nigra and PD-like behaviors in animals. Suppression of prolonged A1R activation potently inhibited sortilin expression and α-synuclein accumulation, and obviously improved host cognition and kinesis. In contrast to the protective role of A1R activation in the traditional view, our findings establish that long-term A1R activation is destructive and involved in α-synuclein accumulation and PD-like behavior in a sor-tilin-dependent manner. The underlying mechanism of sortilin-dependent α-synuclein accumulation should be further elucidated because of the complicated mechanistic link between sortilin-dependent vesicular trafficking and α-synuclein accumulation. Regardless, effective suppression of long-term A1R activation potentially impedes neurodegeneration and neurodegenerative disease development, ultimately benefiting patients.

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of the manuscript.

Institutional review board statement: *The study was approved by the University Committee of Animal Care and Supply at the University of Saskatchewan (approval No. AUP#20070090) in March 2007 and Animals Ethics Committee of University of South China (approval No. LL0387-USC) in June 2017, and performed in accordance with the National Institutes of Health (USA) guidelines for care and use of laboratory animals.*

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Open peer reviewer: *Jin Zhang, Nanjing Sanhome Pharmaceutical Co., Ltd., China.*

Additional file:

Additional Table 1: *Body weight alteration (g) of Sprague-Dawley rats at the beginning and ending of intraperitoneal injection with CPA alone or together with DPCPX for 5 weeks.*

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Additional Table 1 Body weight alteration (g) of Sprague-Dawley rats at the beginning and ending of intraperitoneal injection with CPA alone or together with DPCPX for 5 weeks.

	Control (n = 20)	CPA (n = 20)	CPA+DPCPX (n = 10)
Beginning	199.74±14.39	202.03±15.41	201.56±15.87
Ending	532.14±40.06	527.50±36.89	523.96±38.23

Experimental animals were administered as described in Materials and methods. The data were expressed as the mean ± SD. $^{*}P$<0.05, *vs.* control group (one-way analysis of variance followed by Student-Newman-Keuls post hoc test). CPA: N^6-cyclopentyladenosine; DPCPX: 8-cyclopentyl-1,3-dipropylxanthine.