The regulator of calcineurin 1 increases adenine nucleotide translocator 1 and leads to mitochondrial dysfunctions

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Abstract

The over-expression of regulator of calcineurin 1 isoform 1 (RCAN1.1) has been implicated in mitochondrial dysfunctions of Alzheimer’s disease; however, the mechanism linking RCAN1.1 over-expression and the mitochondrial dysfunctions remains unknown. In this study, we use human neuroblastoma SH-SY5Y cells stably expressing RCAN1.1S and rat primary neurons infected with RCAN1.1S expression lentivirus to study the association of RCAN1 with mitochondrial functions. Our study here showed that the over-expression of RCAN1.1S remarkably up-regulates the expression of adenine nucleotide translocator (ANT1) by stabilizing ANT1 mRNA. The increased ANT1 level leads to accelerated ATP–ADP exchange rate, more Ca2+-induced mitochondrial permeability transition pore opening, increased cytochrome c release, and eventually cell apoptosis. Furthermore, knockdown of ANT1 expression brings these mitochondria perturbations caused by RCAN1.1S back to normal. The effect of RCAN1.1S on ANT1 was independent of its inhibition on calcineurin. This study elucidated a novel function of RCAN1 in mitochondria and provides a molecular basis for the RCAN1.1S over-expression-induced mitochondrial dysfunctions and neuronal apoptosis.

Keywords: Alzheimer’s disease, ANT1, mitochondria dysfunction, neuronal apoptosis, RCAN1.1.

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Mitochondria are the critical regulators of neuronal death which is a key mark in neurodegeneration. And dysfunctions of mitochondria have been suggested in aging and neurodegenerative diseases including Alzheimer’s disease (AD) and Parkinson’s disease (Lin and Beal 2006). Mitochondrial dysfunctions are one of the most early and prominent features in vulnerable neurons in neurodegenerative diseases, including impaired mitochondrial respiration, increased reactive oxygen species generation, mitochondrial DNA damage, decreased mitochondrial mass, and abnormal mitochondrial dynamics (de la Monte et al. 2000; Zhu et al. 2006; Querfurth and LaFerla 2010; Swerdlow et al. 2010). Adenine nucleotide translocator 1 (ANT1), or the ADP/ATP translocator 1, is the most abundant protein in the inner mitochondrial membrane. It forms as a homodimer, a gated channel by which ADP is brought into and ATP brought out of the mitochondrial matrix. In addition to the translocase activity, ANT has regulatory role in mitochondrial permeability transition pore (mPTP) function and is involved in mitochondria-mediated apoptosis (Kokoszka et al. 2004; Sharer 2005; Dahout-Gonzalez et al. 2006). mPTP, a non-specific pore in the mitochondrial inner membrane, opens by

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Abbreviations used: AD, Alzheimer’s disease; ANT, adenine nucleotide translocator; BCL-2, B-cell lymphoma-2; BKA, bongkrekic acid; CATR, carboxyatractyloside; COX IV, cytochrome c oxidase subunit IV; Cyt c, cytochrome c; mPTP, mitochondrial permeability transition pore; RCAN1, regulator of calcineurin 1; ROS, reactive oxygen species; SOD2, superoxide dismutase 2.
the primary trigger of elevated matrix Ca\textsuperscript{2+}, leading to permeability to any molecule of \(< 1.5\) kDa (Halestrap et al. 2002). As the result of this pore opening, the mitochondrial electrochemical hydrogen ion gradient dissipates, the matrix is depleted of pyridine nucleotides, mitochondria swell because of the osmotic uptake of water, cytochrome c is released into cytosol, and eventually leads to cell apoptosis (Soane et al. 2007). mPTP opening and energy crisis have been considered to play important roles in acute and chronic neurodegeneration. There are two conformations of ANT1, matrix conformation (m-conformation) and cytosol conformation (c-conformation), which could be induced and stabilized by specific ligands bongkrekic acid (BKA) and carboxyatractyloside (CATR), respectively (Dahout-Gonzalez et al. 2006). Regulations of mPTP as well as the translocase activity of ANT1 likely lie on the conformational grounds, indicated by the fact that BKA can inhibit mPTP opening while CATR can facilitate it, and both the two ligands can inhibit ATP–ADP exchange (Dahout-Gonzalez et al. 2006). Many studies have demonstrated that appropriate ANT1 level was vital to mitochondrial function and cell survival (Sharer 2005; Kawamata et al. 2011; Liu and Chen 2013), implying that the expression of ANT1 must be tightly controlled to avoid any deleterious effect.

Regulator of calcineurin 1 (RCAN1), also known as MCIPI, DSCR1, adapt78, and calcipressin, is located at 21q22.12 and consists of seven exons and six introns. Alternatively, splicing the first four exons generates four isoforms of RCAN1 that differ only in their N-terminal (Fuentes et al. 1997). Different usage of two translational start codon (AUG) resulted in two isoforms of RCAN1.1, RCAN1.1L and RCAN1.1S with 55 amino acids longer in RCAN1.1L (Wu and Song 2013). RCAN1.1L is the predominant form expressed in the brain. RCAN1.1S and RCAN1.4, differing in 28 amino acids in N-terminus, have tissue-specific expression pattern by usage of two alternative promoters (Sun et al. 2014b). RCAN1.1 isoform is primarily abundant in the fetal and adult brains. Previous data have shown that RCAN1.1 expression is elevated in the cortex of AD patients and the over-expression may contribute to AD pathogenesis (Ermak et al. 2001; Ermak and Davies 2013). We recently report that the degradation of RCAN1.1 is mediated by both chaperon-mediated autophagy and ubiquitin proteasome pathways (Liu et al. 2009); RCAN1.1 is elevated in the brains of AD and DS patients and RCAN1 over-expression facilitates neuronal apoptosis through caspase 3 activation (Sun et al. 2011); the transcription of RCAN1.4 can be activated by NF-κB (Zheng et al. 2014) and RCAN1.4 over-expression exacerbates calcium overloading-induced neuronal apoptosis (Sun et al. 2014b). Our recent paper also showed that RCAN1.1S and RCAN1.4 inhibited NF-κB and suppressed lymphoma growth that is independent of its inhibition on calcineurin (Liu et al. 2015). Our data also showed that RCAN1 was located in ER and promoted N-glycosylation via oligosaccharyltransferase (Wang et al. 2014). These studies suggested that RCAN1 is a multifunctional protein. The RCAN1.1-related mitochondrial dysfunctions include reduction of mitochondrial mass, decline of cellular ATP level, opening of mPTP, and activation of caspase signal pathway (Chang and Min 2005; Sun et al. 2011, 2014a,b; Ermak et al. 2012), but the underlying molecular basis remains to be discovered. A report from Drosophila species proposes that nebula (Drosophila homolog of RCAN1) can interact with ANT1 to modulate mitochondrial function (Chang and Min 2005); however, whether RCAN1 interacts with ANT1 in mammalian system remains elusive.

Our study here showed that the over-expression of RCAN1.1S remarkably elevated ANT1 expression by stabilizing ANT1 mRNA. The increased ANT1 level led to abnormal mitochondrial functions including accelerated ATP–ADP exchange rate, more Ca\textsuperscript{2+}-induced mPTP opening, increased Cyt c release, and eventually cell apoptosis in neuronal cell lines. Furthermore, knockdown of ANT1 expression by shRNA vector brought these mitochondrial perturbations caused by RCAN1.1S back to normal. The study here demonstrated that RCAN1 impeded mitochondrial functions through ANT1.

Materials and methods

The experimental protocols were approved by the Animal Care and Protection Committee of Shandong University and institutional Ethics Committees of Qilu Hospital, and in compliance with ARRIVE guideline.

Cell culture

YD2 cells were generated by stable transfection of pRCAN1.1S-myc into human neuroblastoma SH-SY5Y cells as previously described (Liu et al. 2009). Rat primary neurons were isolated from E18 pregnant rats and cultured as previously described (Sun et al. 2011). Pregnant rats were bought from experimental animal center of Shandong University. All cells were maintained at 37°C in an incubator containing 5% CO\textsubscript{2}.

Plasmids construction and transfection

The RCAN1.1S expression plasmid pcDNA3.1-RCAN1.1S-6myc and the RCAN1 knockdown plasmid pSuper-siRCAN1 were generated as previously described (Liu et al. 2009). RCAN1.1S refers to the shorter isoform of NCBI seq NM_004414. RCAN1.1S refers to the NCBI seq NM_203418. The truncation plasmids pcDNA3.1-RCAN1.1S 1-103-6myc and pcDNA3.1-RCAN1.1S 141-197-6myc were constructed as described previously (Liu et al. 2015). The cDNA of ANT1 was PCR amplified with ANT1-1F (5′-CCGGGATCTTTC-GACATATTTTTTGAT) and ANT1-894R (5′-CCGGGATATGTCAGAATTCGCCACC) and cloned into p3-flagCMV10 vector to generate ANT1 expression plasmid p3 × flagCMV10-ANT1. The two strands of siANT1-147F (5′-GATCCCCGAGTACAAAGGG-GATCATTTGTATTCCAGAGATGACTATTTGAC) and siANT1-894R (5′-GATCCAGATTTCTGATGACTATTTGAC) were relevant to the anti-ANT1 siRNA. The two strands of siANT1-147F and siANT1-894R were annealed to form double-stranded siRNA. The siRNA duplexes were transfected into neurons using Lipofectamine 2000 (Invitrogen) at a concentration of 20 nM. After transfection, the cells were seeded into a 24-well plate and cultured for 24 hours followed by treatment with the drugs.
annealed and ligated into pSuper vector to generate the ANT1
knockdown plasmid pSuper-siANT1. All transfections were carried
out with Lipofectamine™ 2000 (Thermo Fisher Scientific) transfection
reagent according to the manufacturer’s instructions.

Lentivirus construction and Infection
The cDNA of RCAN1.1S and ANT1 were PCR amplified and cloned
into lentivirus vector pWPXL. The lentivirus expression vector was
co-transfected with psPAX2 and pMD2.G into HEK293T cells for
lentivirus production. Lentivirus was harvested from the culture
media 48 h after transfection and precipitated with PEG8000. The
titer of lentivirus produced is about 107 pfu/mL. Rat primary
neurons were infected with a MOI (multiplicity of infection) of 5 for
the mPTP-related assays, metabolically active mitochondrial
dysfunctions were detected. RCAN1-mediated ANT1 upregulation
was determined by Western blot using an mAb recognizing ANT1
antigen. 

Immunoblotting analysis
For immunoblotting analysis, cells or isolated mitochondria
were lysed in radio-immunoprecipitation assay buffer (1% Triton X100,
1% sodium deoxycholate, 4% sodium dodecyl sulfate, 0.15 M NaCl,
0.05 M Tris-HCl, pH 7.2) supplemented with protease inhibitors.
The lysates were resolved by sodium dodecyl sulfate–polyacryl-
lamide gel electrophoresis and the immunoblotting was performed as
described previously (Liu et al. 2009). The primary antibodies used
were mouse 9E10 mAb, M2 mAb, anti-ANT1 mAb, anti-superoxide
dismutase 2 (SOD2) mAb, and anti-β-actin mAb. The endogenous
RCAN1.1 antibody DCT3 (a polyclonal rabbit against the last 20 aa
in the RCAN1 C-terminus) was used to detect endogenous expres-
sion of RCAN1 (Sun et al. 2011). Detection and quantifications were
performed with the Li-Cor Odyssey imaging system and its software
(Liu et al. 2009).

Mitochondrial isolation
All steps were carried out at 0–4°C; mitochondrial isolation from
YD2 and SH-SY5Y cells was performed as described previously
with minor modifications (Sun et al. 2011). The cell homogenate
was centrifuged twice at 800 g for 5 min in order to get rid of
the nuclei and remaining intact cells; the supernatant was
centrifuged at 8000 g for 15 min to pellet crude mitochondria.
The supernatant was transferred to a new tube and centrifuged
for an additional time in order to remove any remaining
mitochondria, and collected as the cytosol fraction. The crude
mitochondria were layered over a 1.0/1.5 M discontinuous
sucrose gradient containing protease and phosphatase inhibitors
and centrifuged at 100 000 g (Beckman Optima MAX-XP
Ultracentrifuge MLS 50 rotor (Beckman Coulter, Krefeld,
Germany) for 1 h. The purified mitochondria were collected from
the 1.0 to 1.5 M sucrose interface by pipetting. 9E10 mAb
was used to detect exogenous myc-tagged RCAN1.1S of YD2
cells, and DCT3 to detect endogenous RCAN1.1 of SH-SY5Y
cells. Several mitochondrial markers were used to quantify mitochondria: B-cell lymphoma-2 (BCL-2) for the outer
membrane, Cyt c for the intermembrane space, SOD2 for matrix, and
Cyt c oxidase subunit IV (COX IV) for the inner membrane. In
the mPTP-related assays, metabolically active mitochondrial
isolation from cultured rat primary neurons was performed
according to the method previously described (Almeida and Medina 1998).

RT-PCR
RT-PCR was performed as previously described (Liu et al. 2009).
Specific primers used to amplify RCAN1.1S gene are RCAN1.1R
(5’-CGACCTGGAGCTTTCAATTGACT) and RCAN1.1R (5’-GCCCA
GTTCCTCGAGTTAGCGATCGGTGA). Specific primers were used to amplify a 125-bp fragment of ANT1 gene are ANT1-202F
(5’-CTCTCCCTCCTGGAGGGTTAAC) and ANT1-327R (5’-GA
ACTGCTATGCCGATCCAC). A 141-bp fragment of human β-
actin amplified with primers actin-F (5’-GACAGGATGCAGAG
AGATTACT) and actin-R (5’-TGATCCACATCTGTGGAGGT
GT) was used as internal control. To verify that the read-out of
RT-PCR results was linear, different amplification cycle numbers
of RCAN1.1S, ANT1, and β-actin genes were performed. Samples
were analyzed on 1.5% agarose gel. Image J software was used to
quantify and analyze the data. Mitochondrial DNA (mtDNA) was
determined to evaluate the mitochondrial content using quantitative
real-time PCR as previously described (Bai and Wong 2005). RPPH1
was used as nuclear gene normalizers for the mtDNA
content (TaqMan Copy Number Reference Assay from ABI
(Waltham, MA, USA).

Degradation of ANT1 mRNA and protein
For the measuring of ANT1 mRNA degradation, SH-SY5Y and YD2
cells were treated with 1 μg/mL actinomycin D (Act D) to inhibit de
novo RNA synthesis for 0, 3, 6, 9, and 12 h. ANT1 mRNA level was
measured by RT-PCR: isolated RNA was treated with recombinant
DNase I before reverse transcription to prevent contamination of
genic DNA, random primer (3801; Takara, Dalian, China) was used
to synthesize the first strand of cDNA, and 18s rRNA was used as an
internal control. The specific primers amplifying a 232-bp fragment
of 18s gene were 18sRF1 (5’-CACCCACCCGAGATTGAG
CA) and 18sR (5’-TAGTAGCAGGCGCCGTTG). The primers amplifying a 140-bp fragment of 18s gene were 18sRF1 (5’-CACCCACCCGAGATTGAG
CA) and 18sR (5’-TAGTAGCAGGCGCCGTTG).

ATP-ADP exchange rate assay
ATP-ADP exchange rate solely mediated by ANT was measured
according to a method developed by Kawamata et al. (2010) by
exploiting the differential affinity of ADP and ATP to Mg2+. A total
quantity of 40 μg/mL digitonin was used to permeabilize cells;
2 mM ATP was added to start the mitochondrial phosphorylation;
magnesium green fluorescence was recorded in, calibrated, and
converted to [ATP] by appearing in the reaction medium using
standard binding equations listed in the published method
(Chinopoulos et al. 2009; Kawamata et al. 2010). The fitted slope
obtained by the linear regression of this time course of [ATP] appearing in the reaction medium reflects ATP-ADP exchange rate.
mediated by the ANT. The ATP–ADP exchange rate mediated by the ANT was validated in this assay by sequentially adding 4 mM CATR to the reaction medium, which resulted in a complete halt of ATP rise in the media after three additions.

**Determination of mPTP opening**

mPTP opening was determined as previously described using the calcine-CoCl2 bleaching assay (Petronilli et al. 1999). Briefly, cultured cells (grown in wells of a 24-well plate) were washed in reaction buffer (140 mM NaCl, 5.0 mM KCl, 10 mM HEPES, 2.0 mM CaCl2, 1.0 mM MgCl2, and 10 mM glucose, pH 7.4), and pre-incubated in fresh buffer A containing 2 μM fluorescence dye calcine-AM and 1 mM CoCl2 at 37°C for 30 min, then washed three times with buffer B, and the initial calcine fluorescent signals was recorded by Varioskan flash instruments (Thermo Scientific, Shanghai, China) at 25°C with the excitation wavelength of 494 nm and emission wavelength of 517 nm. mPTP opening was induced by adding 500 μM CaCl2 along with the 5 μM Mg2+ ionophore ionomycin in the presence or absence of ANT1 ligand, and the fluorescence signals were measured again. The added ANT1 ligand was 1 μM carboxyatractysolose which sensitized the mPTP to Ca2+, or 5 μM BKA which made the mPTP insensitive to Ca2+. The protein concentration for each well was measured by the Bradford protein assay. The fluorescent signals were normalized to total protein content. The decreased percentage of initial fluorescent signals could be interpreted as mPTP opening.

**Swelling of energized mitochondria**

The Ca2+-triggered mitochondrial swelling assay was performed as follows: isolated mitochondria (0.4 mg/mL) were incubated in KCl media (125 mM KCl, 2 mM K2HPO4, 1 mM MgCl2, 20 mM HEPES, 5 mM glutamate, 5 mM malate, and 2 μM rotenone, pH 7.4) for 10 min in the presence or absence of ANT1 ligand (1 μM CATR or 5 μM BKA). Mitochondrial swelling was triggered by the addition of Ca2+ (500 nmol/mg mitochondrial protein); the mitochondrial swelling caused by an influx of solutes across the inner membrane was observed by immediately and continuously recording the decrease in absorbance at 540 nm on Varioskan flash instruments (Thermo Scientific).

**Ca2+ retention capacity of mitochondria**

Mitochondrial Ca2+ retention capacity was determined under energized conditions. Isolated mitochondria (0.4 mg/mL) were suspended in 1 mL KCl media containing 0.5 μM Calcium green-5N in the presence or absence of ANT1 ligand (1 μM CATR or 5 μM BKA). Fluorescence changes were continuously measured at 25°C with the excitation wavelength of 506 nm and emission wavelength of 517 nm. A total quantity of 4 μL aliquots of a 20 mM CaCl2 solution (20 mM CaCl2, 127 mM KCl, 1 mM MgCl2, 20 mM HEPES, 5 mM glutamate, 5 mM malate, pH 7.4) were added every 2 mins to introduce 200 nmol Ca2+/mg protein until mPTP opening indicated a double increase above the baseline reading of fluorescence. The total amount of Ca2+ added can be interpreted as Ca2+ retention capacity.

**Detection of Cyt c release and TUNEL staining**

For detection of Cyt c release, mitochondria and cytosol fractions were separated as mentioned above. Western blot was used to detect Cyt c release from mitochondria to cytosol. SOD2 was used as mitochondrial marker and GAPDH for cytosolic marker. For TUNEL staining, cells were fixed in 4% paraformaldehyde for 40 min, permeabilized with 0.1% TritonX-100 for 10 min, and stained with 1 μg/mL DAPI (4’,6-diamidino-2-phenylindole) (D9542; Sigma-Aldrich, Shanghai, China) at 25°C for 10 min. TUNEL staining was performed using the Roche-In Situ Cell Death Detection Kit according to the manufacturers’ instructions. Results were analyzed by fluorescence microscopy (Leica DM4000B, Wetzlar, Germany).

**Statistical analysis**

All the experiments were repeated at least three times. One representative picture is shown in figures. Quantifications were from three or more independent experiments. Data were analyzed by Student’s t-test. Values represent means ± SE; p < 0.05 was considered as statistically significant.

**Data accessibility**

The original unprocessed data are available in supporting information online.

**Materials**

The materials used were as follows: lipofectamine™ 2000 transfection reagent (Life Technologies, Grand Island, NY, USA), lentivirus vector pWPXL (from Addgene, Cambridge, MA, USA), protease inhibitors (Caltag; 04693116001; Roche Molecular Biologicals, Indianapolis, IN, USA), 9E10 mAb (anti-c-myc; ab32; Abcam, USA), M2 mAb (anti-flag; F1804; Sigma-Aldrich), anti-ANT1 mAb (ab10322; Abcam, Cambridge, MA, USA), anti-SOD2 mAb (12656-RP02; Sino Biological, Beijing, China), BCL-2 (BS1511; Bioworld Technology, Nanjing, China), Cyt c (AC909; Beyotime, Guangzhou China), COX IV (4850; Cell Signaling Technology, Beverly, MA, USA), anti-β-actin mAb (AC-15; Sigma-Aldrich), TaqMan Copy Number Reference Assay (4403136; Life Technologies), FK506 (F4679; Sigma-Aldrich), actinomycin D (A9415; Sigma-Aldrich), DNAsel (2270A; Takara), CHX (S1560; Beyotime), digitonin (D141; Sigma-Aldrich), random primer (3801; Takara), Magnesium Green (M-3733; Life Technologies), carboxyatractysolose (C4992; Sigma-Aldrich), Bongkrekic acid (1820-100; Biovision, Milpitas, CA, USA), COX IV (AC909; Beyotime), Bongkrekic acid (1820-100; Biovision, Milpitas, CA, USA), Calcium green-5N (c-3737; Life Technologies), and DAPI (D9542; Sigma-Aldrich); Roche-In Situ Cell Death Detection Kit (12156792910; Roche Molecular Biologicals).

**Results**

**RCAN1.1S increased ANT1 mRNA and protein levels**

To reveal the mechanism of RCAN1-ANT1 interaction, we examined the expression of ANT1 in YD2 cells and SH-SY5Y cells transiently over-expressing RCAN1.1S. RT-PCR was used to examine the ANT1 mRNA expression. RT-PCR showed that ANT1 mRNA level in YD2 cells was significantly increased to 209.60 ± 6.94% of control cells (p < 0.01, Fig. 1a). **ANT1 mRNA expression was also**
elevated to 256.80 ± 17.45% of control in SH-SY5Y cells transiently transfected with RCAN1.1S expression plasmid (p < 0.01, Fig. 1b). Furthermore, ANT1 mRNA was decreased to 51.79 ± 3.47% of control in cells with RCAN1 knocked down (p < 0.01, Fig. 1d). RCAN1 knockdown effect was also verified by western blot in Fig. 1(c). To further examine whether the increase of mRNA resulted in the increase of its protein expression, western blot assay was performed and showed that the overall levels of endogenous ANT1 (Fig. 1e) as well as the mitochondrial ANT1 (Fig. 1f) were increased by RCAN1.1S over-expression. The results showed that endogenous ANT1 protein in total cell lysates was increased to 149.70 ± 14.80% in YD2 cells (p < 0.05, Fig. 1e, lane 2), and 179.90 ± 14.14% of control in RCAN1.1S transiently transfected SH-SY5Y cells (p < 0.01, Fig. 1e, lane 4). Endogenous ANT1 protein in mitochondria was also increased to 142.50 ± 3.82% in YD2 cells (p < 0.01, Fig. 1f, lane 2) and 179.90 ± 14.14% of control in RCAN1.1S transiently transfected SH-SY5Y cells (p < 0.01, Fig. 1f, lane 4).

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**Fig. 1** Over-expression of RCAN1.1S increased adenine nucleotide translocator (ANT1) mRNA and protein levels. (a) RT-PCR showed that stable over-expression of RCAN1.1S in YD2 cells significantly increased ANT1 mRNA levels. β-actin was amplified as the internal control (row 3). (b) RT-PCR showed that transient (48 h) over-expression of RCAN1.1S in SH-SY5Y cells also remarkably increased ANT1 mRNA levels. (c) pSuper-RCAN1 and pcDNA3.1-RCAN1.1S-6myc were co-transfected into SH-SY5Y cells. Western blot was used to confirm the knockdown of RCAN1.1 by pSuper-RCAN1 48 h after transfection. (d) RT-PCR showed that RCAN1 knockdown in SH-SY5Y cells significantly decreased ANT1 mRNA level. The knockdown effect of pSuper-RCAN1 was also confirmed in RCAN1.1 mRNA level. β-actin was amplified as the internal control. (e and f) Over-expression of RCAN1.1S increased ANT1 protein levels. Endogenous ANT1 protein levels of total cell lysates (e) and mitochondria (f) were detected by anti-ANT1 mAb in YD2 cells and pcDNA3.1-RCAN1.1S-6myc transiently transfected SH-SY5Y cells. Over-expressed RCAN1.1S protein in YD2 and transfected SH-SY5Y cells was also confirmed by 9E10 (anti-myc) antibody. β-actin was used as loading control for whole cell lysate and superoxide dismutase 2 (SOD2) as loading control for mitochondria fractions. Values represent mean ± SE; n = 4 (a–d) n = 5 (e and f), *p < 0.05 by Student’s t-test. (g) Mitochondrial DNA (mtDNA) was determined to evaluate the mitochondrial content using quantitative real-time PCR. RPPH1 gene was used as nuclear gene normalizers for the mtDNA content (TaqMan Copy Number Reference Assay from ABI).
transiently transfected SH-SY5Y cells (p < 0.01, Fig. 1f, lane 4). To confirm that the RCAN1.1S effect was specific for ANT1, we also detected the level of COX IV which was also located at mitochondrial inner membrane and BCL-2 which could interact with ANT1 (Brenner et al. 2000) in total cell lysates and mitochondrial fractions (Fig. 1e and f). Quantitative results showed that the levels of COX IV and BCL-2 were not altered by the over-expressed RCAN1.1S (p > 0.05; quantitative data in supporting information). In addition, mitochondrial content was associated with the level of mitochondrial protein, so we evaluated mitochondrial content by mtDNA copy number; the results demonstrated that the mtDNA content determined by normalized DLOOP copy number significantly decreased to 79.20 ± 11.16% in YD2, to 48.48 ± 8.84% in RCAN1.1S transiently transfected SH-SY5Y cells, and to 75.80 ± 14.07% in ANT1 transiently transfected SH-SY5Y cells (Fig. 1g). These data clearly indicated that ANT1 expression was up-regulated by RCAN1.1S at both the mRNA and protein levels.

**RCAN1.1S retarded the degradation rate of ANT1 mRNA**

To further elucidate the molecular mechanism of the ANT1 mRNA up-regulation by RCAN1, the degradation rate of ANT1 mRNA was measured by actinomycin D (Act D) chase assay. 18s rRNA was chosen as an internal control (Salve et al. 2001). The data showed that ANT1 mRNA was more stable in YD2 cells compared with SH-SY5Y cells (p < 0.05 starting from 3 h point, Fig. 2a and b). To confirm that the increased stability is specific to the ANT1 transcript rather than a change in mRNA stability throughout the cell, we also detected the degradation of DNM1 mRNA whose half-life was approximately 3 h in Act D treated SH-SY5Y and YD2 cells. The results indicated that the stability of DNM1 mRNA was not altered by RCAN1.1 (p > 0.05; Fig. 2a and c), demonstrating that the increased stability is specific to the ANT1 transcript. CHX chase assay

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**Fig. 2** Over-expression of RCAN1.1S stabilized adenine nucleotide translocator (ANT1) mRNA (a). SH-SY5Y and YD2 cells were treated with 1 μg/mL actinomycin D (Act D) for 0, 3, 6, 9, and 12 h. RT-PCR was used to detect the ANT1 mRNA and dynamin 1 (DNM1) mRNA. 18s rRNA was amplified as an internal control. (b). Quantification of ANT1 mRNA in (a). (c). Quantification of DNM1 mRNA in (a). Values represent mean ± SE; n = 5. mRNA and protein levels at 0 h were artificially set to 100%. (d). ANT1 expression plasmid p3 × flagCMV10-ANT1 was transfected in SH-SY5Y and YD2 cells. A total quantity of 100 μg/mL cycloheximide (CHX) was added 48 h after transfection and cells were collected at 0, 12, 24, 36 h after treatment. Western blot was used to detect the ANT1 protein level using M2 mAb and β-actin was used as loading control. (e). Quantification of (d). Values represent mean ± SE; n = 5. mRNA and protein levels at 0 h were artificially set to 100%. 

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was used to measure the degradation rate of ANT1 protein. The data showed that there were no significant difference in the ANT1 protein stability between RCAN1.1S over-expressed cells and control cells (p > 0.05, Fig. 2d and e), implying RCAN1.1S over-expression did not interfere with the degradation of ANT1 protein. The data here supported that RCAN1 up-regulated ANT1 expression by stabilizing the ANT1 mRNA.

RCAN1 increased ANT1 independent of its inhibition on calcineurin
RCAN1 can inhibit calcineurin via its C-terminus 141–197 aa (Chan et al. 2005; Martinez-Martinez et al. 2009). To further investigate whether the effect of RCAN1.1 over-expression on ANT1 was associated with its inhibition on calcineurin, endogenous ANT1 was monitored in SH-SY5Y cells treated with FK506, a pharmacological inhibitor of calcineurin.

**Fig. 3** RCAN1 increased adenine nucleotide translocator (ANT1) independent of its inhibition on calcineurin. (a and c), SH-SY5Y cells were treated with DMSO (control) and 10 μM FK506 (a) and 10 nM FK506 (c) (dissolved in DMSO, FK506+) for 0, 3, 24, and 48 h. Endogenous ANT1 level was detected in whole cell lysate. (b), Quantification of (a). (d) Quantification of (c). (e) SH-SY5Y cells were transfected with pcDNA3.1-RCAN1.1S-6myc, pcDNA3.1-RCAN1.1S-1-103-6myc, pcDNA3.1-RCAN1.1S 141-197-6myc. Forty-eight hours after transfection, mitochondria were isolated and detected with anti-ANT1 antibody. RCAN1 isoforms and truncation forms were detected with anti-myc antibody in the whole cell lysate and β-actin was used as loading control. (f) Quantification of (e). Values represent mean ± SE; n = 3. mRNA and protein levels at con were artificially set to 100%. (g) SH-SY5Y cells were transfected with pcDNA3.1-RCAN1.4-6myc, pcDNA3.1-RCAN1.1S-6myc, pcDNA3.1-RCAN1.1S 1-103-6myc, pcDNA3.1-RCAN1.1S 141-197-6myc. Forty-eight hours after transfection, the total RNA was isolated and real-time PCR was used to detect ANT1 mRNA level. (h) HEK293 cells were transfected with pNFATluc and pcDNA3.1-RCAN1.4-6myc, pcDNA3.1-RCAN1.1S-6myc, pcDNA3.1-RCAN1.1S 1-103-6myc, pcDNA3.1-RCAN1.1S 141-197-6myc, and calcineurin expression vector. Dual luciferase assay was used to measure the luciferase activity to reflect the NFAT transcriptional activity. Values represent mean ± SE; n = 3. *p < 0.05 by Student’s t-test.
calcineurin. The FK506 effect was assessed both as chronic administration (24 and 48 h) and acutely (3 h) to distinguish between long-term effects and short-term effects. A higher dosage of 10 μM (Fig. 3a and b) and lower dosage of 10 nM (Fig. 3c and d) were used for FK506 treatment. The results showed that FK506 did not alter ANT1 level, no matter at chronic or acute administration (p > 0.05; Fig. 3a–d), demonstrating that the effect of RCAN1.1 on ANT1 is independent of calcineurin. The C-terminus 141–197 aa was sufficient for inhibition of calcineurin. To further verify that RCAN1’s effect is independent of its effect on calcineurin, the two isoforms of RCAN1.1S and RCAN1.4 as well as two truncations RCAN1.1S 1–103 aa and 141–197 aa were transfected into SH-SY5Y cells. ANT1 mRNA and protein expression were assayed by RT-PCR and western blot. The results showed that RCAN1.4, RCAN1.1S, and RCAN1.1S 1–103 increased ANT1 protein level (Fig. 3e and f) and mRNA level (Fig. 3g). The C-terminus 141–197 aa can inhibit calcineurin-NFAT signaling while it had no effect on ANT1 expression (lane 4 of Fig. 3e–h), indicating inhibition of calcineurin is not sufficient to increase ANT1. The N-terminus 1–103 aa increased ANT1 expression (lane 5 of Fig. 3e–h) while having no effect on calcineurin, indicating that N-terminus domain is sufficient for its effect on ANT1. The larger isoform 1 of RCAN1.1L also increased ANT1 expression (supplementary Fig. 1), implying that the effect of RCAN1 on ANT1 is a common effect among RCAN1 variants. These experiments demonstrated that RCAN1’s effect on ANT1 is independent of its effect on calcineurin.

**RCAN1.1S accelerated ATP–ADP exchange rate via ANT1**

The fundamental function of ANT1 is the exchange function of bringing ADP into the mitochondrial matrix and bringing ATP out to the cytosol (Dahout-Gonzalez et al. 2006). To find out whether ANT1 function was altered by RCAN1, we measured the ATP–ADP exchange rate in the digitonin-permeabilized cells. Data displayed that the ATP–ADP exchange rate (nmol/s) was accelerated from 10.52 ± 0.56 nmol/s in SH-SY5Y cells to 12.79 ± 0.75 nmol/s in YD2 cells (p < 0.05, Fig. 4b, lane 1 vs. lane 2), and from 10.83 ± 1.04 nmol/s in control cells to 15.55 ± 1.87 nmol/s in RCAN1.1S transiently transfected SH-SY5Y cells (p < 0.05, Fig. 4b, lane 1 vs. lane 2). And the effect of ANT1 over-expression increased the ATP–ADP exchange rate from 10.43 ± 1.04 nmol/s in control cells to 14.57 ± 1.21 nmol/s (p < 0.05, Fig. 4b, lane 5 vs. lane 6). To further verify whether ANT1 mediated the effect of RCAN1 on ATP–ADP exchange, we knocked down the elevated ANT1 expression in YD2 cells using ANT1 shRNA vector. The ATP–ADP exchange rate was decreased from 13.14 ± 0.60 nmol/s to 9.44 ± 0.84 nmol/s (p < 0.01, Fig. 4c, black vs. gray), corresponding with decreased protein expression of ANT1. These data suggested that RCAN1 had an effect on ATP/ADP exchange rate via its interaction with ANT1.

**RCAN1.1S affected Ca2+-induced mPTP opening via ANT1**

In addition to ATP–ADP exchange, ANT1 also plays a central role in modulating the sensitivity of mPTP to Ca2+ (Kokoszka et al. 2004; Dahout-Gonzalez et al. 2006; Leung and Halestrap 2008). CATR stabilizes the c-conformation of the ANT1 and sensitizes the mPTP to Ca2+, while BKA stabilizes the m-conformation of the ANT1 and makes the mPTP insensitive to Ca2+ (Dahout-Gonzalez et al. 2006). To further examine whether RCAN1.1S over-expression affected mPTP opening through its up-regulation on ANT1, mPTP opening was measured by a calcein fluorescence decrease. Though the basal level of calcein fluorescence showed no difference (Fig. 4d, lane 1–6), under the treatment of 5 μM Ca2+ ionophore ionomycin that triggered mPTP opening, the calcein fluorescence was further decreased by RCAN1.1S or ANT1 over-expression (Fig. 4d, lane 7–12). The treatment of BKA alone had no influence on the basal level of calcein (Fig. 4d, lane 13–18), while treatment of CATR alone decreased the calcein fluorescence via inducing mPTP opening, and the fluorescence was also further decreased by RCAN1.1S or ANT1 over-expression (Fig. 4d, lane 19–24). The effect of RCAN1.1S on mPTP opening was abolished by BKA (Fig. 4d, lane 25–30) and intensified by CATR (Fig. 4d, lane 31–36). Furthermore, knockdown of ANT1 by shRNA vector in YD2 cells brought back the mPTP opening induced by RCAN1.1S (Fig. 4e, lane 6, 12, 18 compared to lane 5, 11, 17, respectively). The data here demonstrated that RCAN1 affected mPTP opening through ANT1.

**RCAN1.1S exacerbated Ca2+-induced mitochondrial swelling and compromised Ca2+ retention capacity via ANT1**

mPTP opening leads to a series of consequences, including Ca2+ retention incapacity, massive swelling of mitochondria, rupture of the outer membrane, release of Cyt c or the apoptosis-inducing factor, and eventually cell death (Halestrap et al. 2002; Schwarz et al. 2007; Tsujimoto and Shimizu 2007; Leung and Halestrap 2008). Mitochondrial swelling caused by influx of solutes across the inner membrane could be detected by measuring a decrease in the absorbance at 540 nm. Mitochondria Ca2+ retention capacity was reflected by the total Ca2+ injection pulses in the reaction before mPTP opening. The OD540 showed that YD2 cells had a larger degree of swelling than SH-SY5Y cells (Fig. 5a, curve 2 vs. curve 1 p < 0.05). The exacerbation of mitochondrial swelling was abolished by BKA and further amplified by CATR. Similar results were observed in rat primary neurons infected with RCAN1.1S expression lentivirus (Fig. 5b). Also, the difference can be abolished by the knockdown of ANT1 with shRNA vector (Fig. 5c). In addition, RCAN1.1S expression reduced Ca2+ retention capacity (Fig. 5d, p < 0.05). And, the effect on Ca2+ retention capacity by RCAN1.1S was abolished by BKA and further amplified by CATR (Fig. 5e). Knockdown of...
Fig. 4 Over-expression of RCAN1.1S resulted in accelerated ATP–ADP exchange rate and more Ca\textsuperscript{2+}-induced mitochondrial permeability transition pore (mPTP) opening. (a). Over-expression of RCAN1.1S and adenine nucleotide translocator (ANT1) resulted in accelerated ATP–ADP exchange rate. The representative time course of [ATP] in SH-SY5Y, YD2, SH-SY5Y transfected with pcDNA3.1-RCAN1.1S-6myc (RCAN1.1S), p3 × flagCMV10-ANT1 (ANT1), and their empty vectors (CON). The [ATP] was calculated from the change of the magnesium green fluorescence according to Methods. Thumbnail figure at top right confirmed the ATP/ADP exchange was mediated by ANT1 (b). The calculated ATP–ADP exchange rates were the slopes of the regression lines of the data in (a). Values represent mean ± SE; n > 5. *p < 0.05 by Student’s t-test. Knockdown effect of pSuper-siANT1. RCAN1.1S protein was detected with 9E10 (anti-myc) antibody and endogenous ANT1 was detected with anti-ANT1 antibody. Superoxide dismutase 2 (SOD2) was used as mitochondrial loading control. (d). Over-expression of RCAN1.1 and ANT1 resulted in more Ca\textsuperscript{2+}-induced mPTP opening. Assay of mPTP opening was determined in SH-SY5Y, YD2, SH-SY5Y transfected with RCAN1.1 and ANT1 expression vectors. mPTP opening was indicated as a decrease in the initial calcein fluorescence (fluor.). The cells were treated with 5 \mu M ionomycin, 5 \mu M bongkrekic acid (BKA), 1 \mu M carboxyatractyloside (CATR), 5 \mu M ionomycin + 5 \mu M BKA, 5 \mu M ionomycin + 1 \mu M CATR, respectively. Values represent mean ± SE; n = 5. *p < 0.05 by Student’s t-test. (e). Knockdown of ANT1 in YD2 cells reduced the Ca\textsuperscript{2+}-induced mPTP opening. The increase of calcein fluorescence indicated the reduction of mPTP opening. Values represent mean ± SE; n = 6. *p < 0.05 by Student’s t-test.
ANT1 in YD2 cells brought back the effect on calcium retention capacity (Fig. 5g). Furthermore, similar results were obtained in metabolically active mitochondria from cultured primary rat neurons infected with lentivirus expressing RCAN1.1S and ANT1. The data here demonstrated that RCAN1 affect mitochondrial swelling and calcium retention capacity via its interaction with ANT1.

**RCAN1.1S induced Cyt c release and cell apoptosis via ANT1**

To further verify whether mPTP opening induced by RCAN1 would result in Cyt c release and cell apoptosis, we examined the translocation of Cyt c from mitochondria to cytosol using western blot. A decrease of Cyt c in mitochondria and an increase of Cyt c in cytosol were observed in SH-SY5Y cells transfected with RCAN1.1S (Fig. 6a). ANT1 overexpression displayed similar pattern of Cyt c release from mitochondria to cytosol (lane 5 and 6 of Fig 6b), while knockdown of ANT1 using shRNA vector abolished the translocation of Cyt c induced by RCAN1.1S (Fig. 6c). To further investigate the outcome of Cyt c release, TUNEL was used to monitor cell apoptosis.
Consistent with our previous reports (Sun et al. 2011), TUNEL assay showed more cell apoptosis in cells over-expressing RCAN1.1S (lane 1–4 of Fig. 6e). Similar results were obtained with ANT1 over-expression (lane 5 and 6 of Fig. 6e), and ANT1 knockdown protected cells from RCAN1.1S induced apoptosis (Fig. 6f). The data here suggested that RCAN1 induced Cyt c release and neuronal cell apoptosis through mPTP opening via its interaction with ANT1.

**Discussion**

Our study here showed that RCAN1.1S elevated ANT1 expression, which resulted in mitochondrial dysfunctions...
including ATP/ADP exchange rate, mPTP opening, mitochondrial swelling, and Cyt c release. Furthermore, knockdown of ANT1 in cells over-expressing RCAN1.1 brought back these perturbations to normal. The study suggested that ANT1 and mitochondrial perturbations contributed to the cell apoptosis induced by RCAN1 over-expression. Our previous study has shown that RCAN1 over-expression in primary neurons induced neuronal apoptosis, mediated by Cyt c release, and activation of caspase 9 and caspase 3 (Sun et al. 2011). The study here further provided the molecular mechanism which RCAN1 interacted with ANT1 in mitochondria and facilitated mPTP opening, resulting in Cyt c release and apoptosis.

RCAN1 can physically interact with calcineurin subunit A through its C-terminus aa 141–197 and inhibit calcineurin-NFAT signaling. The effect of RCAN1.1S on ANT1 is independent of its effect on calcineurin-NFAT since ANT1 did not respond to calcineurin inhibitor FK506. And, the RCAN1 C-terminus that inhibited calcineurin-NFAT signaling did not affect ANT1 expression. Furthermore, we identified that the aa 1–103 of RCAN1.1S was responsible for its effect on ANT1 in mitochondria. RCAN1.4 isoform has similar effect on ANT1 with RCAN1.1S isoform. We also had data showing that RCAN1.1L isoform had similar effect on ANT1 in mitochondria. Our data suggested that the effect of RCAN1 on ANT1 in mitochondria is a common function of RCAN1 isoforms and independent of its inhibition on calcineurin.

The abundance of mRNA in the cell is a function of not only its synthesis, processing, and nuclear export rate but also of its degradation rate in the cytoplasm. mRNA degradation rates often change in response to stimulus. RNA binding proteins or non-coding RNAs bind to their cis-acting elements in mRNA and affect the mRNA degradation rates via their ability to recruit or exclude mRNA binding machinery. The 28–103 aa of RCAN1.1S, a common domain among the isoforms RCAN1.1S, RCAN1.4, and RCAN1.1L, was predicted to be an RNA binding domain by RCSB PDB database. It would be of great interest to examine whether this RNA binding activity of RCAN1 would contribute to the stability of ANT1 mRNA in the future.

ANT1 missense mutations have been found in autosomal dominant progressive external ophthalmoplegia with mitochondrial DNA deletions-2 (Kaukonen et al. 2000). Senger’s syndrome and Senger-like syndrome are also associated with severe depletion of ANT1 protein and absence of ANT1 function, but no ANT1 gene abnormality has been identified (Jordens et al. 2002; Morava et al. 2004). It would be interesting to investigate whether RCAN1 played some role in regulating the ANT1 protein expression in these diseases.

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This study was supported by grants from NSFC (81322014). There are no conflicts of interests.

Supporting information
Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. RCAN1 larger isoform increased endogenous ANT1 expression.

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