Regulator of Calcineurin 1 (RCAN1) Facilitates Neuronal Apoptosis through Caspase-3 Activation*

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Individuals with Down syndrome (DS) will inevitably develop Alzheimer disease (AD) neuropathology sometime after middle age, which may be attributable to genes triplicated in individuals with DS. The characteristics of AD neuropathology include neuritic plaques, neurofibrillary tangles, and neuronal loss in various brain regions. The mechanism underlying neurodegeneration in AD and DS remains elusive. Regulator of calcineurin 1 (RCAN1) has been implicated in the pathogenesis of DS. Our data show that RCAN1 expression is elevated in the cortex of DS and AD patients. RCAN1 expression can be activated by the stress hormone dexamethasone. A functional glucocorticoid response element was identified in the RCAN1 isoform 1 (RCAN1-1) promoter region, which is able to mediate the up-regulation of RCAN1 expression. Here we show that overexpression of RCAN1-1 in primary neurons activates caspase-9 and caspase-3 and subsequently induces neuronal apoptosis. Furthermore, we found that the neurotoxicity of RCAN1-1 is inhibited by knock-out of caspase-3 in caspase-3−/− neurons. Our study provides a novel mechanism by which RCAN1 functions as a mediator of stress- and Aβ-induced neuronal death, and overexpression of RCAN1 due to an extra copy of the RCAN1 gene on chromosome 21 contributes to AD pathogenesis in DS.

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The development of DS is caused by the presence of an extra copy of human chromosome 21 (10, 11). The DSCR1 (Down syndrome critical region 1) gene was identified and located on chromosome 21 (12, 13). Disregulation of a regulatory circuit involving DSCR1-calcineurin-nuclear factor of activated T cells (NFAT) plays an important role in DS development (14). DSCR1 proteins physically interact with calcineurin subunit A and inhibit calcineurin activity in vitro and in vivo (15–19). DSCR1 was accordingly renamed as RCAN1 (regulator of calcineurin 1) (20). RCAN1 is phosphorylated at Ser112 by BMK1 (big MAP kinase 1), which is the priming site for the subsequent phosphorylation at Ser112 by GSK-3 kinase (21–24). The phosphorylated form of the RCAN1 could be a substrate of the NFAT complex (23). RCAN1 could also be phosphorylated by NF-κB-activating kinase (NIK), which increases the RCAN1 stability (27). Furthermore, RCAN1 interacts with TAB2 (TAK1 (TGF-β-activating kinase 1)-binding protein 2) to be phosphorylated by TAB1 (28). The phosphorylated form of the RCAN1 could serve as a calcineurin cofactor. NFAT is a major substrate for calcineurin, and dephosphorylation of NFAT facilitates NFAT nuclear translocation and activation of its target genes’ transcription. RCAN1 can repress the NFAT signaling pathway by inhibition of calcineurin (14). Also, as a downstream gene of the
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NFAT signaling pathway, RCAN1 can be activated by a subset of molecules, including VEGF, angiotensin II, G protein-coupled receptor 54, TNF-α, thrombin, and other activators of the calcineurin-NFAT pathway, such as calcium ionophore (29, 30). RCAN1 can also be activated by diphosphorylation in neural cells via calcium current increase through the L-type calcium channel (31).

RCAN1 has been shown to be involved in cardiac valve development, cardiac hypertrophy, inflammation, angiogenesis, and cancer. RCAN1 has also been implicated in learning and memory. However, the role of RCAN1 in neurodegeneration in AD and DS is unknown. In this study, we show that RCAN1 is overexpressed in cortical tissues from AD and DS patients. To investigate the mechanism of RCAN1 overexpression in AD and DS, we characterized human RCAN1 gene promoters and identified a functional glucocorticoid response element (GRE), through which RCAN1 expression is up-regulated by stress hormone dexamethasone. Here we show that RCAN1 mediates glucocorticoid-induced neuronal apoptosis. We found that overexpression of RCAN1-1 in primary neurons activates the caspase-9 and caspase-3 apoptotic pathway, thereby rendering neurons more vulnerable to apoptosis induced by dexamethasone and Aβ. Our data suggest that RCAN1 overexpression may contribute to AD pathogenesis by mediating neuronal death in the brains of DS and AD patients.

**EXPERIMENTAL PROCEDURES**

**Cloning of the RCAN1 Gene Promoter and Construction of Chimeric Luciferase Reporter Plasmids**—A forward primer corresponding to −684 bp (5′-ccgctcagctctctcttttccgccat-ttc-3′) of the transcriptional start site and a reverse primer corresponding to the coding sequence (5′-caacaagtttcacagctcagtc-ccacctc-3′) were used to PCR-amplify the 5′-UTR region of the RCAN1 gene exon 1 from the genome of human neuroblastoma cells SH-SY5Y. The DNA fragment was cloned into pGL3-Basic upstream of the luciferase reporter gene to construct pRCANLuc-G. The fragment from −684 to +46 bp was amplified and cloned using primers 5′-ccgctcagctctcttttccgccattc and 5′-caacaagtttcacagctcagtc-ccacctc, corresponding to +46 bp of the transcriptional start site on RCAN1 gene exon 1. Primers 5′-gcattgagatc-ctgcaggtcgtcctct (core sequence is italic and uppercase) and 3′-cacctc-3′) were used to amplify the region between −1650 and −685 bp in the 5′-UTR of RCAN1 gene exon 1. The amplified fragment was inserted in front of the −684 to 46 bp fragment in pRCANLuc-B to obtain the pRCANLuc-A. Primers were designed to include restriction enzyme sites so that the resulting PCR-amplified fragment could be easily cloned into the multicloning sites of vector pGL3-Basic (Promega). Deletion plasmids pRCANLuc-C, -D, and -E were created from pRCANLuc-A by utilization of restriction enzyme sites PvuII, and EcoRI, respectively.

**Cell Culture**—Neuronal tissues for primary cultures originating from Wistar rat embryos at 17–18 days of gestation were dissected and gently digested with trypsin (0.025% EDTA; Invitrogen). The cells were suspended in neurobasal medium supplemented with B27 (Invitrogen) and plated at a density of 1–2 × 10⁵ cells/well onto poly-d-lysine (0.01 mg/ml; Sigma)-coated 24-well plates (Nunc). The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and used for experiments after 7–14 days. Primary neuronal cultures derived from caspase-3 knock-out (caspase-3−/−) (32) and wild type newborn mice were isolated and cultured as described (33). SH-SY5Y cells and human embryonic kidney 293 cells (HEK293) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 units/ml penicillin G sodium, and 50 μg/ml streptomycin sulfate (Invitrogen). All cells were maintained in a 37 °C incubator containing 5% CO₂.

**Preparation of β-Amyloid Fibrils**—Synthesized Aβ(1–40) was dissolved in sterile double-distilled H₂O to 1 mM. The dissolved Aβ was incubated at 37 °C for 1 h and diluted again with the same volume of PBS (1:1 dilution). Aβ was aged at 37 °C for 4 days, allowing the stable growth of Aβ fibrils.

**Transfection and Luciferase Assay**—Cells were grown to ~70% confluence and transfected with 2 μg of plasmid DNA/35-mm plate using Lipofectamine 2000 (Invitrogen) as described (34). The pH110 β-galactosidase expression plasmid was co-transfected to normalize for transfection efficiency. Cells were harvested 48 h after transfection and lysed in 200 μl of 1× Reporter Lysis Buffer (Promega). The luciferase and β-galactosidase assays were performed according to the manufacturer’s protocols (Promega) as described (35).

** Primer Extension Assay**—A primer extension assay was performed to determine the transcription initiation site. Total neuronal RNA was extracted from SH-SY5Y cells using TRI-Reagent (Sigma). Yeast tRNA was used as a control. A reverse primer, corresponding to positions +46 to +28 bp, 5′-cagagtccttcagactcagcgc, was synthesized and radioactively end-labeled with [γ-32P]ATP (Amersham Biosciences). Primer extension and sequencing were performed as described (34).

**Gel Shift Assay**—Oligonucleotide RCAN1−272−237bp (5′-cagagtccttcagactcagcgc) and its antisense strand (5′-aatctgagatc-ctgcaggtcgtcctct (core sequence is italic and uppercase)) were synthesized and radioactively end-labeled with [γ-32P]ATP by T4 polynucleotide kinase. Gel shift assays were performed as described (36). The samples were analyzed by a 4% nondenaturing PAGE gel.

**Virus Infection**—The RCAN1 expression plasmid pcDNA3.1-RCAN1mycHis was generated as described (37). RCAN1-1 CDNA was cloned into a Semliki Forest virus vector pSFV to generate pSFV-RCAN1, and a green fluorescent protein cDNA was cloned into pSFV to generate pSFV-GFP. To generate the virus, pSFV1-EGFP, pSFV1-RCAN1myc, pSFV-RCAN1GFP, and helper virus pHelper-2 DNA were linearized by Spel or Sau3A. Viral particles were generated according to the manufacturer’s instructions (Invitrogen). For infection, the viral particles activated with chymotrypsin (Sigma) were added to neuronal cultures at a 1:10 dilution with culture medium. Cells were infected for 1 h at 37 °C, followed by replacement of the infection media with conditioned culture medium and incubated for 12–18 h prior to use. Then the infected neurons were monitored for expression of GFP or immunostained for detection of...
Myc tag by 9E10 antibody to visualize RCAN1 expression in SFV-RCAN1myc-infected neurons.

Antibodies and Immunoblotting—The rabbit anti-RCAN1 polyclonal antibody was raised against the C terminus of RCAN1 protein (RPKPIIQTTRPETYPHL5). The antibody was characterized as being able to specifically detect RCAN1. Brain tissues from AD patients were obtained from the Department of Pathology, Columbia University (New York). Brain tissues of DS abortuses (17–21 gestational weeks) were obtained from University of Maryland Brain and Tissue Bank for Developmental Disorders. The brain tissues or cells were lysed in radioimmune precipitation assay lysis buffer (1% Triton X-100, 1% sodium deoxycholate and 4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with protease inhibitors (Complete, Roche Applied Science). The lysates were resolved by 12% SDS-PAGE for detecting RCAN1 and 16% Tris-Tricine PAGE for caspase-3 and caspase-9. The immunoblotting was performed as described (33). Rabbit anti-RCAN1 polyclonal antibody (1:1000 dilution) was used to detect RCAN1 expression. Caspase-3 and its cleaved form P20 were detected with a rabbit polyclonal antibody against amino acids 29–43 of caspase-3 (Sigma, catalog no. C9598, 1:1000). Caspase-3 cleavage was further confirmed with an anti-cleaved caspase-3 antibody (catalog no. 9661) from Cell Signaling. Internal control β-actin was analyzed using monoclonal anti-β-actin antibody AC-15 (Sigma, 1:1000). Caspase-9 was detected with anti-caspase-9 antibody specifically stains the granule cell layer in the hippocampus and the pyramidal neuronal layers in the cortex (Fig. 1A). These data clearly demonstrate that our DCT3 antibody is able to specifically detect RCAN1 protein.

RESULTS

RCAN1 Expression Is Elevated in the Brains of DS Fetus and AD Patients—Previous studies have shown that RCAN1 mRNA is elevated in the brains of DS and AD patients (17, 38), indicating that RCAN1 gene expression is up-regulated at the transcriptional level. To further examine whether RCAN1 is also elevated at the protein level, an antibody specific to RCAN1, DCT3, was used to detect RCAN1 proteins in the brain tissue lysates from AD patients and DS abortuses as well as their age-matched controls. A synthetic peptide DCT with sequence RPKPIIQTTRPETYPHL5 corresponding to the C terminus of the human RCAN1 protein was used to immunize a rabbit, and a polyclonal antibody DCT3 was raised against the human RCAN1 protein C terminus. To characterize this RCAN1 antibody, HEK293 cells were transfected with empty vector or pRCAN1-mycHis plasmid. Fig. 1A shows that Myc-tagged RCAN1 proteins were detected by both mouse monoclonal 9E10 anti-Myc antibody and rabbit polyclonal antibody DCT3. Overexpressed RCAN1 protein could not be detected with the preimmunization serum pre-DCT. Furthermore, preincubation of DCT3 with excess DCT peptides resulted in clearance of RCAN1-specific antibody, and the precleared antibody could not detect RCAN1 protein (Fig. 1A). These data clearly demonstrate that our DCT3 antibody is able to specifically detect RCAN1 protein.

To examine the localization of RCAN1 protein expression in human brain, human hippocampal and cortical slices were immunostained with DCT3 antibody specifically targeting RCAN1. Immunohistochemistry studies show that DCT3 antibody specifically stains the granule cell layer in the hippocampus and the pyramidal neuronal layers in the cortex (Fig. 1B). RCAN1 expression is detected in both the nucleus and cytosol of neurons. Higher magnification reveals that RCAN1 is preferentially localized in nucleus of pyramidal neurons (Fig. 1B).
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These results show that RCAN1 is highly and specifically expressed in neurons in the hippocampus and cortex. Because neurons of the hippocampus and cortex are specifically affected in Alzheimer disease, the high and specific expression of RCAN1 in neurons of the hippocampus and cortex suggests that RCAN1 may be involved in AD pathogenesis.

RCAN1 proteins were examined in the brain tissue lysates from AD patients and DS abortuses and patients as well as their age-matched controls. Western blot analysis using DCT3 antibody showed that RCAN1 protein levels are elevated in brains from AD and DS patients. The result is consistent with previous reports (39, 40). Because nearly all DS patients will develop AD after their 30s, up-regulation of RCAN1 in both DS and AD suggests that RCAN1 may be involved in AD pathogenesis. Increased RCAN1 levels in DS may be attributed to its triplication on chromosome 21. However, the mechanism of RCAN1 up-regulation in AD is unknown.

Cloning and Functional Analysis of the Human RCAN1 Gene Promoter—To investigate the molecular mechanism by which RCAN1 gene expression is up-regulated in AD pathogenesis, we cloned and functionally analyzed a 2000-bp fragment of the 5′-flanking region of the first exon of the human RCAN1 gene.

The sequence was deposited to GenBank™ under accession number EF577083 (Fig. 2A). To determine the transcription start site of the human RCAN1 gene, a primer extension assay was performed. A 32P-labeled antisense primer (5′-tgctcagcagtctccagc-3′) located 302 bp upstream of the translational start site AUG was used to hybridize with neuronal RNA. The primer extension assay yielded a 45-bp major cDNA product. DNA sequencing gel analysis indicates that the major transcription start site is located at 348 bp upstream from the translational start site. Transcription starts with guanine, and this site was designated as +1 (Fig. 2B). A computer-based transcription factor binding site search revealed that this 2.0-kb 5′-flanking region contains several putative regulatory elements, such as AP1, AP2, GATA, OCT1, and USF (Fig. 2A).

To determine if the 5′-flanking fragment obtained from genomic DNA contains the promoter of the RCAN1 gene, we cloned the fragment into a promoterless plasmid vector, pGL3-Basic (Fig. 3A). The pGL3-Basic vector lacks eukaryotic promoter and enhancer sequences upstream of a reporter luciferase gene. Luciferase activity in cells transfected with this plasmid depends on insertion and proper orientation of a functional promoter upstream from the luciferase gene. Luciferase activity is indicative of promoter activity. pRCANluc-A plasmid was constructed to contain a 1.70-kb 5′-flanking region from −1651 to +45 bp of the RCAN1 gene upstream of the luciferase reporter gene. Plasmid DNA was transfected into HEK293 cells and SH-SY5Y cells, and luciferase activity was measured by a
luminometer to reflect promoter activity. Compared with an empty vector control, pRCANluc-A-transfected cells had significant luciferase activity at 554.2/11006 44.48 RLU in HEK293 and 461.6/11006 16.82 RLU in SH-SY5Y cells (p < 0.001 relative to controls) (Fig. 3, C and D, lane A and Basic). However, plasmid pRCANluc-R-I containing the RCAN1 promoter sequence from 1651 to 45 bp in the reverse orientation (Fig. 3C, lane F) and plasmid pRCANluc-F containing the sequence from 1651 to 686 bp lacking the transcription initiation site (Fig. 3C, lane I) have little or no luciferase activity. These results indicate that the 1.70-kb fragment contains the functional promoter of the human RCAN1 gene. To analyze the transcriptional activity of the RCAN1 promoter, a series of luciferase reporter gene plasmids containing different upstream deletions of the RCAN1 promoter were constructed and transfected into HEK293 and SH-SY5Y cells. Deletion of an additional 413 bp from 685 had no significant effect on the promoter activity in HEK293 cells. pRCANluc-C and pRCANluc-D had similar promoter activity to pRCANluc-B in HEK293 cells, 449.1/11006 33.45 and 442.5/11006 40.73 RLU, respectively (p < 0.05) (Fig. 3C, lane C and D). However, deletion of an additional 413 bp from 685 resulted in an increased promoter activity in SH-SY5Y cells from 352.2/11006 7.10 to 457.4/11006 8.75 RLU (p < 0.005) (Fig. 4D, lanes B and C). The promoter activ-
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FIGURE 3. Characterization of the RCAN1 promoter. A, schematic diagram of the promoter constructs. RCAN1 promoter deletion constructs were cloned into a promoterless vector, pGL3-Basic, in front of the reporter luciferase gene (Luc). The arrow denotes the direction of transcription. The numbers represent the end points of each construct. The corresponding deletion plasmids were confirmed by sequencing and restriction enzyme digestion. Digested samples were analyzed on a 1.0% agarose gel. The size of the vector is 4.7 kb, and the RCAN1 gene promoter activity, and dexamethasone had no effect on promoter activity. The values represent mean ± S.E. *(error bars) (n = 4).* p < 0.001 by analysis of variance with post hoc Newmann-Keuls test.

ities of pRCANLuc-C and pRCANLuc-A were not significantly different in SH-SY5Y cells (p > 0.05) (Fig. 3D). Further deletion of 40 bp from −312 reduced promoter activity to 353.0 ± 12.49 RLU in SH-SY5Y cells (p < 0.005, Fig. 3D lane D versus lane C). These data suggested that the RCAN1 promoter has cell-specific transcriptional activity.

To identify the minimal promoter region required for RCAN1 gene expression, additional deletion plasmids were constructed. Further deletion of 95 bp from −272 to −167 bp in pRCANLuc-D to −167 bp in pRCANLuc-E significantly reduced luciferase activity from 442.5 ± 40.73 to 170.7 ± 2.68 RLU in HEK293 cells (p < 0.0005) and from 353.0 ± 12.49 to 138.8 ± 3.61 RLU in SH-SY5Y cells (p < 0.0001) (Fig. 3, C and D). These results indicate that a 317-bp fragment from −272 to +45 bp contains the minimal human RCAN1 gene promoter.

The RCAN1 Promoter Contains a Glucocorticoid Response Element (GRE)—It was previously reported that RCAN1 mRNA was markedly increased by glucocorticoid induction in pre-B human leukemia cells (41). Neurons in hippocampus and cortex express high levels of glucocorticoid receptor (GR) (42). To investigate if glucocorticoids also activate the RCAN1 gene promoter, a GR expression plasmid was cotransfected with pRCANLuc-A into HEK293 cells, and the cells were subjected to 100 nM dexamethasone treatment for 0, 12, 24, 48, and 72 h. A luciferase assay showed that the RCAN1 gene promoter activity was significantly up-regulated in a time-dependent manner by dexamethasone, with 143.6 ± 10.63, 175.4 ± 13.38, 237.0 ± 19.95, and 306.1 ± 47.53% increases at 12, 24, 48, and 72 h (p < 0.0001) (Fig. 4A). Similar results were also observed in SH-SY5Y cells. Up-regulation of RCAN1 promoter activity by dexamethasone suggests that the RCAN1 promoter may contain a GRE.

To identify the GRE site in the RCAN1 promoter, we examined the effect of dexamethasone on the deletion constructs containing various RCAN1 promoter regions, including pRCANLuc-A, -B, -C, -D, and -E. pGL3-Basic and pGL3-Promoter, which do not contain any GRE sites, were used as negative controls. Luciferase activity in the cells transfected with pGL3-Basic and pGL3-Promoter plasmids was not affected by dexamethasone treatment (p > 0.05), whereas luciferase activity in cells transfected with pRCANLuc-A, -B, -C, and -D plasmids was markedly increased (Fig. 4B). Dexamethasone increased promoter activity by 254.45 ± 27.32, 183.48 ± 9.89, 213.32 ± 4.85, and 273.09 ± 16.28% in cells transfected with pRCANLuc-A, -B, -C, and -D plasmids, respectively (p < 0.001). However, further deletion of 95 bp from −272 to −167 bp in pRCANLuc-D to −167 bp in pRCANLuc-E abolished the up-regulatory effect of dexamethasone on RCAN1 promoter activity, and dexamethasone had no effect on RCAN1 promoter activity of pRCANLuc-E, 83.13 ± 14.81% of control (p > 0.05) (Fig. 4B, lane E). Thus, the results suggest that a GRE is located in the region of −272 to −167 bp in the RCAN1 gene promoter.

To further confirm that the −272 to −167 bp region of the RCAN1 promoter contains a GRE, gel shift assays were performed. Double-stranded oligonucleotide probes corresponding to −272 to −237 bp, −251 to −217 bp, −231 to −197 bp,
and −211 to −166 bp of the RCAN1 promoter were synthesized and end-labeled with $^{32}$P radioactive isotope. A shifted DNA-protein complex band was detected after hybridizing the $^{32}$P-labeled RCAN1−272−237bp probe with HeLa nuclear extract (Fig. 4C, lane 2) but not with other labeled oligonucleotide probes. These data suggest that the GRE site may be located in the −272 to −237 bp region of the RCAN1 promoter. To determine the binding specificity, GRE consensus oligonucleo-
tides (5'-agaggacctgtacagatgtctaga-3') were added to compete for GR binding with the labeled RCAN1-272-237bp probe. The binding intensity of this shifted band was partially reduced after adding a 10- or 50-fold molar excess of unlabeled GRE consensus competition oligonucleotides, and the shifted band was abolished by the addition of a 100-fold excess of GRE consensus oligonucleotides (Fig. 4C, lanes 3–5). These results confirm that the shifted band represents the complex of RCAN1-272-237bp oligonucleotide bound with GR, and the human RCAN1 promoter contains a GRE in the region of −272 to −237 bp.

To investigate if this element plays an important role in transcription of the human RCAN1 gene, the effect of dexamethasone on endogenous RCAN1 gene expression was examined. Quantitative RT-PCR was performed to amplify RCAN1-1 mRNA by a pair of gene-specific primers (5′-gccacattgaggaggtggacctg and 5′-tgctgagggagatgccgt). Dexamethasone treatment increased the RCAN1-1 mRNA level more than 5-fold in GR-transfected SH-SY5Y cells (p < 0.0001) (Fig. 4, D and E). To further confirm the regulatory effect of GRs on RCAN1 gene expression, endogenous levels of RCAN1 protein were examined with anti-RCAN1 antibody DCT3. Consistent with our data showing that dexamethasone up-regulated RCAN1-1 expression at the transcription level, endogenous RCAN1-1 protein level was also markedly elevated by dexamethasone treatment (225.49 ± 5.78% relative to control, p < 0.0001) (Fig. 4, F and G). Taken together, these results demonstrate that the RCAN1-1 gene promoter contains a functional GRE in the region of −272 to −237 bp, through which glucocorticoids exert an up-regulatory effect on RCAN1 gene expression.

RCAN1 has a tissue-specific expression pattern. The RCAN1 gene spans ~45 kb of genomic DNA and contains seven exons and six introns. Two major isoforms, RCAN1-1 and isoform 4 (RCAN1-4), are generated by alternative splicing of the first four exons. RCAN1-1 has a large form with 252 amino acids (RCAN1-1L) and a short form, RCAN1-1S. RCAN1-1S and RCAN1-4 both consist of 197 amino acids. The isoforms differ only at their N terminus, and the last 168 amino acids of the C terminus, encoded by exons 5–7, are the same in all forms of the isoforms. RCAN1-1 is highly expressed in the central nervous system (CNS), whereas RCAN1-4 is mostly expressed in heart muscle and fetal kidney (13, 43). It was reported that the calcineurin-dependent isoform 4 is also highly expressed in areas of the brain in which calcineurin is highly expressed (44, 45).

We have cloned a 1.7-kb region upstream of the first exon of the human RCAN1 gene containing a functional promoter that controls the transcription of RCAN1-1 (Fig. 2). It has been reported that there is an alternative promoter upstream of exon 4 of the RCAN1 gene, which responds to the calcineurin-NFAT signaling pathway (30, 46). Therefore, the RCAN1 gene contains two promoters and two translation initiation codons: one in the 5′-UTR of exon 1 and the other in the 5′-UTR of exon 4, which are responsible for transcriptional control of RCAN1-1 and RCAN1-4, respectively. To examine whether the effect of glucocorticoids is specific for the RCAN1 exon 1 promoter, a 1200-bp DNA fragment upstream of RCAN1 exon 4 was cloned into promoterless luciferase reporter plasmid pGL3-Basic to generate pDE4Luc. Dexamethasone markedly increased isoform 1 promoter activity (from 175.33 ± 3.74 to 268.86 ± 14.2 RU, p < 0.0001), whereas it had no effect on isoform 4 promoter activity (from 117.47 ± 2.51 to 112.17 ± 2.39 RU, p > 0.05) (Fig. 4H). The data suggest that glucocorticoids specifically up-regulate RCAN1-1 expression but not RCAN1-4 expression.

Overexpression of RCAN1 Induces Neuronal Apoptosis—Our results thus far have shown that RCAN1 expression can be activated by dexamethasone, which has been shown to induce apoptosis in primary neurons. Previous studies have shown that overexpression of RCAN1 deters melanoma tumor growth and suppresses metastasis in mice (29, 47, 48). Although muscle-specific overexpression of RCAN1 does not always result in embryonic lethality (49), there was a report that RCAN1 overexpression could lead to early embryonic fatality in transgenic mice (50), suggesting that RCAN1 may be proapoptotic. RCAN1 is preferentially localized in neurons of the hippocampus and cortex, where there is a marked neuronal loss in the brains of AD patients (38). To investigate whether RCAN1 overexpression could induce apoptosis or exacerbate neuronal death induced by apoptotic inducers, cDNA encoding for the RCAN1 isoform 1 was cloned into a Semliki Forest virus vector, pSFV, to generate pSFV-RCAN1. A green fluorescent protein vector, pSFV-GFP, was used as control. Rat E18 primary neurons were infected with SFV-RCAN1 or SFV-GFP. The infection efficiency is about 30–40%. A colorimetric MTT assay and Hoechst and TUNEL staining were performed to determine the cell viability of primary neurons. Hoechst staining revealed that there is more nuclear condensation and fragmentation in RCAN1-overexpressing neurons exposed to H2O2 (Fig. 5A, compare A2 with A1). The apoptotic features of RCAN1-overexpressing neurons with dexamethasone treatment were confirmed by TUNEL staining (Fig. 5A, A3–A8). Compared with SFV-GFP control, overexpression of RCAN1-1 renders more neuronal death, a 57.90 ± 5.31% apoptosis ratio relative to 33.87 ± 4.86% in control by Hoechst staining (p < 0.005) (Fig. 5B, lane 2 versus lane 1). RCAN1-1 overexpression also exacerbated the toxicity of H2O2 on neurons, 97.02 ± 0.20% compared with 70.18 ± 0.93% in control (p < 0.005) (Fig. 5B, lane 4 versus lane 3), and significantly increased the dexamethasone-induced neuronal apoptosis (80.37 ± 2.80%, compared with 53.81 ± 5.80% in control, p < 0.001) (Fig. 5B, lane 6 versus lane 5). The proapoptotic effect of RCAN1 was further confirmed by an MTT assay. RCAN1 overexpression resulted in a 54.01 ± 5.17% reduction in A540 nm relative to control (p < 0.0001) (Fig. 5C). To examine whether RCAN1 overexpression could further exacerbate neuronal death induced by Ab, a 10 μM concentration of aggregated Ab peptides was added to SFV-infected neurons for 12 h. Consistent with a previous report (51), Ab induced neuronal apoptosis, as indicated by A540 nm (69.11 ± 1.45% of control) in the Ab-treated neuronal culture (p < 0.001) (Fig. 5C, lane 3 versus lane 1). The addition of Ab to the RCAN1-1-overexpressing neurons further increased neuronal apoptosis, with the MTT assay showing a reduction to 26.39 ± 1.14% of control (p < 0.0001) (Fig. 5C, lane 4 versus lane 3). These data clearly indicate that RCAN1-1 facilitates neuronal apoptosis.
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Previous studies reported that dexamethasone can induce cortical neuronal death, an effect rescued by a caspase inhibitor (52). Our results also showed that exposure of RCAN1-1-overexpressing neurons to 1 μM dexamethasone rendered more neuronal death, as indicated by the MTT assay (Fig. 5A). To further determine if RCAN1 mediated apoptosis, RCAN1 expression was inhibited by using RCAN1-specific antisense oligonucleotides (5′-ezefgtcttgctcfzfog) (Fig. 5D). Knockdown of endogenous RCAN1 expression with the antisense oligonucleotides inhibited dexamethasone-induced neurotoxicity (100 ± 11.02% versus 83.50 ± 5.84% in controls, p < 0.0001) (Fig. 5E). Furthermore, endogenous RCAN1 knockdown was also able to rescue neurons from Aβ-induced neuronal death (100 ± 3.41% versus 86.90 ± 4.29% in controls, p < 0.01) (Fig. 5F) and H₂O₂-induced neuronal death (100 ± 8.73% versus 71.87 ± 2.76% in controls, p < 0.05) (Fig. 5G). These results demonstrate that RCAN1 exacerbates the neuronal apoptosis induced by dexamethasone, Aβ, and H₂O₂.

RCAN1 Activates the Caspase-3 Apoptotic Pathway—The mechanism by which RCAN1 overexpression induces neuronal apoptosis is unknown. The caspase family of proteins is the principal molecular machinery that executes apoptosis (53–56). Altered expression of apoptosis-related proteins, such as Par-4, Bak, Bad, Bax, Bcl-2, p53, caspase-3, and Fas, has been reported in AD brains (57–61). To investigate if the neuronal death induced by RCAN1 overexpression is mediated by the caspase signaling pathway, the caspase inhibitor benzylxycarbonyl-VAD-fluoromethyl ketone was added to primary neuronal cultures. The caspase inhibitor benzylxycarbonyl-VAD-fluoromethyl ketone blocked the neuronal apoptosis induced by SFV-RCAN1 (100.0 ± 2.30%, compared with 83.44 ± 1.60%, p < 0.0001) (Fig. 6A), suggesting that the caspase pathway is indeed involved in neuronal death induced by RCAN1 overexpression. Caspase-3 is a major executor of the caspase signaling pathway (62). To investigate whether caspase-3 activation is involved in the proapoptotic effect of RCAN1, the activities of caspase-3/7 in SFV-RCAN1- or SFV-GFP-infected primary neurons were measured by the Caspase-Glo® 3/7 assay. Overexpression of RCAN1 increased caspase-3/7 activity by 136.63 ± 6.4% relative to vector control (p < 0.0001); exposure to dexamethasone further up-regulated caspase-3/7 activity from 189.28 ± 10.12 to 218.41 ± 3.11% (p < 0.0001) (Fig. 6B). The results also indicate that dexamethasone could activate caspase-3/7 (189.28 ± 10.12% of control, p < 0.0001) (Fig. 6B, lane 3 versus lane 1). Activation of caspase-3 results in the cleavage of caspase-3 from its pro form to the P17 and P12 fragments. To further confirm that caspase-3 is activated by RCAN1 isoform 1 overexpression, Western blot assays were performed to detect the cleavage of caspase-3 in primary neurons infected by SFV-RCAN1 or SFV-GFP in the presence of Aβ and H₂O₂ treatment (Fig. 6B). Generation of the cleaved caspase-3 fragment P17 was markedly increased in RCAN1-1-overexpressing primary neurons compared with controls by 2.34 ± 0.01-fold in Aβ-treated neurons (p < 0.0001) and 8.20 ± 0.10-fold in H₂O₂-treated neurons (p < 0.0001) (Fig. 6C). In the absence of insults, the caspase-3 cleavage was not detectable by Western blot, probably due to the low activity of the caspase pathway (data not shown). These data demonstrate that RCAN1 overexpression...
activates the caspase-3 signaling pathway, thereby inducing neuronal apoptosis.

To further confirm the involvement of caspase-3 in RCAN1-mediated neuronal apoptosis, primary neuron cultures were derived from caspase-3 knock-out (caspase-3−/−) and wild type newborn mice (32). Caspase-3−/− and wild type neurons were infected with SFV-RCAN1 and further subjected to dexamethasone treatments. TUNEL staining showed markedly reduced neuronal apoptosis in neurons derived from the caspase-3−/− mice compared with neurons derived from wild type mice (0.60 ± 0.59% apoptosis ratio in caspase-3−/− mice compared with 23.91 ± 3.48% in wild type mice (p < 0.0001)) (Fig. 6, E (E1 and E2) and F). Similar results were observed in the presence of dexamethasone treatments (1.36 ± 0.67% versus 27.99 ± 2.39%, p < 0.0001) (Fig. 6, E (E3 and E4) and G). Furthermore, an MTT assay showed that the neuronal death induced by RCAN1 isoform 1 overexpression was abolished in caspase-3−/− primary neurons with or without dexamethasone treatment (Fig. 6H). These data demonstrate that disruption of the caspase-3 gene blocks dexamethasone-induced and RCAN1-mediated neuronal apoptosis in caspase-3−/− mice, and furthermore, caspase-3 is required for dexamethasone-induced and RCAN1-mediated neuronal apoptosis.

RCAN1 Overexpression Activates Caspase-9—Our data suggested that overexpression of RCAN1 isoform 1 can activate
Caspase-9 is another upstream initiator of caspase-3 and mainly associated with inflammation in murine (64, 65). Caspase-11 and caspase-3 activation have been observed in the spinal cord of mice with amyotrophic lateral sclerosis (66). To test if the activation of caspase-9 and caspase-3 by RCAN1 has a specific effect or if it has a panactivation effect on all of the caspase families, caspase-11 was examined in rat glial C6 cells transduced with SFV-GFP and SFV-RCAN1. Spleen tissue lysates derived from mice treated with LPS (40 mg/kg) were used as caspase-11 protein markers (67). A monoclonal anti-caspase-11 antibody was used to detect caspase-11, and our study showed no significant difference in SFV-GFP- and SFV-RCAN1-transduced C6 cells (data not shown). These results suggest that overexpression of RCAN1 can specifically activate caspase-9 and caspase-3 but not caspase-11.

Activation of caspase-9 requires cytochrome c release from the mitochondria. To investigate whether RCAN1 overexpression affects cytochrome c release, a Western blot assay was used to examine cytochrome c levels in cytosolic and mitochondrial fractions (Fig. 7D). RCAN1-1 overexpression in HEK293 cells resulted in significantly reduced levels of cytochrome c in the mitochondria and markedly increased levels in the cytosol ($p < 0.01$) (Fig. 7E). Taken together, our data suggest that the cytochrome c, caspase-9, and caspase-3 signaling pathway is activated by RCAN1-1 overexpression and mediates neuronal apoptosis induced by RCAN1-1 overexpression.

**DISCUSSION**

Prominent neuronal death, neuritic plaques, and neurofibrillary tangles are neuropathological hallmarks in AD brains (68). The neuronal loss is closely correlated to memory impairment in AD patients. Most studies in AD have been focused on the initial steps leading to neuritic plaque and neurofibrillary tangle formation in AD brains, whereas the mechanism underlying neuronal death still remains elusive. RCAN1 has been shown to play an important role in memory and learning. Although disruption of RCAN1 in mice does not show overt abnormalities in vivo (69), both disruption and overexpression of *nebula*, a RCAN1 ortholog in *Drosophila*, lead to severe learning and memory deficits (70). RCAN1 is highly expressed in neurons in the brain and is overexpressed in the brains of AD and DS patients (38, 40, 43). Recently, we reported that degradation of RCAN1 is mediated by both the chaperone-mediated autophagy and ubiquitin proteasome pathways, and alteration of the autophagy and ubiquitin proteasome pathways could result in dysfunction of RCAN1 signaling (71). Knock-out of RCAN1 in mice resulted in increased enzymatic calcineurin activity and cleaved calcineurin fragments and caused learning and memory deficits and impaired late phase long term potentiation (45). The effect of RCAN1 on AD and DS pathogenesis is unknown.

Epidemiological studies have revealed that risk factors for AD, including aging, atherosclerosis, stroke, and diabetes, will induce stress and increase oxidative free radicals in the brain, resulting in neuronal death in patients. Furthermore, hippocampal atrophy and severity of cognitive impairment in AD patients are correlated with dysfunctions of the HPA axis (72). Corticosteroids can facilitate neuronal apoptosis in the hip-
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DS patients show AD-associated neuropathological changes in their 30s, which is 20–30 years earlier than sporadic AD cases (80). We previously reported that Aβ is abnormally elevated in brains of DS patients (81). In addition to overproduction and accumulation of Aβ, our current study implies that RCAN1, a gene localized on chromosome 21 and overexpressed in DS, may further contribute to the early onset of AD pathogenesis in DS due to its proapoptotic effect. Abnormal autophagy lysosome and ubiquitin proteasome pathways, resulting in protein accumulation, have been implicated in AD pathogenesis, and our recent report showed that RCAN1 degradation is mediated by both chaperone-mediated autophagy and ubiquitin proteasome pathways (71). Overexpression of RCAN1, due to its triplication in DS, can render neurons more vulnerable to stress and neurotoxic insults leading to hippocampal and cortical neuronal loss. We have demonstrated that Aβ further increases neuronal apoptosis in RCAN1-overexpressing neurons. AD pathology is characterized by a specific demise of hippocampal and cortical neurons; however, how the specific population of cells is affected remains elusive. Our immunohistochemistry data showed that RCAN1 is specifically expressed in cortical and hippocampal neurons. The specific expression pattern of RCAN1 may explain the particular population of neuronal death in AD because RCAN1 overexpression in neurons leads to neuronal death. Our study provides a novel mechanism by which RCAN1 functions as a mediator of stress and Aβ-induced neuronal death, and overexpression of RCAN1 due to an extra copy of the RCAN1 gene on chromosome 21 contributes to AD pathogenesis in DS. Our study suggests that inhibition of RCAN1 signaling may have pharmaceutical potential for reducing neuronal loss and treating cognitive impairments, thereby benefiting AD and DS patients.

It has been reported that the RCAN1 isoform 4 promoter can be regulated by the calcineurin-NFAT signaling pathway, thus forming a negative feedback loop in RCAN1 isoform 4 gene regulation because RCAN1 can inhibit calcineurin activity (14). The negative feedback loop in RCAN1 gene regulation implies that tight regulation of RCAN1 is vital to cell survival. The negative loop may also serve a self-protective function in cells by way of preventing RCAN1 overexpression, leading to cell demise. Previous studies showed that RCAN1 physically binds to calcineurin and inhibits its function (15, 17, 24, 82). Activation of caspase-3 by RCAN1 is probably not due to this known function because previous studies show that calcineurin overexpression induces caspase-3 activation and apoptosis (83, 84). Calcineurin promotes apoptosis via BAD dephosphorylation, and there is also evidence indicating that calcineurin inhibitors such as FK506 are neuroprotective (85, 86). This suggests that the proapoptotic effect of RCAN1 is independent of its function of inhibiting calcineurin and that RCAN1 is a multifunctional protein. A previous report showed that nebula, the RCAN1 homolog in Drosophila, is crucial for mitochondrial integrity and function (87). Our fractionation and confocal microscopy imaging data indicate that RCAN1 is localized in cytosol, mitochondria, and nucleus, suggesting that RCAN1 is a multifunctional protein. The mitochondrial localization of RCAN1 may contribute to its proapoptotic effect. The underlying mechanism by which RCAN1 overexpression triggers cytochrome c release and initiates cell death remains unknown. Future studies will elucidate the involvement of RCAN1 in the mitochondrial apoptotic pathway.

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Regulator of Calcineurin 1 (RCAN1) Facilitates Neuronal Apoptosis through Caspase-3 Activation

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