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Tau binds both subunits of calcineurin, and binding is impaired by calmodulin

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ABSTRACT

Calcineurin, an important protein Ser/Thr phosphatase which acts on tau in vivo, is a heterodimer of a catalytic subunit, calcineurin A, and a regulatory subunit, calcineurin B, and is unique in being regulated by calmodulin. Here, we find that both subunits of calcineurin bind tau, and calmodulin interferes with the association between calcineurin and tau. The domains of both subunits of calcineurin and tau involved in binding are mapped. We also investigate the functional consequences of the interactions between both subunits of calcineurin, tau and calmodulin, and reveal the interactions affect dephosphorylation of tau by calcineurin and contribute to the balance of phosphorylation and dephosphorylation of tau in vivo. Our findings may be of potential significance in neuronal physiology and also in neurodegenerative disorders. They shed some light on how the interactions might control the phosphorylation state of tau under physiological conditions, and provide new insights into the treatment of tauopathies such as Alzheimer's disease.

1. Introduction

Calcineurin (CN), also termed protein phosphatase 2B (PP-2B), is the only known protein Ser/Thr phosphatase whose activity is regulated by calmodulin (CaM) [1]. CN is involved in many critical physiological and pathological processes, including T-cell activation, apoptosis, ion channel regulation, cardiac myocyte hypertrophy and Alzheimer's disease (AD) [2,3].

CN has a very narrow substrate specificity in vivo, in contrast to other phosphatases. A number of potential neuronal target proteins for CN have been suggested, such as tau, whose abnormal phosphorylation is a key pathological characteristic of AD [4]. As the major microtubule (MT)-associated protein (MAPs) in vivo, the most important function of tau is to modulate the assembly, behavior and spatial organization of microtubules [5,6]. But tau is also able to associate with many important cellular components, and participates in many key physiological processes [7–9]. It was shown previously that tau associate with protein phosphatase 2A (PP2A), a homologous protein Ser/Thr phosphatase of CN, via the former's MT-binding domain; as a consequence, MTs are able to inhibit the tau phosphatase activity of PP2A [10]. Although the PP2A holoenzyme associates with tau, the separate A, B, C subunits and the AC dimer are unable to do so [11]. As a heterodimeric protein, CN consists of a 61 kDa catalytic subunit A (CNA) and a 19 kDa regulatory subunit B (CNB) [12]. It is interesting to know whether just like PP2A, both subunits of CN are required for associating with tau or not, and what role CaM plays in the interaction between CN and tau.

In the present study, we found that not only CNA, but also CNB bound tau, and CaM impaired the association between CNA–CNB and tau. The domains involved in the associations involving tau, CNA and CNB were located. We also investigated the functional consequences of the interactions between CNA, CNB, tau and CaM, and revealed how the interactions affected dephosphorylation of tau by CN.

2. Materials and methods

2.1. Materials

The following were from our laboratory collection: cDNAs for human tau40, rat CNAα and its truncation mutants, rat CNB and its truncation mutants, and human CaM; also the vectors pGEX-4T-3 and pET-31b(+) as well as the PC12 rat pheochromocytoma cell line. The Bal b/c mice were from the animal center of Peking University. RII peptide was obtained from BioMoL Research Laboratories Inc. (Plymouth, USA). [γ-^{32}P] ATP was from Beijing Furi Biologic and Medicinal Engineering Co. (Beijing, China). The catalytic subunit of cAMP-dependent protein kinase was obtained from Promega Chemical Co. (Madison, USA). Rabbit polyclonal anti-tau, mouse polyclonal anti-CNA and anti-CNB, CNB–sepharose-4B and CaM–sepharose-4B were produced in our laboratory. Fluoresceinisothiocynate (FITC)-

Abbreviations: AD, Alzheimer's disease; AID, autoinhibitory domain of CNA; BBH, CNB-binding domain of CNA; CaM, calmodulin; CBA, recombinant single chain CNA–CNB–CaM complex; CBD, CaM-binding domain of CNA; CN, calcineurin; CNA, catalytic subunit of CN; CNAa, a catalytic domain of CNA; CNAab, CNAa-BBH; CNAabc, CNAa-BBH-CBD; CNB, regulatory subunit of CN; DN, N-terminal domain of CNB; DC, C-terminal domain of CNB; FITC, fluoresceinisothiocynate; MAPs, MT associated protein; MBR, microtubule binding repeats domain of tau; MT, microtubule; PF, projection fragment region of tau; PP-2A, protein phosphatase 2A; PP-2B, protein phosphatase 2B; PRB, proline-rich and basic region of tau; TRITC, tetramethyl rhodamine isothiocyanate

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conjugated goat anti-rabbit IgG, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG and peroxidase-conjugated goat anti-rabbit and horse anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, USA). Rabbit polyclonal anti-MAP2 and protein G plus-agarose beads were from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Glutathione-agarose beads and His·bind purification columns were from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Chemiluminescent substrate kits were obtained from Pierce Chemical Co. (Rockford, USA). All other reagents were of standard laboratory grade and the highest quality available from commercial suppliers.

2.2. GST pull-down assay

Glutathione-agarose beads (50 µl each) coated with GST or GST-tau were incubated with 100 µl aliquots of lysates of cells including CNA or CNB (1 mg/ml protein in buffer A [50 mM Tris–HCl, 50 mM NaCl, 1 mM Ca^{2+}, 0.1% β-ME, 0.2 mM PMSF, pH 7.4]) for 3 h at 4 °C with end-over-end shaking. The beads were recovered by centrifugation and washed three times with buffer B (50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1 mM Ca^{2+}, 0.1% β-ME, 0.2 mM PMSF). They were mixed with 50 µl SDS-PAGE sample buffer, boiled, and centrifuged, and 10 µl samples were immunoblotted with anti-CNA or anti-CNB antibody.

2.3. Immunoprecipitation

Mouse brain extract (0.5 ml) or cell lysates (0.1 ml) containing 1 mg/ml protein in buffer A were precleared with 25 µl protein G plus-agarose beads equilibrated in buffer A, and divided in half. To one half, 25 µl preimmune serum was added, and to the other, 25 µl anti-tau, anti-MAP2, anti-CNA or anti-CNB antiserum. Both halves were shaken end-over-end at 4 °C. After 4 h of shaking, 25 µl protein

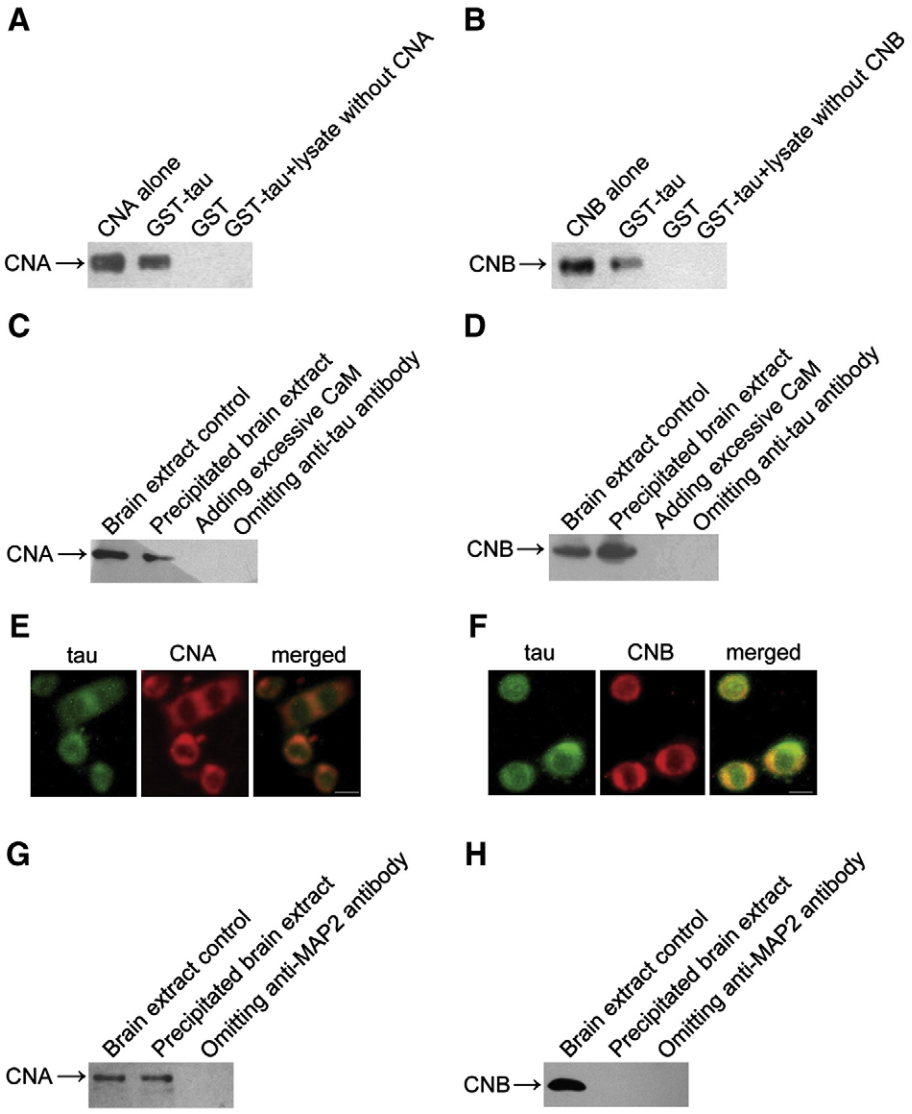

Fig. 1. Tau binding properties of CNA and CNB. (A, B) CNA-binding (A) and CNB-binding (B) of GST-tau were detected by pull-down experiments. The sample order is CNA alone or CNB alone, CNA or CNB incubated with GST-tau, CNA or CNB incubated with GST control, and control cell lysates without CNA or CNB incubated with GST-tau. (C, D) Tau was immunoprecipitated from fresh mouse brain extracts with anti-tau antibody. The resulting anti-tau antibody immune complexes were immunoblotted using anti-CNA (C) and anti-CNB antibody (D). The sample order is brain extract control, brain extracts precipitated by anti-tau antibody, brain extracts precipitated by anti-tau antibody in the presence of excess CaM, and control omitting anti-tau antibody. (E, F) Immunofluorescence staining of cultured PC12 cells for tau and CNA (E) or CNB (F). Separate staining and merged images are shown. Tau was detected with FITC-conjugated goat anti-rabbit IgG (green) and CNA or CNB was detected by TRITC-conjugated goat anti-mouse IgG (red). Co-localization is indicated by yellow staining. (G, H) MAP2 was immunoprecipitated from fresh mouse brain extracts with anti-MAP2 antibody. The resulting anti-MAP2 antibody immune complexes were immunoblotted using anti-CNA (G) and anti-CNB antibody (H). The sample order is brain extract control, brain extracts precipitated by anti-MAP2 antibody, and control omitting anti-MAP2 antibody. Scale bar, 10 µm. All immunoblot and immunofluorescence images are based on at least three independent experiments; representative examples are shown.

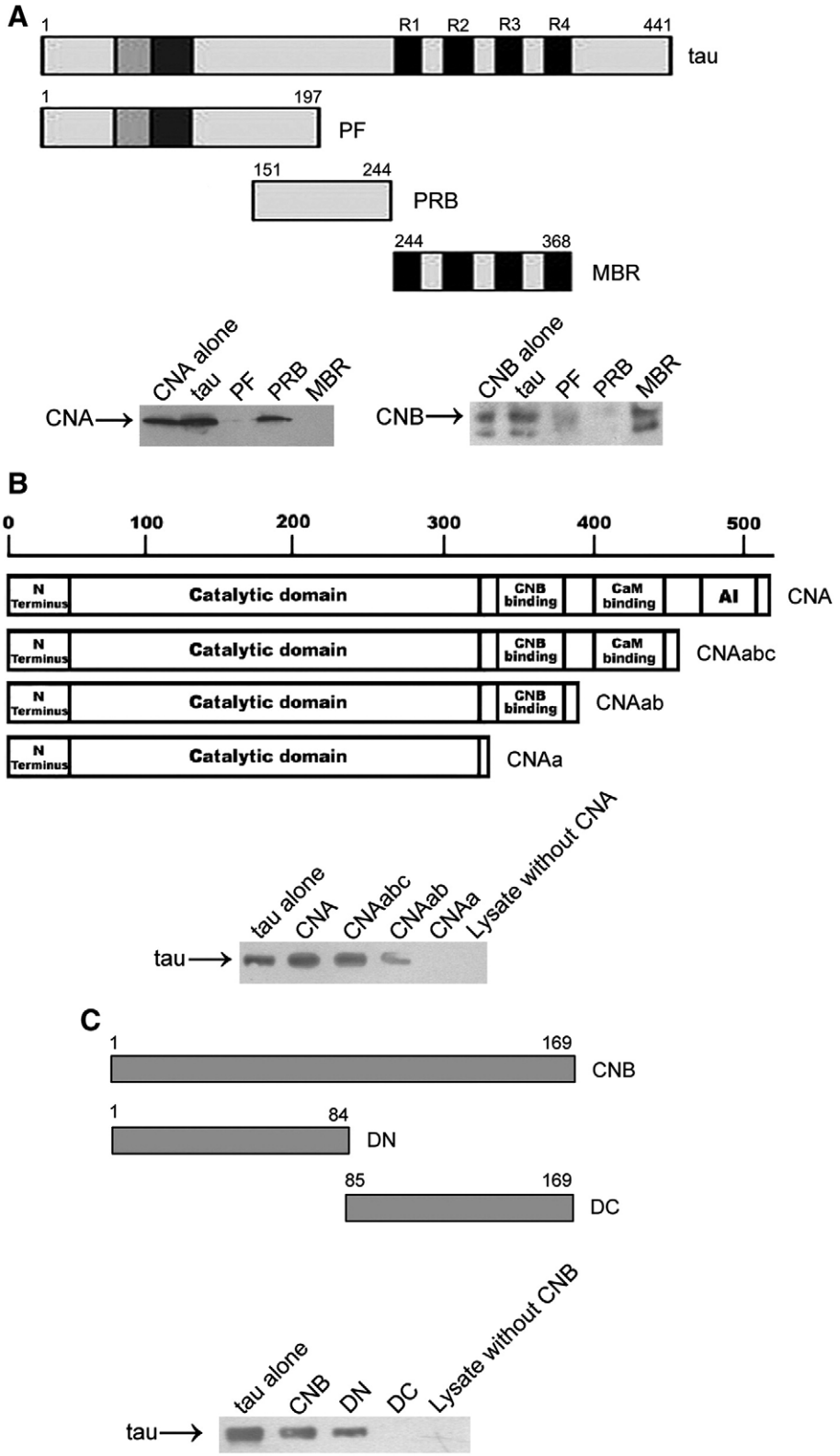

Fig. 2. Localization of the interacting regions within tau, CNA and CNB. (A) Localization of CNA and CNB-binding domains within tau. Lysates of cells expressing tau or its truncation mutants were mixed with lysates of cells expressing CNA or CNB, and precipitated with anti-tau. A schematic diagram of the tau species used in the assay, and the immunoblot analysis of the precipitates with anti-CNA or anti-CNB are shown. (B) Localization of tau binding domain within CNA. Lysates of cells expressing tau were mixed with lysates of cells expressing CNA or its truncation mutants, and precipitated with anti-CNA. A schematic diagram of the CNA species used in the assay and the immunoblot analysis of the precipitates with anti-tau are shown. (C) Localization of tau binding domain within CNB. Lysates of cells expressing tau were mixed with lysates of cells expressing CNB, DN or DC, and precipitated with anti-CNB. A diagram of the CNB species used in the assay and the immunoblot analysis of the precipitates with anti-tau are shown. All immunoblot images are from at least three independent experiments; a representative example is shown.

G plus-agarose beads pre-equilibrated in buffer A were added to each half, and shaking was continued for another 1 h. The protein G plus-agarose beads were collected by centrifugation, washed five times with ice-cold buffer B, and immunoblotted using anti-CNA, anti-CNB or anti-tau antibody.

2.4. Immunofluorescence staining

PC12 cells were immunofluorescently stained as described [13] except for some modifications. The cells were grown on polylysine glass coverslips. For fixation without detergent extraction, the cells were washed with PBS (10 mM phosphate buffer, pH 7.4, 137 mM NaCl), and incubated with ice-cold methanol for 5 min. After washing five times with PBS, they were incubated with incubation buffer (5% defatted milk powder in PBS) at 37 °C for 1 h. Anti-tau antibody and anti-CNA or anti-CNB antibody were added, and incubation continued for another 2 h at 37 °C. The cells were washed three times with PBS, and then incubated with FITC-conjugated goat anti-rabbit IgG and TRITC-conjugated goat anti-mouse IgG in incubation buffer for 30 min at 37 °C. After washing three times with PBS, the coverslips were mounted in 90% (vol/vol) glycerol and 10% (vol/vol) PBS, and the cells were photographed with an Olympus fluorescence microscope.

2.5. CNB and CaM affinity chromatography

CNB–sepharose-4B or CaM–sepharose-4B affinity chromatography medium was incubated with lysates of cells expressing protein I (tau or CNA in buffer A [50 mM Tris–HCl, 50 mM NaCl, 1 mM Ca^{2+}, 0.1% β-ME, 0.2 mM PMSF, pH 7.4]) for 2 h at 4 °C with end-over-end shaking. The sepharose-4B was recovered by centrifugation, washed three times with ice-cold buffer A and incubated with lysates of cells expressing protein II (tau, CNB or CaM in buffer A) for 2 h at 4 °C with end-over-end shaking. The sepharose-4B was again recovered by centrifugation, washed three times with ice-cold buffer A and incubated with lysates of cells expressing protein III (tau) for 2 h at 4 °C with end-over-end shaking, washed as before. It was then mixed with 50 µl SDS-PAGE sample buffer, boiled, and centrifuged, and a 10 µl sample was immunoblotted with anti-tau antibody.

2.6. Protein phosphatase assay

RII peptide was ^{32}P-labeled using the catalytic subunit of cAMP-dependent protein kinase as described [14]. Radioactive labeling of tau was performed in the same way except that incubation was prolonged to 4 h. In these conditions tau was labeled at several main sites including Ser214, Ser262, Ser324, Ser356 etc [15]. The ^{32}P-labeled RII peptide and tau were used to measure CN phosphatase activity as described [16] with some modifications. Purified CNA was diluted in 2×assay buffer (100 mM Tris–HCl, 0.2 mg/ml BSA, 1 mM DTT and 1 mM Mn^{2+}, pH 7.4; with or without 0.2 mM $CaCl_2$, 0.6 µM CaM and/or 0.2 µM CNB). 10 µl of diluted enzyme solution was mixed with 10 µl of 10 µM ^{32}P-labeled RII peptide or tau dissolved in milliQ water, and incubated for 10 min at 30 °C. The reaction was terminated by adding 180 µl of 83.3 mM H_3PO_4 and the released inorganic ^{32}P was quantified by liquid scintillation counting. Phosphatase activities are expressed as picomoles of phosphate released/mg of protein/min at 30 °C.

3. Results and discussion

3.1. CNB as well as CNA binds tau

To illuminate how CN associates with tau, we investigated the interaction between CNB and tau, as well as between CNA and tau, using GST pull-down assays. We were surprised to find that not only CNA, but also CNB was precipitated with GST-tau from the respective cell lysates (Fig. 1A, B). To gain more support for the above result, we immunoprecipitated tau from fresh mouse brain extracts. Both CNA and CNB were immunoprecipitated with tau (Fig. 1C, D). Further, we showed by immunofluorescence microscopy that endogenous CNA and CNB colocalized with tau in PC12 cells (Fig. 1E, F). Evidently both subunits of CN are able to associate with tau in vitro and in vivo, which are distinct from the association between PP2A and tau.

Tau related MAPs, MAP2 is also the known physiological substrate of CN [17]. In order to test the specificity of tau binding to CN, the interaction between both subunits of CN and MAP2 was studied by co-immunoprecipitation. MAP2 binds to CNA, but not to CNB (Fig. 1G, H), which are different from the above exposed association between tau and CN. This suggests that tau binding to CNB is of tau specificity.

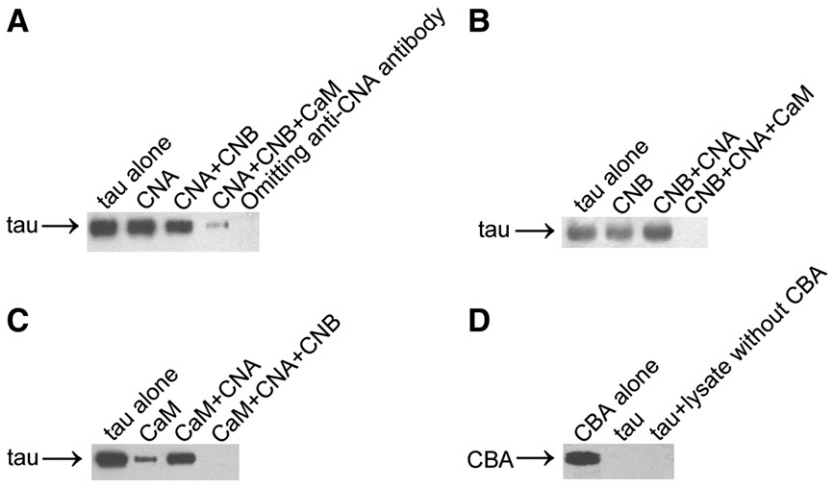

Fig. 3. Effects of CaM on tau binding capacity of CNA–CNB. (A) Tau binding detected by co-immunoprecipitation. The first lane is tau alone. The solid phase consisted of protein G plus-agarose pre-incubated with anti-CNA and CNA in the middle three lanes, and the liquid phases were as followings: tau; CNB added first, followed by tau; CNB followed by CaM, and followed by tau. The final lane is same as the 4th lane except that anti-CNA was omitted. (B) Tau binding detected by CNB affinity chromatography. The first lane consisted of tau alone. The solid phase was CNB–sepharose-4B in all the other lanes, and the liquid phases were as followings: tau; CNA added first, followed by tau; CNA followed by CaM, followed by tau. (C) Tau binding detected by CaM affinity chromatography. The first lane was tau alone. Solid phases were all CaM–sepharose-4B in the other lanes, and the liquid phases were as followings: tau; CNA added first, followed by tau; CNA followed by CNB, followed by tau. (D) Tau binding of CBA detected by co-immunoprecipitation. The sample order was CBA alone; CBA precipitated by tau; control cell lysate without CBA precipitated by tau. All immunoblot images are from at least three independent experiments; representative examples are shown.

3.2. Localization of binding domains within tau, CNA and CNB

To localize the CNA and CNB-binding domains within tau, we constructed tau truncation mutants in three regions that are responsible for binding tau to other proteins [18,19]: the PF (projection fragment region, 1–197 Aa), PRB (proline-rich and basic region, 151–244 Aa) and MBR (microtubule binding repeats domain, 244–368 Aa). Immunoprecipitation of tau and its truncation mutants showed that the PRB is involved in the interaction between CNA and tau and that the MBR is required for CNB-binding by tau, indicating that CNB and the MTs interact with the same functional domain of tau (Fig. 2A). It has been observed previously that, in the presence of Ca^{2+}, the CNB-homologous protein, CaM, also binds weakly to the MBR of tau [20].

CNA contains four functional domains: a catalytic domain (CNAa), a CNB-binding domain (BBH), a CaM-binding domain (CBD), and an autoinhibitory domain (AID) [21]. The BBH is responsible for the interaction between CNA and RII peptide [16]. In order to determine which functional domains of CNA participate in tau binding we immunoprecipitated CNA and its three truncation mutants: CNAa, CNAab (CNAa-BBH) and CNAabc. The results in Fig. 2B show that CNAabc and CNA bind tau with almost equivalent efficiencies. By comparison, CNAab was clearly less able to bind tau, and no binding of CNAa was detected. These observations indicate that the CBD, in addition to the BBH, is involved in the binding of tau to CNA.

CNB is composed of two global Ca^{2+} binding domains: DN (N-terminal domain) and DC (C-terminal domain), each of which contains two Ca^{2+}-binding EF-hand motifs [22]. DN and DC were immunoprecipitated to localize the tau binding domain within CNB. Fig. 2C shows that the DN domain is responsible for tau binding of CNB. It was shown previously that the DC domain played a central role in the interaction between CNB and CNA [23], which suggests that CNA and tau do not compete significantly for binding to CNB.

3.3. CaM impairs binding between CNA–CNB and tau

To clarify the role of CaM in the interaction between CN and tau, we carried out interaction experiments involving CaM, CNA, CNB and tau. Co-immunoprecipitation of CNA and tau showed that their interaction was almost unaffected by CNB, but CaM almost completely blocked the interaction between CNA–CNB and tau (Fig. 3A). CNB affinity chromatography revealed that both CNB–sepharose-4B itself and CNB–sepharose-4B pre-incubated with CNA were able to bind tau. However, when the CNA–CNB–sepharose-4B complex formed was incubated with CaM, the resulting CNA–CNB–CaM complex failed to bind tau (Fig. 3B). CaM affinity chromatography also showed that the presence of CaM impaired tau binding by CNA–CNB (Fig. 3C). We then used the recombinant single chain CaM–CNB–CNA complex (CBA) thought to have uniform structure with native CNA–CNB–CaM ternary complex [24] to confirm the above results. Fig. 3D shows that no interaction between CBA and tau was detected by co-immunoprecipitation. In addition, tau failed to co-immunoprecipitate with CNA or CNB in mouse brain lysates in the presence of excess CaM (Fig. 1C, D).

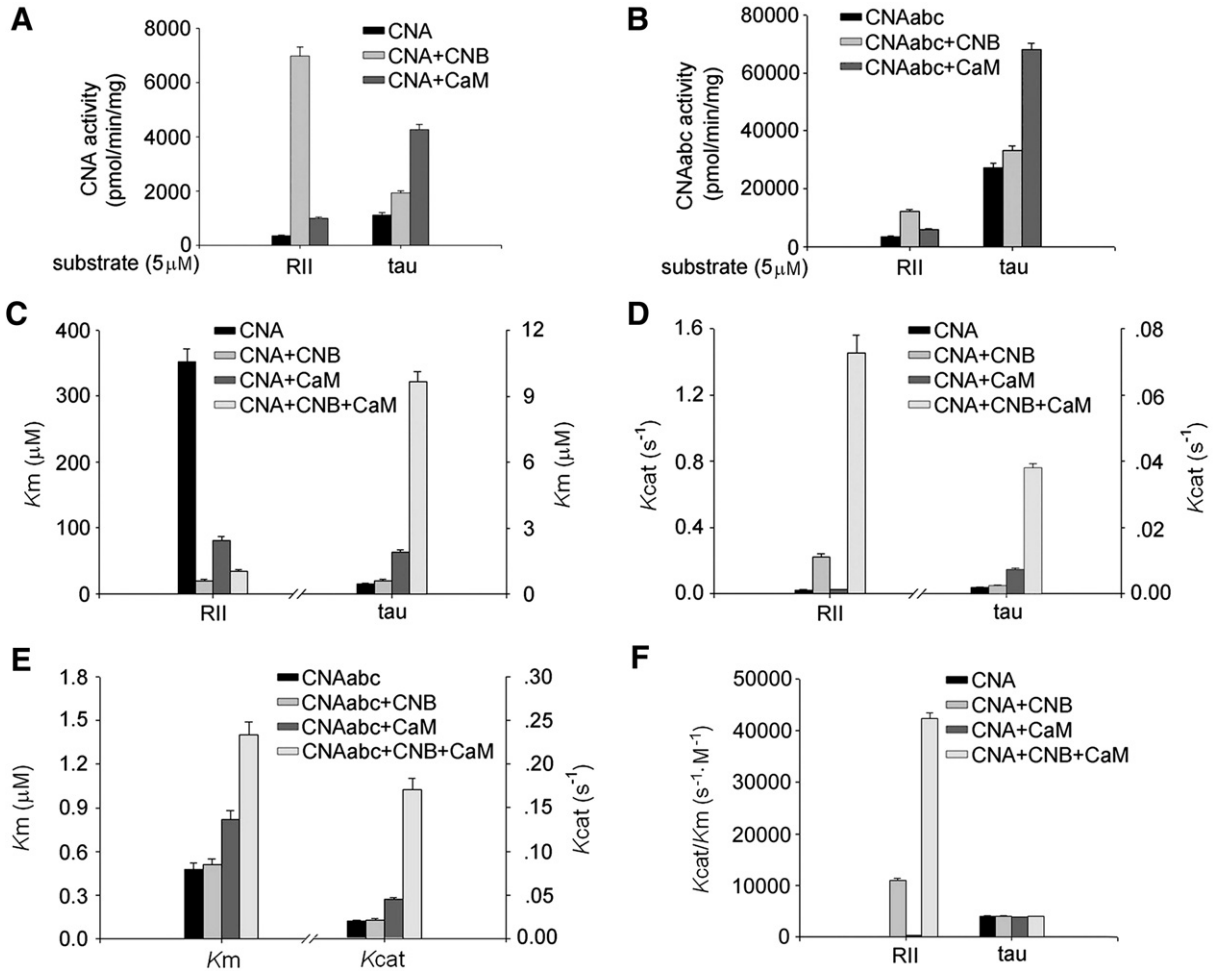

Fig. 4. Activity assays and kinetic analyses of CNA and CNAabc. (A, B) With RII peptide or tau as substrate, CNA (A) and CNAabc (B) activities were determined in the absence or presence of CNB or CaM. (C, D) The K_m (C) and K_{cat} (D) of CNA for RII peptide and tau were determined in the absence or presence of CNB and/or CaM. (E) The K_m and K_{cat} of CNAabc for tau were determined in the absence or presence of CNB and/or CaM. (F) The K_{cat}/K_m of CNA for RII peptide and tau was determined in the absence or presence of CNB and/or CaM. Error bars are s.d. ($n=3$).

Taken together, these results demonstrate that formation of the CNA–CNB–CaM ternary complex impedes the interaction of CNA and CNB with tau. It may be that the association between CNA, CNB and CaM sterically blocks their respective sites for tau binding.

3.4. Protein interactions affect dephosphorylation of tau by CN

Previously, the abnormal phosphorylation of tau at multiple Ser/Thr sites was attributed to defective CN activity in early AD [3,25–27]. The above results revealed that both subunits of CN associated with tau, and CaM impaired the binding between CN and tau. It is interesting to know whether the interactions between CNA, CNB, tau and CaM participate in regulating the phosphorylation levels of tau under normal physiological or pathological conditions in vivo. In vitro, with RII peptide or *p*-nitrophenyl phosphate as substrate, the catalytic and regulatory mechanisms of CN have been investigated. However, only by using tau as substrate, would it be possible to establish the actual in vivo relationship between CN and tau phosphorylation.

By a series of chromatographic steps, we purified, to electrophoretic purity, recombinant tau, CNA, and CNAabc (which contains CNAa, the BBH and the CBD of CNA), as well as recombinant CNB and CaM (data not shown).

We investigated the potential activating effects of CNB and CaM on the activity of CNA and its truncation mutant CNAabc with phosphorylated RII peptide and tau as substrates. In agreement with previous results [16] we found that, with RII peptide as substrate, CNB activated CNA 19.4 fold and CaM activated it 2.8 fold. With tau as substrate, CaM activated CNA 3.8 fold, which was similar to the stimulation with RII peptide as substrate, but CNB only activated CNA 1.7 fold (Fig. 4A). This indicated that using tau as substrate, the regulatory effect of CNB on CNA differed with that using RII peptide as substrate. Maybe, it is the interaction between CNB and tau that leads to the different regulatory effect. The results with CNAabc confirmed this notion (Fig. 4B).

In order to understand how the interactions between CNA, CNB, tau and CaM affect the dephosphorylation of tau by CN, we performed kinetic analyses of the affinity of CN for RII peptide and tau. Interestingly, the K_m of tau for CNA on its own was a mere 0.5 μM, lower than its K_m for the CNA–CNB, CNA–CaM or CNA–CNB–CaM complex. These data were quite different from those obtained with RII peptide. They suggested that CNB and CaM are not required for the association between CNA and tau, which confirms the results of the above protein interactions. The association is very strong, about 700 fold stronger than that between CNA and RII peptide (Fig. 4C). In addition, the kinetic analyses indicated that CNB reduced the K_m of CNA for RII peptide, and increased the K_{cat} of CNA. However, CNB alone had hardly any effect on the kinetic constants of CNA for tau (Fig. 4C, D). Our kinetic analyses also indicated that CNB together with CaM decreased the K_m of CNA for RII peptide. In contrast, the K_m for tau was increased (Fig. 4C), which confirmed the impairment of CaM for the association between CN and tau. The kinetic analyses of the interactions of CNAabc and tau confirmed the above results (Fig. 4E). These results thus demonstrate that the associations between CNA, CNB, tau and CaM affect the regulatory actions of CNB and CaM on CNA.

We are surprised to find that the efficiency (K_{cat}/K_m) with which CN dephosphorylates tau is constant, about 4000 $M^{-1}.^{-1}$, in the absence or presence of CNB and/or CaM (Fig. 4F), which may contribute to the balance between in vivo phosphorylation and dephosphorylation of tau under normal physiological conditions in vivo. Although CNB and CaM play different roles in the above interactions, both of them are responsible for the constant efficiency. Our findings may be of potential significance not only in neuronal physiology, but also in neurodegenerative disorders. In AD, the defect in CN's ability to dephosphorylate tau has been attributed to a loss of CNA activity [28,29]. However, based on the above results we think that changes in the ability of CNB or the CNA–CNB–CaM complex to bind to tau may also play a role. Correcting any binding defects should contribute to restoring CN's ability to dephosphorylate tau. Furthermore, this effect could be specific because it should not influence the ability of CN to dephosphorylate other substrates in vivo. Thus, the specific interactions between CNB and tau, as well as between the CNA–CNB–CaM complex and tau are promising targets for new therapeutic drugs against tauopathies such as AD.

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