Cyclic AMP-dependent Protein Kinase Regulates the Alternative Splicing of Tau Exon 10

A MECHANISM INVOLVED IN TAU PATHOLOGY OF ALZHEIMER DISEASE

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Hyperphosphorylation and deposition of tau into neurofibrillary tangles is a hallmark of Alzheimer disease (AD). Alternative splicing of tau exon 10 generates tau isoforms containing three or four microtubule binding repeats (3R-tau and 4R-tau), which are equally expressed in adult human brain. Dysregulation of exon 10 causes neurofibrillary degeneration. Here, we report that cyclic AMP-dependent protein kinase, PKA, phosphorylates splicing factor SRSF1, modulates its binding to tau pre-mRNA, and promotes tau exon 10 inclusion in cultured cells and in vivo in rat brain. PKA-Cα, but not PKA-Cβ, interacts with SRSF1 and elevates SRSF1-mediated tau exon 10 inclusion. In AD brain, the decreased level of PKA-Cα correlates with the increased level of 3R-tau. These findings suggest that a down-regulation of PKA dysregulates the alternative splicing of tau exon 10 and contributes to neurofibrillary degeneration in AD by causing an imbalance in 3R-tau and 4R-tau expression.

Tau is a neuronal microtubule-associated protein, the function of which is to stimulate microtubule assembly and stabilize microtubules. Hyperphosphorylation of tau leads to its aggregation into neurofibrillary tangles, a hallmark of Alzheimer disease (AD) and related neurodegenerative diseases called tauopathies (1–4). Adult human brain expresses six different tau isoforms from a single gene by alternative splicing of its pre-mRNA (5). The exon 10 encodes the second microtubule binding repeat (6). Alternative splicing of exon 10 generates tau with three or four microtubule binding repeats (3R-tau or 4R-tau), which is under developmental and cell type-specific regulation. Only 3R-tau is expressed during embryogenesis, whereas 3R-tau and 4R-tau are expressed in approximately equal amounts in adult human brain (6, 7). Several mutations in tau gene result in either an increase or a decrease in 4R-tau expression and cause frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), one of the tauopathies (8). Thus, alteration in the 3R-tau/4R-tau ratio is sufficient to trigger neurodegeneration in frontotemporal dementia and might also play a role in other neurodegenerative disorders such as Pick’s disease, progressive nuclear palsy, or corticobasal degeneration in which the 3R-tau/4R-tau ratio is markedly altered (9–12). Thus, the regulation of alternative splicing of human tau exon 10 has been of critical interest. However, results of studies of the alternative splicing of tau exon 10 in AD brain have been contradictory (13–15). Recent studies have shown that aggregation and deposition of 3R-tau may be associated with more advanced stages (16, 17).

Alternative splicing of tau exon 10 is regulated by several trans-acting factors, including serine- and arginine-rich (SR) proteins, and their phosphorylation (18–24). Splicing factor 2/alternative splicing factor (ASF/SF2), now named SRSF1 (serine/arginine-rich splicing factor 1) (25), is a prototypic SR protein that participates in both constitutive and alternative splicing (26). SRSF1 acts on a polypurine enhancer (PPE) of exon 10 located at tau exon 10 and plays essential and regulatory roles in the alternative splicing of tau exon 10 (24). Overexpression of SRSF1 promotes exon 10 inclusion (20, 24, 27). SRSF1 contains two copies of an N-terminal RNA recognition motif (RRM) and a C-terminal RS domain. The serine residues of the RS domain are targets of phosphorylation by multiple kinases, including SRPK1 (28), SRPK2 (29), Cik/Sty (30), DNA topoisomerase I (31), AKT (32), and Dyrk1A (37). Phosphorylation of SRSF1 regulates its translocation between the cytoplasm and the nucleus or within the nucleus and affects its function (27, 28, 30, 33–36).

Cyclic AMP (cAMP)-dependent protein kinase, PKA, has emerged as a key kinase that is able to interact with many of the proteins involved in the etiology of AD as well as other tauopathies. It has been shown that PKA phosphorylates tau at several sites and primes phosphorylation of tau by glycogen synthase kinase-3β (37). PKA is a tetrameric holoenzyme consisting of two catalytic (C) subunits and two regulatory (R) subunits in the absence of cAMP. Stimulation by cAMP dissociates the holoenzyme and causes translocation to the nucleus of a fraction of the C subunit. Apart from regulation of transcription, little is known about the role of PKA in the regulation of tau expression and alternative splicing. In this study, we have demonstrated that PKA, which is known to be strongly increased in AD brain (38), regulates the alternative splicing of tau exon 10 in cultured cells and in vivo in rat brain. PKA-Cα, but not PKA-Cβ, interacts with SRSF1 and elevates SRSF1-mediated tau exon 10 inclusion. In AD brain, the decreased level of PKA-Cα correlates with the increased level of 3R-tau. These findings suggest that a down-regulation of PKA dysregulates the alternative splicing of tau exon 10 and contributes to neurofibrillary degeneration in AD by causing an imbalance in 3R-tau and 4R-tau expression.

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known about the function of the C subunit in the nucleus. It is known that PKA phosphorylates several splicing factors and is involved in the pre-mRNA splicing (30, 38, 39). We recently found that in AD brain, the activity of PKA is down-regulated as a result of proteolysis of the regulatory subunit by over-activated calpain I (40). However, the role of PKA in the alternative splicing of tau exon 10 was unclear. In the present study we demonstrate that PKA phosphorylates SRSF1 and thereby enhances the inclusion of tau exon 10 and that down-regulation of PKA in AD brain correlates with increase in 3R-tau expression. These results suggest that PKA is involved in the tau pathology in AD via regulation of tau exon 10 splicing.

EXPERIMENTAL PROCEDURES

Human Brain Tissue—Medial frontal cortices from 15 AD and 15 age-matched normal cases used for this study (Table 1) were obtained from the Sun Health Research Institute Donation Program (Sun City, AZ); all cases were histopathologically confirmed. The tissue was stored at −70 °C until used. The use of frozen human brain tissue was in accordance with the National Institutes of Health guidelines and was approved by our institutional review committee.

Plasmids, Antibodies, and Other Reagents—The expression constructs for human PKA-Cα and PKA-Cβ were generated by reverse transcription PCR from RNA isolated from normal human neuronal progenitor cells and confirmed by DNA sequence analysis. Both PKA-Cα and PKA-Cβ tagged with HA were cloned into pCI-Neo vector via Sall and NotI sites. pCEP4-SRSF1-HA was a gift from Dr. Tarn of the Institute of Biomedical Sciences, Academia Sinica, Taiwan. pEGFP-N1-SRSF1 was generated by inserting the cDNA of SRSF1 without a stop code into pEGFP-N1. pGEX-2T-SRSF1, pGEX-2T-SRSF1ΔRS2, pGEX-2T-SRSF1ΔRS3, pGEX-2T-SRSF1ΔRRM, and pGEX-2T-SRSF1ΔRS5 were constructed by PCR amplification from pCEP4-SRSF1-HA and subcloning into pGEX-2T to express GST fusion proteins of SRSF1 and its deletion mutants. For mammalian vectors, SRSF1 mutants were cloned into pCI-Neomax vector via Sall and NotI sites. pCEP4-SRSF1-HA was a gift from Dr. Tarn of the Institute of Biomedical Sciences, Academia Sinica, Taiwan. pEGFP-N1-SRSF1 was generated by inserting the cDNA of SRSF1 without a stop code into pEGFP-N1. pGEX-2T-SRSF1, pGEX-2T-SRSF1ΔRS2, pGEX-2T-SRSF1ΔRS3, pGEX-2T-SRSF1ΔRRM, and pGEX-2T-SRSF1ΔRS5 were constructed by PCR amplification from pCEP4-SRSF1-HA and subcloning into pGEX-2T to express GST fusion proteins of SRSF1 and its deletion mutants. For mammalian vectors, SRSF1 mutants were constructed by digestion of those SRSF1 mutants in pGEX-2T vector and inserting them into pCEP4 to generate pCEP4-SRSF1 deletion mutants tagged with HA. pCI-S19/L1101 containing a tau minigene, S19/L1101, comprising tau exons 9, 10, and 11 and part of intron 9 and the full-length of intron 10, was a gift from Dr. Jianhua Zhou of the University of Massachusetts Medical School. pFC-CRE-luciferase was bought from Stratagene (La Jolla, CA). The catalytic subunit of PKA, the monoclonal anti-HA, anti-PKA-C, and anti-PKA-C/β were obtained from Sigma. Monoclonal anti-3R-tau (RD3) and anti-4R-tau (RD4) were from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-human tau (43D2) and polyclonal anti-tau (R134d) were described previously (41). Polyclonal Anti-PKA-Cα, polyclonal anti-PKA-Cβ, and monoclonal anti-SRSF-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated anti-mouse and anti-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA); Alexa 488-conjugated goat anti-mouse IgG, and Alexa 555-conjugated goat anti-rabbit IgG and TO-PRO-3 iodide (642/661) were from Invitrogen (Invitrogen). The ECL kit was from Thermo Fisher Scientific (Rockford, IL), and [γ-32P]ATP and [32P]orthophosphate were from MP Biomedicals (Irvine, CA). The dual-luciferase reporter assay system was purchased from Promega (Madison, WI).

Cell Culture and Transfection—COS-7, HEK-293T, HEK-293FT, HeLa, and CHO cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C. All transfections were performed in triplicate with FuGENE 6 (Roche Diagnostics) in 12-well plates. The cells were transfected with FuGENE 6 for 48 h according to the manufacturer’s instructions.

In Vitro Phosphorylation of SRSF1 by PKA—GST-SRSF1, GST-SRSF1 mutants, or as a control, GST (0.2 mg/ml) was incubated with various concentrations of PKA catalytic subunit in a reaction buffer consisting of 50 mM HEPES, pH 6.8, 10 mM β-mercaptoethanol, 10 mM MgCl2, 1.0 mM EGTA, and 0.2 mM [γ-32P]ATP (500 cpm/pmol). After incubation at 30 °C for 30 min, the reaction was stopped by boiling with an equal volume of 2× Laemmli sample buffer. The reaction products were separated by SDS-PAGE. Incorporation of 32P was detected by exposure of the dried gel to phosphor-image system.

Phosphorylation of SRSF1 in Cultured Cells—CHO cells were transfected with pCEP4-SRSF1-HA and cultured in DMEM supplemented with 10% fetal bovine serum. After 45 h of transfection, the medium was replaced with [32P]orthophosphate (10 μCi) in DMEM (without phosphate) supplemented with 10% fetal bovine serum. After a 3-h incubation, the cells were harvested in lystate buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM Na2VO4, 50 mM okadaic acid, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.25% sodium deoxycholate, 2 mM EDTA, 1 mM PMSF, and 10 μg/ml of aprotinin, leupeptin, and pepstatin). Insoluble materials were removed by centrifugation, and the supernatant was incubated with anti-HA pre-coupled to protein G-conjugated agarose for 4 h at 4 °C. After being washed with TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), the immunoprecipitated SRSF1-HA by anti-HA was analyzed by immunoblotting and autoradiography.

GST Pulldown of PKA by SRSF1—GST, GST-SRSF1, and GST-SRSF1 deletion mutants were purified by affinity purification with glutathione-Sepharose but without elution from the beads. These beads coupled with GST, GST-SRSF1, and GST SRSF1 deletion mutants were incubated with crude extract from rat brain homogenate in buffer (50 mM Tris-HCl, pH 7.4, 8.5% sucrose, 50 mM NaF, 1 mM Na2VO4, 0.1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). After 4 h of incubation at 4 °C, the beads were washed with washing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM DTT) 6 times, the bound proteins were eluted by boiling in Laemmli sample buffer, and the samples were subjected to Western blot analysis.

Co-immunoprecipitation of PKA by SRSF1—HEK-293FT cells were transfected with pCEP4-SRSF1-HA for 40 h as described above and treated with 10 μM forskolin for 8 h, and then the cells were washed twice with PBS and lysed by sonication in lystate buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM Na2VO4, 2 mM EDTA, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin). The cell lysate was centrifuged at 16,000 × g for 10 min and incubated
with anti-HA antibody overnight at 4 °C, and then protein G beads were added. After 4 h of incubation at 4 °C, the beads were washed with lysis buffer twice and with TBS twice, and bound proteins were eluted by boiling in Laemmli sample buffer. The samples were subjected to Western blot analysis with the indicated primary antibodies.

Co-localization of PKA with SRSF1—HeLa cells were plated in 24-well plates onto coverslips 1 day before transfection at 50–60% confluence. The cells were then transfected with pEGFP-N1-SRSF1 as described above. After 40 h of transfection, the cells were treated with 10 μM forskolin for 30 min to activate PKA, and then the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After washing with PBS, the cells were blocked with 10% goat serum in 0.2% Triton X-100, PBS for 2 h at 37 °C and incubated with mouse anti-PKA-α (1:50) overnight at 4 °C. The cells were then washed and incubated for 1 h with secondary antibody (Alexa 488-conjugated goat anti-mouse IgG, 1:1000) plus TO-PRO-3 iodide at room temperature. The cells were washed with PBS, mounted with Fluromount-G, and observed with a Nikon TCS-SP2 laser-scanning confocal microscope.

Knockdown of SRSF1 or PKA Catalytic Subunits, Ca and Cb, with RNA Interference—For inhibition of SRSF1 expression, HEK-293T cells cultured in 12-well plates were transfected with various amounts of short interfering RNA (siRNA) using Lipofectamine 2000. After 48 h transfection, cells were lysed, and protein and RNA were extracted as described above. siRNA is a pool of 3 target-specific 20–25-nucleotide siRNAs to knock down target gene expression (Santa Cruz Biotechnology). Both strands of siRNAs had 3′-dTdT tails. The same amount of scramble siRNA was used for controls.

Quantitation of tau Exon 10 Splicing by Reverse Transcription-PCR (RT-PCR)—Total cellular RNA was isolated from cultured cells by using the RNeasy Mini Kit (Qiagen, GmbH, Germany). One microgram of total RNA was used for first-strand cDNA synthesis with oligo-(dT)15–18 by using the Omniscript Reverse Transcription kit (Qiagen). PCR was performed by using PrimeSTAR™ HS DNA Polymerase (Takara Bio Inc., Otsu, Shiga, Japan) with forward primer 5′-GGTGTTCCACCTC-CAGTTCA-3′ and reverse primer 5′-CCTGTTTTA-TGATGGATGTGCCTAATGAG-3′ to measure alternative splicing of tau exon 10. The PCR conditions were: 94 °C for 3 min, 98 °C for 10 s, and 68 °C for 40 s for 30 cycles and then 68 °C 10 min for extension. The PCR products were resolved on 1.5% agarose gels and quantitated using the Molecular Imager system (Bio-Rad).

Electrophoretic Mobility Shift Assay (EMSA)—The RNA oligonucleotides of tau cover PPE at the tau exon (5′-GGTGTTCCACCTC-CAGTTCA-3′), tau-RNA (5′-GGTGTTCCACCTC-CAGTTCA-3′), tau-RNA (5′-GGTGTTCCACCTC-CAGTTCA-3′), and deleted SC35-like element (5′-GGTGTTCCACCTC-CAGTTCA-3′) was labeled with [γ-32P]ATP (3000 Ci/mm, Amersham Biosciences) using T4 polynucleotide kinase (New England Biolabs) and subsequently purified with MicroSpin G-25 column (Amersham Biosciences). To perform the EMSA assay, we immunoprecipitated SRSF1-HA from HEK-293T cells transfected with pCEP4-SRSF1 by using anti-HA cross-linked to protein G-agarose with a Seize X Protein G affinity kit. The immunopurified SRSF1 in 50 mm Tris–HCl buffer, pH 7.5, containing 50 mm NaCl, 1 mm EDTA, and 1 mm dithiothreitol was mixed with [32P]-labeled tau-RNA or tau-RNA (5′-GGTGTTCCACCTC-CAGTTCA-3′), and primer set 2 (forward 5′-ATGCCCTTACAGCACCCCCTCTATCC-3′) and reverse (5′-AGCGGGATCCGGCTTGCCTACCTAC-3′). The PCR products were resolved in 2.0% agarose gels.

Intracerebroventricular Injection—Sprague-Dawley rats at postnatal day 10 (P10) were first anesthetized by wrapping in ice pack for 5 min, and then 2 μl of 1 mm forskolin in artificial CSF was injected into the left lateral ventricle of the brain at around 2.5 mm depth. The control animals were treated identically but with vehicle only. Rats were killed 3 days after injec-
tion. The brains were immediately removed and processed for measuring the tau exon 10 splicing by RT-PCR.

Statistical Analysis—Where appropriate, the data are presented as the means \pm S.D. Data points were compared by the unpaired two-tailed Student’s t test, and the calculated p values are indicated in the figures. For analysis of the correlation between levels of PKA-C and 3R-tau or 4R-tau in human brain homogenates, the Pearson product-moment correlation coefficient r was calculated.

RESULTS

Activation of PKA Promotes Tau Exon 10 Inclusion in HEK-293T Cells and in Rat Brain—To elucidate the regulation of the alternative splicing of tau exon 10 by PKA, we transfected mini-tau gene pCI/S19/L10 into HEK-293T cells and treated them with 10 \( \mu M \) forskolin for 8 h. The cell lysates were analyzed for phosphorylation of CREB at Ser-133 and total CREB by Western blots or luciferase activity with a commercial assay system (Promega). b and c, promotion of tau exon 10 inclusion by forskolin is shown. HEK-293T cells transfected with pCI-S19/L10 mini-tau gene were treated with 10 \( \mu M \) forskolin for various times (b) or with various concentrations of forskolin for 8 h (c). The total RNA was then extracted and subjected to RT-PCR to measure the tau exon 10 splicing. Results represent the mean \pm S.D.

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PKA-C\( \alpha \) and C\( \beta \) of PKA (40, 43). PKA-C\( \beta \) is expressed in testis only (44). To understand which isoform of PKA regulates tau exon 10 splicing, we co-transfected PKA-C\( \alpha \) or -C\( \beta \) with mini-tau gene pCI/S19/L10 into HEK-293FT cells and analyzed the tau exon 10 splicing. We found that overexpression of PKA-C\( \alpha \) significantly promoted tau exon 10 inclusion, but overexpression of PKA-C\( \beta \) slightly inhibited tau exon 10 inclusion (Fig. 1d). Knock-down of PKA-C\( \alpha \) expression (supplemental Fig. 1) did not affect the alternative splicing at basal level but significantly inhibited forskolin-promoted tau exon 10 inclusion (Fig. 1e). Down-regulation of PKA-C\( \beta \) by siRNA (supplemental Fig. 1) increased the tau exon 10 inclusion at basal level but did not influence forskolin-promoted tau exon 10 splicing. We found that overexpression of PKA-C\( \alpha \) significantly promoted tau exon 10 inclusion, but overexpression of PKA-C\( \beta \) slightly inhibited tau exon 10 inclusion (Fig. 1f). Knock-down of PKA-C\( \alpha \) expression (supplemental Fig. 1) did not affect the alternative splicing at basal level but significantly inhibited forskolin-promoted tau exon 10 inclusion (Fig. 1f). Down-regulation of PKA-C\( \beta \) by siRNA (supplemental Fig. 1) increased the tau exon 10 inclusion at basal level but did not influence forskolin-promoted tau exon 10 inclusion (Fig. 1f). To determine that overexpression of PKA-C\( \alpha \) significantly decreased PKA activity, and overexpression of PKA-C\( \alpha \) or PKA-C\( \beta \) increased PKA activity, as determined by phosphorylation of CREB (supplemental Fig. 2). Taken together, these...
results suggest that PKA-Cα promotes and PKA-Cβ suppresses tau exon 10 inclusion and that promotion of forskolin on tau exon 10 inclusion is dependent on PKA-Cα but not PKA-Cβ.

Adult murine brain only expresses 4R-tau, and both 3R-tau and 4R-tau are expressed during P5 to P30 (supplemental Fig. 3). To determine the correlation of developmental expressions of PKA-Cα and tau isoforms, we measured the level of PKA-Cα in the rat brain, and we found that PKA-Cα expression coincided with the expression of 3R-tau and 4R-tau (supplemental Fig. 3). To test whether PKA regulates the alternative splicing of tau exon 10 in vivo, we treated forskolin (2 μl of 1 mm) into the rat left lateral ventricle at p10 and then measured the tau exon 10 splicing by RT-PCR 3 days after the injection. We detected a significant increase of 4R-tau expression in forskolin-treated rats (Fig. 1f), which provides evidence that PKA regulates the alternative splicing of tau exon 10 in vivo and activation of PKA enhances tau exon 10 inclusion.

PKA Enhances SRSF1-mediated Tau Exon 10 Inclusion—Splicing factor SRSF1 plays a critical role in the alternative splicing of tau exon 10. Overexpression of SRSF1 promoted tau exon 10 inclusion in a time-dependent manner (supplemental Fig. 4). To determine whether PKA modulates SRSF1-promoted tau exon 10 inclusion, we treated HEK-293T cells with 10 μM forskolin for 8 h and then measured exon 10 splicing. We observed that treatment with forskolin as well as SRSF1 overexpression promoted tau exon 10 inclusion, and forskolin treatment further enhanced SRSF1-promoted tau exon 10 inclusion (Fig. 2a). In contrast, knockdown of the expression of SRSF1 (supplemental Fig. 5) by siRNA suppressed tau exon 10 inclusion and eliminated the effect of forskolin on tau exon 10 inclusion (Fig. 2b). These findings suggest that PKA modulates SRSF1-promoted tau exon 10 inclusion.

To determine the isoform-specific role of PKA catalytic subunits on SRSF1-mediated tau exon inclusion, we overexpressed PKA-Cα and -Cβ in pCEP4/SRSF1-transfected HEK-293FT cells and determined tau exon 10 splicing. Consistent with the findings shown in Fig. 1e, we found that PKA-Cα, but not PKA-Cβ, further enhanced the SRSF1-promoted tau exon 10 inclusion (Fig. 2c).

To confirm the isoform-specific promotion of PKA-C on SRSF1-mediated tau exon 10 inclusion, we knocked down the expression of PKA-Cα or PKA-Cβ in SRSF1-expression cells by RNA interference and measured the splicing products of tau exon 10. Overexpression of SRSF1 promoted tau exon 10 inclusion. Similar to the results in Fig. 1e, down-regulation of PKA-Cα did not affect the alternative splicing of tau exon, but down-regulation of PKA-Cβ increased tau exon 10 inclusion. Knockdown of the PKA-Cα, but not PKA-Cβ, reduced the tau exon 10 inclusion by SRSF1 (supplemental Fig. 6a).

To determine whether the promotion of tau exon 10 inclusion by forskolin is also isoform-specific, we knocked down the expression of PKA catalytic subunits (supplemental Fig. 1) and then treated cells with forskolin. We found that an increase of tau exon 10 inclusion by forskolin treatment was diminished by siPKA-Cα but not by siPKA-Cβ (supplemental Fig. 6b). Further enhancement of SRSF1-promoted tau exon 10 inclusion by forskolin treatment was also suppressed by siPKA-Cα but not siPKA-Cβ (supplemental Fig. 6b). These results further support that PKA-Cα, but not PKA-Cβ, promotes SRSF1-mediated tau exon 10 inclusion.

Alternative splicing is regulated by splicing factors and their phosphorylation, which results from the relative activity of the kinases and phosphatases. Cells from different sources and spe-
cies and at different culture conditions have different basal activity of splicing factors or their regulators, resulting in the different splicing patterns in basal condition (Fig. 1, a, b, and c, and Fig. 2, a, b, and c) (27). Nevertheless, SRSF1 or PKA promotes tau exon 10 inclusion consistently.

SRSF1 promotes tau exon 10 inclusion by acting on the PPE at exon 10 (24). To confirm SRSF1 acting on PPE, we overexpressed SRSF1-HA in the HEK-293FT cells and immunopurified SRSF1 with anti-HA cross-linked to protein G-agarose. We got two elution fractions, E1 and E2, by elution buffer. We found that E1 had slower mobility than E2 on the SDS-PAGE, suggesting that phosphorylation of SRSF1 in E1 was higher than in E2 (Fig. 2d, left). Then, we incubated the purified SRSF1 with 32P-labeled partial tau exon 10 RNA oligonucleotides and carried out EMSA. We found that SRSF1 in E1, but not in E2, made tau RNA oligonucleotides shift up (Fig. 2d, right), suggesting that binding of SRSF1 requires certain phosphorylation.

To determine whether SRSF binds onto PPE, immunopurified SRSF1 was incubated with a 32P-labeled part of tau exon 10 RNA oligonucleotides (tau-RNA) containing SC35-likes and PPE elements. We found that SRSF1 slowed the mobility shift of tau-RNA (Fig. 2e). However, deletion of PPE, but not SC35-like element, abolished the mobility shift (Fig. 2e), suggesting SRSF1 binds to the PPE of tau exon 10 mRNA.

To elucidate whether PKA modulates the binding of SRSF1 to tau exon 10, we performed, using anti-HA, RNA immunoprecipitation from pCEP4-SRSF1 and pCI/SI9-L110 co-transfected HEK-293FT cells and amplified the precipitated RNA with RT-PCR by using two sets of primers (Fig. 2f). We found that treatment with forskolin increased the level of tau pre-mRNA co-immunoprecipitated with HA-SRSF1 by anti-HA (Fig. 2f), suggesting that PKA activation enhances the binding of SRSF1 to exon 10 of tau.

PKA Phosphorylates SRSF1 in Vitro and in Cultured Cells—The biological function of SRSF1 is highly regulated by its phosphorylation (23, 27, 34). To test whether PKA phosphorylates SRSF1, we incubated GST-SRSF1 with PKA in vitro. We observed that GST fused with different deletion mutants of SRSF1 were incubated with PKA in vitro for 30 min at 30 °C. 32P incorporation into GST-SRSF1 mutants was measured by autoradiography after the separation of the phosphorylation products by SDS-PAGE. Quantitation of the 32P incorporation after being normalized by the protein level is shown in the bar graph. Results represent the mean ± S.D.; *p < 0.05; **, p < 0.01.

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FIGURE 3. PKA phosphorylates SRSF1 in vitro and in cultured cells. a, recombinant GST-SRSF1 or GST was incubated with various concentrations of PKA in the presence of [32P]ATP at 30 °C for 30 min, and the reaction mixture was then separated by SDS-PAGE and visualized with Coomassie blue staining (lower panel) or autoradiograph (upper panel). Quantitation of 32P incorporation after normalization by the protein level is shown in the graph. PSL, photostimulated luminescence. b, CHO cells were transfected with pCEP4-SRSF1-HA for 45 h and then treated with 10 μM forskolin (Fors). At the same time, [32P]orthophosphate was added to label the phosphoproteins. After 3 h of treatment and phospho-labeling, the cell lysates were subjected to immunoprecipitation with anti-HA. The immunoprecipitated SRSF1-HA as well as whole cell lysates was analyzed by autoradiography. c, GST fused with different deletion mutants of SRSF1 were incubated with PKA in vitro for 30 min at 30 °C. 32P incorporation into GST-SRSF1 mutants was measured by autoradiography after the separation of the phosphorylation products by SDS-PAGE. Quantitation of the 32P incorporation after being normalized by the protein level is shown in the bar graph. Results represent the mean ± S.D.; *p < 0.05; **, p < 0.01.
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Expression in AD Brain—Previously we have shown that PKA is down-regulated in AD brain as a result of increased degradation by over-activated calpain I (40). To investigate whether the down-regulation of PKA in AD brain causes a dysregulation in the alternative splicing of tau exon 10, we measured the levels of 3R-tau and 4R-tau. Western blot analysis. We observed that in AD brain, the total tau level was increased by 3-fold, and 3R-tau was increased about 4-fold, but 4R-tau level was not significantly changed (Fig. 5, a and b), leading to an increase in the ratios of 3R-tau/total tau and 3R-tau/4R-tau (Fig. 5c). These results suggest a change in 3R-tau/4R-tau ratio and probably alternative splicing of tau exon 10 in AD brain.

To study whether down-regulation of PKA-Cα (Fig. 5, a and d) correlated with the dysregulation of tau exon 10, we determined levels of PKA-Cα and the ratio of 3R-tau to 4R-tau in AD brain and analyzed their correlation. We found a strong inverse correlation between the ratio of 3R-tau/4R-tau and PKA-Cα levels (Fig. 5e), suggesting that the decreased PKA-Cα in AD brain might contribute to the change in the ratio of 3R-tau and 4R-tau.

### Table 1: Human brain tissue of AD and control (Con) cases used in this study

| Case | Age at death | Gender | PMIa | Braak stageb | Tangle scores | Years | h |
|------|--------------|--------|------|-------------|---------------|-------|---|
| AD 1 | 89           | F      | 3    | V           | 14.5          |       |   |
| AD 2 | 80           | F      | 2.25 | VI          | 14.5          |       |   |
| AD 3 | 85           | F      | 1.66 | V           | 12.0          |       |   |
| AD 4 | 78           | F      | 1.83 | VI          | 15.0          |       |   |
| AD 5 | 95           | F      | 3.16 | VI          | 15.0          |       |   |
| AD 6 | 86           | M      | 2.25 | VI          | 13.5          |       |   |
| AD 7 | 83           | F      | 3.00 | VI          | 12.40         |       |   |
| AD 8 | 74           | M      | 2.75 | VI          | 14.66         |       |   |
| AD 9 | 79           | F      | 1.50 | VI          | 14.46         |       |   |
| AD 10| 73           | F      | 2.00 | VI          | 15.00         |       |   |
| AD 11| 76           | M      | 2.33 | VI          | 15.00         |       |   |
| AD 12| 72           | M      | 2.50 | VI          | 15.00         |       |   |
| AD 13| 74           | F      | 2.83 | VI          | 15.00         |       |   |
| AD 14| 76           | M      | 4.00 | VI          | 15.00         |       |   |

a PMI = postmortem interval.

b Neurofibrillary pathology was staged according to Braak and Braak (60).

c Tangle score was a density estimate and was designated as none, sparse, moderate, or frequent (0, 1, 2, or 3 for statistics), as defined according to CERAD Alzheimer disease criteria (61). Five areas (frontal, temporal, parietal, hippocampal, and entorhinal) were examined, and the scores were combined for a maximum of 15.

d Mortem delay-matched normal human brains (Table 1) by Western blot analysis. We observed that in AD brain, the total tau level was increased by 3-fold, and 3R-tau was increased about 4-fold, but 4R-tau level was not significantly changed (Fig. 5, a and b), leading to an increase in the ratios of 3R-tau/total tau and 3R-tau/4R-tau (Fig. 5c). These results suggest a change in 3R-tau/4R-tau ratio and probably alternative splicing of tau exon 10 in AD brain.

to study whether down-regulation of PKA-Cα (Fig. 5, a and d) correlated with the dysregulation of tau exon 10, we determined levels of PKA-Cα and the ratio of 3R-tau to 4R-tau in AD brain and analyzed their correlation. We found a strong inverse correlation between the ratio of 3R-tau/4R-tau and PKA-Cα levels (Fig. 5e), suggesting that the decreased PKA-Cα in AD brain might contribute to the change in the ratio of 3R-tau and 4R-tau.
DISCUSSION

The present study shows for the first time that the alternative splicing of tau exon 10 is probably dysregulated in AD brain, resulting in an imbalance between the levels of 3R-tau and 4R-tau. The increase in the level of 3R-tau was negatively correlated with PKA-Cα level, which is down-regulated in AD brain due to activation of calpain I (40). Activation of PKA enhanced the binding of SRSF1 to tau pre-mRNA and promoted SRSF1-mediated exon 10 inclusion in cultured cells and in vivo in rat brain. PKA-Cα, but not PKA-Cβ, interacted with and phosphorylated both the RS and RRM domains of SRSF1. Overexpression PKA-Cα, but not PKA-Cβ, enhanced SRSF1-mediated tau exon 10 inclusion. Knockdown of PKA-Cα inhibited SRSF1 function in promotion of tau exon 10 inclusion. Thus, down-regulation of PKA-Cα in AD brain (40) might be responsible for the increase of 3R-tau expression, resulting in an increase in 3R-tau/4R-tau ratio, which may contribute to neurofibrillary degeneration (Fig. 6).

The finding of increased 3R-tau level in AD brain is inconsistent with previous studies, which showed either an increase in 4R-tau expression in brain regions affected by sporadic AD (15, 45) or no change in tau isoforms in AD brain (13, 46). Most of these studies detected mRNA levels of 3R-tau and 4R-tau. However, RNA can be degraded very easily. Many studies have shown that the postmortem interval is related to the quality of RNA, but it is pH that affects RNA quality the most strongly. In postmortem tissue, the protein was found to be much more stable and its level unchanged, even when RNA was degraded (47, 48). By immunohistochemical analysis, Espinoza et al. observed that tangles appear with both 3R-tau and 4R-tau in the
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hippocampus in AD, and some advanced cases had large amounts of thioflavin-S-positive neurofibrillary tangles only detected by anti-3R-tau antibody but not anti-4R-tau antibody (16). In addition, they found that the pathology appeared to be more severe and displayed more abundant 3R-tau-positive tangles in the anterior as compared with the posterior hippocampus from the same cases (16). These results suggest that aggregation and deposition of 3R-tau may be associated with more advanced stages. In the present study we measured the protein levels of 3R-tau and 4R-tau in the temporal cortices of AD and control brains with a very short postmortem interval (<3 h) and found an increase in 3R-tau level. Previously, we have demonstrated that in AD brain, overactivation of calpain I due to calcium dysregulation causes degradation of the regulatory subunit of PKA, PKA-RII (40). A decrease in PKA-RII at basal conditions provides less protection to PKA-C from degradation. The C subunits of PKA are also decreased in AD brain, including Ca (40), which could lead to tau exon 10 exclusion, resulting in an increase in 3R-tau expression.

PKA is a Ser/Thr protein kinase and is involved in many biological pathways. Under non-stimulated conditions, PKA is present as an inactive heterotetramer consisting of two C subunits and two R subunits. There are three isoforms of C subunits, Ca, Cβ, and Cγ, and four isoforms of R subunits, RIIa, RIIβ, RIIα, and RIIβ. The Ca isoform is ubiquitously expressed, whereas the Cβ isoform is expressed only in brain (43). PKA-Cγ is expressed only in the testis (44). Although the Rα isoforms are ubiquitously expressed, the Rβ isoforms are predominated in the nervous and adipose tissues. When a signal arrives at the cell surface, it activates the corresponding receptor, which in turn leads to the transient elevation of intracellular cAMP and consequently activates PKA by dissociating the C subunits and then becomes vulnerable to degradation. The C subunits of PKA are also decreased in AD brain, including Ca (40), which could lead to tau exon 10 exclusion, resulting in an increase in 3R-tau expression.

PKA not only phosphorylated the RS domain but also phosphorylated the RRM motif. The RS domain has been shown to mediate protein-protein (49) and protein-RNA interactions (50), to function in nuclear import (51–53), and to play a role in the targeting of proteins such as SC35 to nuclear speckles (54), whereas RRM determines their RNA binding specificity. It is known that SRSF1 binds to the PPE at exon 10 and promotes tau exon 10 inclusion. Tau deletion mutation AK280 significantly decreases the SRSF1 binding and leads to tau exon 10 exclusion (24). In the present study we found that with EMSA, the hyperphosphorylated SRSF1, but not the hypophosphorylated SRSF1, bound to oligonucleotides of tau exon 10 and that with RNA immunoprecipitation assay, activation of PKA enhanced the binding of SRSF1 binding to PPE at exon 10. These data suggest that phosphorylation of SRSF1 by PKA might promote its binding to RNA.

Although hyperphosphorylation of tau plays a fundamental role in the development of Alzheimer-type neurofibrillary degeneration, imbalance in the cellular levels of 3R- and 4R-tau is emerging as an important concept in this pathology. Several lines of evidence, from transgenic mouse models to human tauopathies, emphasize the importance of a critical 3R-tau/4R-tau ratio in neurons. Disturbances of the 3R-tau/4R-tau ratio may lead to the characteristic neurofibrillary pathology. Close to 50% of all mutations in the tau gene causing human FTDP-17 affect tau exon 10 splicing and alter 3R/4R tau ratio (55). In addition to FTDP-17, dysregulation of exon 10 splicing may also contribute to other human tauopathies, such as Pick’s disease (with a predominant increase in 3R-tau), progressive supranuclear palsy (4R-tau up-regulation), corticobasal degeneration (4R-tau up-regulation) (12), and Down syndrome (3R-tau up-regulation) (27). The neuronal tau expression levels and isoform content is highly cell- and region-specific both during development and in the mature brain (56). 3R-tau and 4R-tau are not functionally equivalent with respect to interactions with microtubules. In vitro, 4R has an ~3-fold higher binding affinity for microtubules than that of 3R-tau (57, 58). In addition, 4R-tau is better at initiating and promoting microtubule assembly than 3R-tau (6, 59). We observed an ~4-fold increase in 3R-tau and an insignificant increase in 4R-tau in the AD brain with overactivation of calpain I, resulting in an increase of 3R-tau/4R-tau. Imbalanced tau apparently offers a good substrate for the phosphorylation and aggregation in neurons, leading to neurofibrillar degeneration.

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