An N-terminal motif unique to primate tau enables differential protein–protein interactions

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Running title: N-terminal motif in tau mediates interactions

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Keywords: Alzheimer’s disease, Tau, N-terminal motif, protein-protein interactions, iTRAQ, mass spectrometry, coimmunoprecipitation, neurodegenerative disease, neurodegeneration

Abstract

Compared with other mammalian species, humans are particularly susceptible to tau-mediated neurodegenerative disorders. Differential interactions of the tau protein with other proteins are critical for mediating tau’s physiological functions as well as tau-associated pathological processes. Primate tau harbors an 11-amino-acid-long motif in its N-terminal region (residues 18–28), which is not present in non-primate species and whose function is unknown. Here, we used deletion mutagenesis to remove this sequence region from the longest human tau isoform, followed by glutathione-S transferase (GST) pulldown assays paired with isobaric tags for relative and absolute quantitation (iTRAQ) multiplex labeling, a quantitative method to measure protein abundance by mass spectrometry. Using this method, we found that the primate-specific N-terminal tau motif differentially mediates interactions with neuronal proteins. Amongst these binding partners are proteins involved in synaptic transmission (Synapsin-1, Synaptotagmin-1) and signaling proteins of the 14-3-3 family. Furthermore, we identified an interaction of tau with a member of the Annexin family (AnnexinA5) that was linked to the 11-residue motif. These results suggest that primate tau has evolved specific residues that differentially regulate protein–protein interactions compared with tau proteins from other non-primate mammalian species. Our findings provide in vitro insights into tau’s interactions with other proteins that may be relevant to human disease.

Alzheimer’s disease (AD), the most common form of dementia, is characterized by amyloid-β (Aβ)-containing plaques and intracellular neurofibrillary tangles, which predominantly contain hyperphosphorylated forms of the neuronal microtubule-associated protein tau (1). Both aggregated tau and soluble tau species contribute to pathomechanisms in AD (2). Tau shows largely axonal localization in neurons (3). However, a smaller pool of tau localizes to the somatodendritic compartment of tau (1). Mechanisms of neuronal dysfunction mediated through tau include mitochondrial, synaptic and axonal transport deficits (4).

Protein-protein interactions are critical for tau’s physiological functions, and contribute to its role in disease (5). Tau was shown to interact with key neuronal proteins, for example scaffolding proteins Grb2 (6), C-Jun-amino-terminal kinase-interacting protein 1 (7) and postsynaptic density protein 95 (8), signalling molecules such as Fyn (9), phospholipase C (6) and protein phosphatase 2A (10) as well as pre-synaptic factors such as alpha-Synuclein (11). However, the full interactome of tau remains incompletely resolved. Furthermore, localized pools of tau appear to undergo different interactions,
driving diverse functions mediated by tau. Thus, classical axonal interactions of tau with microtubules affect axonal transport (12). Dendritic tau, on the other hand, interacts with post-synaptic protein complexes that mediate signals downstream of post-synaptic neurotransmitter receptors that contribute to Aβ toxicity in AD (8). In addition, tau protein interactions are dynamically regulated, likely reflecting changes in neuronal states of excitation/inhibition or other cellular processes. Post-translational modification of tau, such as phosphorylation, can affect tau complex formation with resulting changes in tau downstream signalling, imparting tau with detrimental (13,14), but also beneficial (15) properties in relation to Aβ toxicity. Finally, in familial forms of tauopathies (=neurological diseases with tau pathology), tau mutations affect protein interactions with tau (16). Thus, deciphering tau protein-protein interactions may be a path to understanding tau physiology and associated pathomechanisms.

Here, we speculated that primate (including human) tau has evolved to undergo species-specific interactions that depend on protein features inherent to primate tau. We report a primate-specific amino acid sequence segment between residues 18 to 28 in the N-terminus of human tau that is present only in other primate species, but absent from other mammalian species. In this study, we use deletion mutagenesis in human tau to identify interaction partners using isobaric tags for relative and absolute quantitation (iTRAQ) multiplex labelling. We found that this primate specific N-terminal feature of tau differentially mediates interactions with neuronal proteins of the vesicle associated machinery and synaptic transmission.

Results

Human tau harbours an 11-amino acid, primate-specific N-terminal sequence

Alignment of human and murine tau primary sequences revealed 11 consecutive amino acids (aa) in the extreme N-terminal region of human tau with no sequence homology to mouse (Fig. 1A). Extending the alignment study to other mammalian tau sequences, and using the longest human tau isoform (441aa) as a template, showed that this motif comprising aa 18 to 28 of tau was conserved in primates, and did not share sequence homology with other mammalian tau N-termini, including bovine, goat, cat and rodent tau (Fig. 1A and 1B). The only notable exception was canine tau, with 54% sequence identity to human tau in these 11 aa within its N-terminus. For comparison, non-mammalian tau showed no homology for the primate 11 aa sequence. To study the requirement of this 11-aa region within N-terminal tau for function and involvement in protein interactions, we used site-directed mutagenesis to delete corresponding codons from the longest isofrom of human tau, resulting in a deletion variant of tau we termed tauΔ18-28 (Fig. 1C). DNA sequencing confirmed successful deletion mutagenesis. Expression of tauΔ18-28 protein in cells showed a lower molecular weight band compared to full length tau (tauFL), as shown by SDS-PAGE and Western blotting with a tau-specific antibody (Fig. 1D).

Differential interaction mediated by aa 18-28 of human tau

Protein interactions involving the microtubule-binding repeats or the poly-proline region of tau have been reported before (17,18). However, N-terminal tau interactions and its sequence-dependent requirements remain poorly understood. Presence of a unique 11 aa segment in primate tau N-terminus suggested that human tau may engage in interactions distinct from murine (and other non-primate mammalian) tau. To address differential interaction in dependence of these 11 aa, we produced human tauFL or tauΔ18-28 as a glutathione-S-transferase (GST) fusion protein for glutathione (GSH)-mediated pull down experiments. GST fusion proteins were produced in E. coli and purified on GSH-beads (Fig. 2A). Purified GST-tauFL and GST-tauΔ18-28 fusion proteins were then used as bait protein for pull down experiments and incubated with murine cortical lysate from tau-deficient (tau”) mice (Fig. 2B). Lysates from tau” mice were chosen to avoid competition of bait protein with endogenous murine tau for potential interaction partners. To enable unbiased quantitative comparison of proteins present in multiple samples, we used labelling of peptides with isobaric isotope tags (iTRAQ) for quantitation (19). GST pulldown samples for tauFL and tauΔ18-28 from 4 independent (biological) replicates were multiplexed with different iTRAQ tags (Fig. 2B). Cortical lysates
from 4 different age- and gender matched tau−/− mice were used for GST pulldown replicates. Supporting Table S1 summarizes the results of the multiplex comparison of pulldowns of tauFL and tauA18-28, including the number of proteins identified at the 1% false discovery rate (FDR). One-hundred-and-thirty-three proteins were identified in both tauA18-28 and tauFL samples, across the 4 biological replicates (Supporting Table S1). Only murine proteins with a minimum unused score of >1.3 (≥95% confidence in sequence identification) and at least two distinct peptides detected by mass spectrometry were included in further analysis. ProteInPilot requires a minimum of 40 counts of iTRAQ reporter ion intensity to calculate iTRAQ ratios. Proteins identified with iTRAQ tag ion intensities below this threshold were not quantified. We calculated differential detection ratios using tauFL as denominator in ProteInPilot, thus reflecting proteins differentially bound to tauA18-28 versus tauFL (Table 1). A list of 8 proteins fulfilled the statistical criteria for differential binding regulated by aa 18-28 of tau. Proteins that were identified without differential binding between tauA18-28 and tauFL included known interaction partners of tau (e.g. tubulin).

**Tau interactors regulated by aa 18-28 form gene ontology clusters**

Performing gene ontology (GO) analysis of the differentially bound proteins specific to aa 18-28, revealed significant enrichment of several clusters. Specifically, we found enriched GO term clusters included axon, dendrite, postsynaptic density and synapse (Fig. 3A; Supporting Table S2). Next, we plotted differential interactors identified by ProteinPilot analysis of the iTRAQ data in a network map using STRING (Fig. 3B; Supporting Table S3). We also included tau to obtain information on how our list of aa 18-28-linked N-terminal binding candidates relate to previous evidence on tau interactions. Three distinct clusters emerged; One centred around tau and the phospho-protein binding factors 14-3-3β and 14-3-3η, one including synaptic vesicle membrane-associated proteins Synapsin-1 (Syn1) and Synaptotagmin-1 (Syt1) and a third cluster of Neuromodulin/Gap43 and Neurogranin. The interaction of tau and AnnexinA5/Lipocortin-IV had not been previously described. Taken together, these results suggest that the N-terminal human-specific residues aa 18-28 in tau contribute to interactions that may affect distinct cellular and molecular targets of synapse function.

**Co-immunoprecipitation validates differential interaction linked to aa 18-28 of tau**

To address interactions of tau identified by mass spectrometry using an independent method, we selected several candidates (Table 1) for co-immunoprecipitation experiments using transiently transfected cells. Both, previously unreported interactions as well as a known interaction partner of tau (i.e. GSK3β) were chosen. Where expression constructs were not available, coding sequences were amplified from reverse transcribed murine cortical mRNA. Transiently transfecting candidates 14-3-3β, 14-3-3η, Syt1 and AnnexinA5/Lipocortin V in 293T cells together with V5-tagged tauFL or tauA18-28 followed by co-immunoprecipitation confirmed differential affinity to tau that was affected by the presence of aa 18-28 of the human tau N-terminus (Fig. 4A-E). Lower levels of AnnexinA5, 14-3-3β, and 14-3-3η were found bound to tauA18-28 as compared with tauFL (Fig. 4A-C). Interaction of tau with Syt1 was confirmed by co-immunoprecipitation, however, with increased binding affinity to tauA18-28 as compared with tauFL (Fig. 4D).

For comparison, co-immunoprecipitation from cells co-transfected with tauA18-28 or tauFL together with a previously reported tau interaction partner GSK3β showed comparable interaction of tauA18-28 and tauFL (Fig. 4E), suggesting that aa 18-28 regulate tau protein interactions selectively. Taken together, we could corroborate differential interactions of several binding partners identified by iTRAQ screening that are modulated by the N-terminal aa 18-28 of human tau.

**Discussion**

In the present study, we revealed that a 11 aa sequence unique to the extreme N-terminus of primate tau is linked to differential interaction of tau with a small subset of partners implicated in synaptic function. All confirmed interaction partners showed differential interactions with a human tau variant that lacks aa18-28, suggesting this unique sequence modulates these interactions. Our results suggest that primate tau may differ in its
interactome, and thus in its molecular function as compared with non-primate tau.

Disease mechanisms involving tau as well as physiological functions of tau remain incompletely understood. Protein-protein interactions of tau have been shown to be critical for the role of tau in Aβ toxicity in Alzheimer’s disease (AD) (8). Furthermore, interactions of tau with other factors that mediate intracellular signals can be modulated through post-translational modifications on tau or intrinsic factors in tau (7,15,20,21). The N-terminal region of human tau is defined by splicing-dependent inclusions of 2 primary sequence segments, that contribute to differential functions of tau regarding protein interactions or localization (20).

When aligning primary amino acid sequences of N-terminal tau from different mammalian species, we observed that primate tau (human, chimp, macaque) contains a segment from aa18 to 28 that is not present in other species tested. This confirms observations by others (22,23). Furthermore, antibodies raised against this segment of human tau do not show immunoreactivity with tau from rat, mouse and bovine brains (24), confirming human/primate-specific differences in the very N-terminal region of tau on the protein level. We postulated that this segment may affect protein interactions of tau and confirmed this hypothesis using pulldown experiments coupled to mass spectrometric binding partner identification.

Seeking to identify potential molecular determinants engaging this segment in N-terminal tau, we employed a deletion approach in the longest human isoform of tau (441aa) to address requirement of this segment for tau protein-protein interactions. We based our approach on the idea that deletion would lead to a change in the behaviour of the entire tau molecule thus altering interactions – possibly – by effects on more distal parts of the protein. This is supported by findings of intramolecular interactions of tau N- and C-terminal regions that are affected through post-translational modifications in either region (25). Thus, binding partners identified by a deletion approach in tau in comparison with full-length tau (tauFL) may reflect the binding propensity of the tau molecule in complex formation rather than direct binding via aa 18-28.

Consistent with this idea, the small N-terminal alteration to tau (=Δ18-28) did not lead to a large change in numbers of binding partners from cortical brain lysates. We and others previously identified differentially expressed proteins using iTRAQ in AD mouse and human samples (20,26,27). Using stringent criteria employed in our previous studies using iTRAQ, we identified a small number of dysregulated binding partners of tau with the tauΔ18-28 variant.

The identified proteins were enriched for synaptic and vesicle membrane proteins. Our iTRAQ data suggests differential interaction of synaptic vesicle proteins Syt1, Syn1/2 with the tauΔ18-28 variant. Using co-immunoprecipitation as an independent method to address interactions, we confirmed interaction of Syt1 and tau in cells (Fig. 4D), with an increased affinity to tauΔ18. Our results suggest that the primate tau N-terminus modulates tau’s interaction with proteins on synaptic vesicles. Vesicular protein interactions with tau have been reported for Syn1, Syt1 and syntaxin-1B (20,28), supporting our own findings. Pathological human tau interacts with presynaptic vesicles though its N-terminal region (28). Though physiological implications of tau interactions with vesicular proteins are unknown, human/primate tau may show different functional involvement with synaptic vesicles as compared with rodent tau due to different binding affinities, potentially differentially affecting synaptic functions across species.

We previously identified Lipocortin IV/Annexin A5 among mitochondria-associated proteins that are dysregulated in AD mouse model (26). AnxA5 is a circulating protein with anti-inflammatory and phospholipid binding properties (29). Interestingly, evidence from cultured neurons and a mouse model suggests AnxA5 as biomarker for AD (30). We observed reduced binding of tauΔ18-28 to AnxA5, which was verified by co-immunoprecipitation. This is the first evidence of an interaction of tau and AnxA5. Virtual loss of interaction of AnxA5 with tauΔ18-28 suggests that the presence of the N-terminal aa18-28 segment in primate tau critically regulates this interaction unique to primate tau. Interestingly, another member of the Annexin family, Annexin A2, was shown to interact with tau and tether it to the cell cortex.
in neurons (31). Together with our finding that tau interacts also with ANxA5 in the present study, it is an intriguing possibility that tau engages in different functional interactions with multiple Annexin family members.

Our iTRAQ data showed a reduced affinity of tauΔ18-28 to Gap43/Neuromodulin, a protein involved in axonal outgrowth and synaptic development (32,33). In cultured neurons, Gap43 shows synaptic localization in dependence of synaptic maturation (34). Interestingly, its synaptic localization inversely correlates with presence of synapsin/synaptotagmin during maturation of axonal synapses. Therefore, tau may contribute to axonal maturation of primate neurons by differential interaction between Syn/Syt1 and Gap43, regulated by the N-terminal aa 18 to 28.

With Nrgn, a postsynaptic protein implicated in synaptic plasticity (35), we identified another tau interaction partner whose affinity to tau is mediated by the aa 18-28 segment in the N-terminus of tau. Nrgn had recently been suggested as cerebrospinal fluid biomarker for AD (36,37). Forced expression of Nrgn enhances local synaptic plasticity in mice (38). Nrgn associates with the postsynaptic density (39) and with neuronal exosomes (14). Other neuronal exosome-associated proteins identified in our study are synapsin-1 (14) and potentially Syt1. Synaptotagmins can be released in presynaptic exosomes to facilitate postsynaptic signals important in synaptic plasticity and development (40). Interestingly, tau pathology can propagate between neurons via a mechanism that may involve exosomes (41,42). Increased affinity of primate tau to binding partners associated with exosomes may help explain mechanisms of tau propagation seen in human tauopathies.

Finally, we identified two members of the 14-3-3 protein family, 14-3-3β and 14-3-3η, with differential affinity to tauΔ18-28 and tauFL. In brain lysates, both were detected with iTRAQ ratios > 1.2, suggesting increased abundance of these proteins bound to tauΔ18-28 compared with tauFL. In contrast, when testing complex formation with tau in cultured cells, we found lower binding of 14-3-3β/η to tauΔ18-28 compared with tauFL. Interactions with 14-3-3 proteins are dependent on phosphorylation status of binding targets (43), and tau was previously shown to bind 14-3-3 proteins in a phosphorylation-dependent manner (44). However, tau phosphorylation status may vary depending on incubation conditions and exposure to kinases in cell culture as compared with brain tissue derived lysates. Given that recombinant tau used for GST-pulldown and mass spectrometry was not phosphorylated may explain the discrepancies in 14-3-3 protein binding between our in vitro and cell culture experiments. Nevertheless, our results indicate that aa 18-28 modulate the binding of tau to 14-3-3β and 14-3-3η. 14-3-3 binding is normally depending on phosphorylation of partners at SP/TP sites in conserved motifs (43). Since the segment of aa 18 to 28 harbours does not harbour SP/TP phosphorylation site, this sequence may modulate the interaction of tau with 14-3-3β and 14-3-3η in a non-conventional way.

It is noteworthy that canine tau, is the only non-primate species to harbor a 1-3-terminal sequence with some, but low similarity to the primate aa 18-28 sequence. Interestingly, there are reports of human-like AD and tau pathology in dogs (45,46), while AD mouse models with Aβ do not develop human-like tau pathology (i.e. NFTs) unless human tau is co-expressed (47). Although we have no direct evidence that this species difference depends on the aa 18-28 motif, it is an intriguing possibility that distinct sequence features that contribute to specific functions of human/primate tau also render it susceptible to disease-driving changes.

To this end, our results define a small, but specific feature within the N-terminus of human/primate tau that is linked to differential interaction with synaptic and vesicle-associated proteins, possibly providing a basis for species-specific insights into tau relevant to human disease. Further studies are required to define the specific role of this motif in vivo.
Experimental procedures

Mice. Tau knockout mice with a targeted allele for Mapt were described previously (48). Mice were housed in individually ventilated cages with food *ad-libitum* and a 12h/12h light-dark cycle. Genotypes of offspring were determined at postnatal day 16 by PCR using primers listed in Supporting Table S4. All animal experiments were approved by the University of New South Wales Animal Care and Ethics Committee.

Plasmid constructs. Codons encoding amino acids 18-28 were deleted from the coding sequence of human full-length tau (hTau40 or tau 441aa) using Q5 site directed mutagenesis kit (NEB, MA, USA) thus generating the tauΔ18 construct. TauFL and tauΔ18 were cloned into pGEX-4-T-1 for recombinant protein production using conventional restriction enzyme cloning. Murine coding DNA (cDNA) encoding AnxA5 was amplified from murine mRNA isolated from cortical brain samples using first strand cDNA synthesis kit (NEB, MA, USA) for reverse transcription. AnxA5 was amplified with a C-terminal HA tag and cloned into pcDNA3.1. Plasmid encoding 14-3-3η was a kind gift from Dr Yue Xiong (Addgene plasmid #19957), 14-3-3β was a kind gift from Dr Michael Yaffe (Addgene plasmid #13270). 14-3-3β and 14-3-3η were subcloned into pENTR-SD-TOPO, and then transferred into a pcDNA3.2-myc mammalian expression destination vector (kind gift from Dr Romanic) by LR-clonase reaction (Invitrogen, ThermoFisher, Sydney, Australia). EGFP-E-Syt1 was a kind gift from Dr Pietro De Camilli (Addgene plasmid #66830). Primers for cloning and mutagenesis are listed in Supporting Table S5. All constructs were confirmed by sequencing. Expression of proteins was confirmed by immunoblotting after transfection into 293T cells.

Cell culture. Human embryonic kidney 293T cells were cultured in Dulbecco’s Modified Eagle’s Medium High Glucose (Sigma-Aldrich, Sydney, Australia) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin at 37°C and 5% CO₂. Cells were transfected using calcium precipitation of plasmid DNA as previously described (49). Briefly, cells were plated out 24 h prior to transfection and media was replaced 2 hr prior to transfection. Plasmid DNA (5 µg) was used per 6 cm dish and 2.5M calcium chloride (CaCl₂) and 2x HBS (281 mM sodium chloride (NaCl), 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 mM disodium hydrogenphosphate (Na₂HPO₄), pH 7.05-7.09)) was used to precipitate DNA. Cells were lysed and sonicated on ice 48 h post transfection, in RIPA buffer (1M tris(hydroxymethyl)aminomethane (TRIS) pH8.0, 150mM NaCl, 5mM Ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P40, 0.1% SDS, 10mM sodium fluoride (NaF), 1mM sodium vanadate (Na₃VO₄), 1mM sodium pyrophosphate (NaPP), 0.1% Glycerophosphate, 1x Complete Mini protease inhibitor (Roche Applied Science, Sydney, Australia)), then centrifuged (10 min, 16,000g, 4°C). Protein concentration was determined using a Bradford assay (BioRad, Sydney, Australia).

Antibodies. Antibodies against the following epitopes were used: Glyceraldehydephosphatase dehydrogenase (GAPDH; clone 6C5, 1:5,000, Invitrogen, ThermoFisher, Sydney, Australia), Tau (cat. # A0024, 1:5,000, DAKO, Sydney, Australia), green fluorescent protein (GFP; cat. # ab290, 1:1,000, Abcam, Sydney, Australia), glycogen synthase kinase-3 (GSK3; cat. # ab131356, 1:1,000, Abcam, Sydney, Australia), haemagglutinin tag (clone HA-7; 1:5,000, Sigma, Sydney, Australia), myc tag-horseradish peroxidase conjugate (MYC-HRP; cat. # R951-25, 1:5,000, Invitrogen, ThermoFisher, Sydney, Australia), V5 tag (cat. # PA1-993, 1:500, Invitrogen, ThermoFisher, Sydney, Australia). HRP-coupled secondary antibodies donkey-anti-rabbit (1:5,000, Santa Cruz, ThermoFisher, Sydney, Australia).

Co-immunoprecipitation and Immunoblotting. Western blotting was performed as previously described (50). Briefly, for immunoprecipitation 500µg of protein lysates were incubated with 0.5µl (0.5 µg) V5 antibody at 4°C for 16 hr with rotation, before the addition of 20µL RIPA-buffer equilibrated Protein G beads (NEB, MA, USA) and incubation with Protein G Beads for 1h at 4°C. Samples were washed 3 times in RIPA buffer and then resuspended in 40µL SDS loading buffer, then incubated (5 min, 95°C). The supernatant was separated from beads using a
magnetic holder and for input samples, 5 µg of protein was used. Samples were separated on 8% SDS-PAGE and following protein transfer, membranes were blocked with 3% BSA in TRIS-buffered saline solution with 0.1% Tween-20 (TBS-T) before probing with primary antibodies overnight at 4 °C.

Preparation of recombinant proteins. pGEX-4T-1-tauFL, pGEX-4T-1-tauA18 or empty pGEX-4T-1 were transformed into E. coli BL21 DE3 pLys (Fermentas, ThermoFisher, MA, USA) and proteins were induced in log phase (OD600=0.4) with 0.5 mM IPTG (Sigma-Aldrich, MI, USA) for 2h at 37°C. Cells were pelleted by centrifugation, washed and lysed by sonication in bacterial lysis buffer (50 mM TRIS pH 7.5, 300 mM NaCl, lysozyme (Sigma, MI, USA), 2 mM EDTA, 0.5% TritonX100, 10% glycerol, 0.25 M dithiothreitol (DTT), 1 µg/mL DNAseI, 10 mM NaF, 1 mM Na3VO4, 1 mM NaPP, 0.1% Glycero phosphate, 1x Complete Mini protease inhibitor (Roche Applied Science, Penzberg, Germany)). After clearing lysates by centrifugation (16,000 g, 4°C, 10 minutes), buffer-equilibrated glutathione-sepharose beads (GE Healthcare, IL, USA) were used to affinity purify GST fusion proteins. Glutathione-sepharose beads were washed 3 times with TBS buffer (Tris 10 mM, 150 mM NaCl). Bacterial lysis, clearing and affinity purification was monitored on SDS-PAGE with Coomassie Brilliant Blue staining (Sigma-Aldrich, MI, USA).

Glutathione-S-transferase (GST) pull down. Murine cortical tissue was dissected from brain after transcardial perfusion with cold phosphate-buffered saline pH 7.4 (PBS). Tissue was homogenized in lysis buffer (50 mM Tris 7.5, 50 mM NaCl, 5 mM EDTA, 0.3% Sucrose, 0.05% Tween-20, 10 mM NaF, 1 mM Na3VO4, 1 mM NaPP, 0.1% Glycero phosphate, 1x Complete Mini protease inhibitor (Roche Applied Science, Penzberg, Germany)) using a dounce homogenizer (Heidolph Douncer, Schwabach, Germany) on ice. Insoluble materials were removed by centrifugation (16,000 g, 4°C, 10 min). Total protein concentration was determined by BCA assay (BioRad, CA, USA). Tissue extract (1 mg) and GST fusion proteins (10 µg) were incubated with buffer-equilibrated GSH beads overnight (16 hours) at 4°C with gentle agitation (6 rpm) on a rotator. Beads were recovered by gentle centrifugation at 400 g and washed three times with GST-buffer. For SDS-PAGE analysis, bound proteins were eluted from the beads by the addition of SDS loading buffer, followed by incubation (5 min, 95°C). Supernatant proteins were then separated by molecular weight using SDS-PAGE (8%).

iTRAQ sample processing, mass spectrometry and data analysis Figure 2 shows iTRAQ labels and corresponding samples, briefly labels 114, 116, 118 and 121 represent tauA18, while 113, 115, 117 and 119 represent tauFL pulldown samples. Labelling of protein samples was performed according to the Applied Biosystems iTRAQ manual. Briefly, GST pull down samples were desalted and buffer exchanged using 3kDa amicon filter units (EMD Millipore) and 50mM sodium bicarbonate. For the 8-plex iTRAQ labelling, 100 µg of protein sample was reduced by adding 2 µL Tris(2-carboxyethyl)phosphine (TCEP, Sigma) and incubating samples at 60°C for 1 hour. Next, samples were treated with 1 µL iodoacetamide (37 µg/mL) for alkylation/cysteine blocking (10 minutes, ambient temp.). Protein samples were digested using 4 µg reconstituted Trypsin (sequencing grade, low autolysis trypsin; Promega, WI, USA), at 37°C for 16 hours. Digested samples were briefly spun in a microfuge and pH if necessary the pH was adjusted to ca 9-10 with a few µL sodium carbonate (500 mM Na2CO3).

iTRAQ 8-plex reagents were reconstituted in 50 µL neat isopropanol (Sigma, Sydney, Australia) and the whole contents of one vial was transferred to one sample (Figure 2B shows the iTRAQ labels and corresponding samples) and incubated at ambient temperature for 1 hour. The labelled samples were then combined into one sample tube, vortexed and spun briefly. To reduce the concentration of buffer salts and organics, the sample mixture was diluted 10-fold with cation exchange load buffer (10 mM potassium phosphate in 25% acetonitrile at pH 3.0) before loading sample mixture onto a strong cation exchange cartridge (9.5 mL/hr). The flow through was discarded. To elute the peptides from the cation exchange cartridge, 500 µL of cation exchange elution buffer (10 mM potassium phosphate in 25% acetonitrile/350 mM potassium chloride, pH 3.0) was injected and the eluent was collected.
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in a 1.5mL polypropylene tube and then dried in a vacuum centrifuge (Speedvac ThermoFischer Scientific, Sydney, Australia). An additional clean-up step was performed by resuspending the dried sample in 500µL of 0.2% heptafluorobutyric acid (Sigma, Sydney, Australia), then loading onto an Oasis HLB cartridge (Waters, Sydney, Australia). The peptides were eluted in 700µL of 0.1% formic/50% neat acetonitrile, dried under vacuum and dissolved in 400µL 0.05% HFBA/1.0% formic acid. Samples were run in triplicate (6µL injected per run) on a tripleTOF 5600+ LCMSMS system (ABSciex, Foster City, USA), as previously described (51). Chromatographic separation of peptides was performed on a ~12cm C18 column (350µm i.d., Reprosil-Pur, 1.9 µ, 200 Å, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using a 240 min gradient (Dionex UltiMate 3000 RSLCnano pump, ThermoFischer Scientific Dionex, Waltham, USA), with buffer A (H₂O:CH₃CN of 98:2 containing 0.1% formic acid) to buffer B (H₂O:CH₃CN of 20:80 containing 0.1% formic acid) at 200 nL/min. Data were processed with ProteinPilot v4.0 software (ABSciex, Foster City, USA). Reporter ion peak area ratios of tauΔ18 were expressed relative to tauFL for the 4 biological replicates. Cut-off for differential expression was set at a ratio of >1.2 for increased interactions and <0.82 for reduced interaction based on previous iTRAQ experiments (26). Only proteins with ≥ 2 annotated peptides and an unused score of >1.3 in ProteinPilot were used for further analysis. Quantitative ratios from ProteinPilot were further analysed using a custom script using MATLAB (vR2016b) by averaging over the 4 biological replicates and comparisons applying a p-value (Student t-test) criterion of p<0.05 to determine the statistically significant differentially bound proteins.

Bioinformatics. Protein ontology was analysed using DAVID gene ontology (v6.8) software using gene ontology annotations for the entire murine proteome. Protein-protein interactions were analysed using STRING (v10.5).
Acknowledgments
This work was supported by funding from the National Health & Medical Research Council (NHMRC), the Australian Research Council (ARC) and Dementia Australia. Y.K. is a NHMRC Career Development Fellow, and L.I. is a NHMRC Principal Research Fellow.

Conflict of interest
The authors declare no conflict of interest.

Author contributions
K.S. performed pull down experiments. K.S., A.V., Y.K. and A.I. generated constructs. K.S. and J.B. generated recombinant proteins. K.S. performed and A.P. supervised mass spectrometry experiments. K.S., A.P., L.I. and A.I. analysed data. L.I. and A.I. designed the study. Y.K., L.I. and A.I. obtained funding. K.S., L.I. and A.I. wrote the manuscript with input from all authors. A.I. supervised the study.
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N-terminal motif in tau mediates interactions

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Table 1. iTRAQ-based relative ratios of peptides/proteins identified by GST-pulldown. Cross-comparison ratios of iTRAQ-labelled peptides were determined by ProteinPilot software and analysed using MATLAB. Ratios are averaged across 4 biological replicates. A protein had to show the same deregulation trend in at least 2 of the 4 replicates to be considered as deregulated with a $p$-value $<0.05$. Ratios of $>1.2$ for increased interactions or $<0.82$ for reduced interactions were used as cut-off from our previous work using iTRAQ (26).

| UNIPROT ID | Protein name                          | Total (ID confidence) | Distinct peptides | tauΔ18-28:tauFL mean ratio (n=4) | $p$-value (n=4 replicates) |
|------------|--------------------------------------|-----------------------|-------------------|----------------------------------|---------------------------|
| 1433E_MOUSE | 14-3-3 eta                           | 8.28                  | 6                 | 1.68                             | 0.016                     |
| 1433B_MOUSE | 14-3-3 beta                          | 10.12                 | 10                | 1.51                             | 0.014                     |
| NEUG_MOUSE | Neurogranin                          | 4.00                  | 2                 | 0.78                             | 0.003                     |
| SYT1_MOUSE | Synaptotagmin-1                      | 2.85                  | 2                 | 0.77                             | 0.025                     |
| NEUM_MOUSE | Neuromodulin/Growth associated protein 43 | 6.42                 | 3                 | 0.77                             | 0.044                     |
| SYN1_MOUSE | Synapsin-1                           | 7.54                  | 4                 | 0.73                             | 0.038                     |
| ANXA5_MOUSE | Annexin A5/Lipocortin V             | 2.09                  | 2                 | 0.59                             | 0.014                     |
Figure legends

**Figure 1.** A unique 11-amino acid motif in the N-terminal region of primate tau. (A) Alignment of mammalian tau primary sequences. Primary sequences of N-terminal regions of human, non-human primate, macaque, bovine, goat, dog, cat and rodent tau proteins were aligned using ClustalOmega ([http://www.ebi.ac.uk/Tools/msa/clustalo/](http://www.ebi.ac.uk/Tools/msa/clustalo/)) with marked up similarities (grey shading). Note absence of a motif between amino acids 18 to 28 of primate tau in other mammalian tau sequences. Only canine tau contains a 10-amino acid motif of lesser similarity to primate tau. (B) Dendrogram of N-terminal mammalian tau protein sequences. Dendrogram analysis of sequences between the indicated residues in A (dashed lines) was performed using ClustalOmega ([http://www.ebi.ac.uk/Tools/msa/clustalo/](http://www.ebi.ac.uk/Tools/msa/clustalo/)). Note that canine tau clusters with primate (Human, *Pan troglodytes*, *Macaca mulatta*) tau N-terminal sequences due to its 10-amino acid motif albeit with weaker similarity. (C) Amino acids 18-28 were deleted in human tau40 to generate the tauΔ18 construct. (D) tauΔ18 and tau full length (tauFL) were transfected into 293T cells. Immunoblot of cell lysates from tauFL, tauΔ18 or untransfected control was detected with anti-human tau antibody. tauΔ18 shows reduced retention, consistent with deletion of amino acids 18-28. Probing for GAPDH served as loading control.

**Figure 2.** iTRAQ-based mass spectrometry to identify tau protein-protein interactions modulated by aa18-28. (A) Production of recombinant tau proteins. GST-tagged tau full length (tauFL) and delta18-28 tau (tauΔ18-28) were produced in *E. coli*. Samples of washed and eluted protein were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue. (B) Experimental setup for quantitative comparison of tau protein-protein interactions in dependence of the 11-aa motif in tau’s N-terminal region using 8-plex iTRAQ. Technical replicates of GST-pulldown with GST-tagged tauFL and tauΔ18-28 were subjected to tryptic digest and iTRAQ labelled (labels 113, 114, 115, 116, 117, 118, 119, 121). Peptides were detected by mass spectrometry (5600 TripleTOF mass spectrometer, SCIEX). Cross-comparison ratios of iTRAQ-labelled peptides were determined by ProteinPilot v4.0 software.

**Figure 3.** Gene ontology and protein network analysis of differential protein-protein interactions modulated by presence of the aa 18 to 28 N-terminal tau motif. (A) Protein ontology was analysed using DAVID (v6.8) gene ontology. (B) Protein interaction network of deregulated protein-protein interactions with tauΔ18-28 was analysed using STRING (v10.5). Network edges are defined by confidence indicating the strength of data support by line thickness.

**Figure 4.** Validated interactions modulated by primate-specific N-terminal tau motif. (A-E) Selected tau protein interaction partners AnnexinA5/Lipocortin-IV (AnxA5) (A) 14-3-3β (B), 14-3-3η (C), Synaptotagmin-1 (D) and GSK3β (E) were expressed in 293T cells together with V5-tagged human tauFL or tauΔ18-28. Protein-protein interaction was assessed by co-immunoprecipitation using a V5-specific antibody and detected by immunoblotting for HA, myc, GFP, or GSK3. Results from 3 independent experiments are represented as means ± SD. (Student t-test) ** p < 0.05 ns, not significant
Figure 1
Figure 2

GST-tauFL

GST-tauΔ18-28

Cortical lysates

tau−/−

GST pull-down

Tryptic digest

iTRAQ labelling

GST-tauFL

GST-tauΔ18-28

113 115

117 119

114 116

118 121

LC-MS/MS

Differential binding ratios
Figure 3
Figure 4

A

B

C

D

E

Input | IP V5
---|---
tauFL-V5 | - - + - -
taxΔ18-V5 | - + - - +
HA-Anxa5 | + + - - +

Relative levels (relative to input)

** 1.5
1.0
0.5
0.0

GAPDH

tau | - - + - -
HA-Anxa5 | + + - - +

** 1.5
1.0
0.5
0.0

Relative levels (relative to input)

ns

GAPDH

taxFL-V5 | - - + - -
taxΔ18-28-V5 | + - - - +
HA-Anxa5 | + + - - +

Relative levels (relative to input)

ns

GAPDH

taxFL-V5 | - - + - -
taxΔ18-28-V5 | + - - - +
MYC-14-3-3β | + + - - +

Relative levels (relative to input)

ns

GAPDH

taxFL-V5 | - - + - -
taxΔ18-28-V5 | + - - - +
GFP-Syt1 | + + - - +

Relative levels (relative to input)

ns

GAPDH

taxFL-V5 | - - + - -
taxΔ18-28-V5 | + - - - +
HA-GSK3β | + + - - +

Relative levels (relative to input)

ns

GAPDH

Figure 4
An N-terminal motif unique to primate tau enables differential protein–protein interactions
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J. Biol. Chem. published online January 30, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.001784

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