Microtubule-associated protein tau is essential for long-term depression in the hippocampus

Tetsuya Kimura1,†, Daniel J. Whitcomb2,3,4,†, Jihoon Jo2,3,†, Philip Regan2,4,5,
Thomas Pierś3,4, Seonghoo Heo3, Christopher Brown2,3,
Tsutomu Hashikawa6, Miyuki Murayama6, Heon Seok2,7,
Ioannis Sotiropoulos2,8, Eunjoon Kim9, Graham L. Collingridge4,5,10,
Akihiko Takashima1,6 and Kwangwook Cho2,4

1Department of Aging Neurobiology, Center for Development of Advanced Medicine for Dementia, National Center for Geriatrics and Gerontology, 35 Gengo, Morioka, Obu, Aichi 474-8522, Japan
2Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, School of Clinical Sciences, Faculty of Medicine and Dentistry, University of Bristol, Whitson Street, Bristol BS1 3NY, UK
3Chonnam-Bristol Frontier Laboratory, Biomedical Research Institute, Chonnam National University Hospital, Gwangju 501-757, South Korea
4Centre for Synaptic Plasticity, and 5School of Physiology and Pharmacology, University of Bristol, University Walk, Bristol BS8 1TD, UK
5Laboratory for Alzheimer’s Disease, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan
6Department of Biomedical Engineering, Jungwon University, 85 Munmu-ro, Goesan-gun, Chungcheongbuk-do 367-805, South Korea
7Department of Biomedical Engineering, Jungwon University, 85 Munmu-ro, Goesan-gun, Chungcheongbuk-do 367-805, South Korea
8Life and Health Sciences Research Institute (ICVS), School of Health Sciences, Universidade do Minho, Campus de Gualtar, Braga 710-057, Portugal
9Center for Synaptic Brain Dysfunctions, Institute for Basic Science and Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea
10Department of Brain and Cognitive Sciences, College of Natural Sciences, Seoul National University, Gwanak-gu, Seoul 151-746, South Korea

The microtubule-associated protein tau is a principal component of neurofibrillary tangles, and has been identified as a key molecule in Alzheimer’s disease and other tauopathies. However, it is unknown how a protein that is primarily located in axons is involved in a disease that is believed to have a synaptic origin. To investigate a possible synaptic function of tau, we studied synaptic plasticity in the hippocampus and found a selective deficit in long-term depression (LTD) in tau knockout mice in vivo and in vitro, an effect that was replicated by RNAi knockdown of tau in vitro. We found that the induction of LTD is associated with the glycogen synthase kinase-3-mediated phosphorylation of tau. These observations demonstrate that tau has a critical physiological function in LTD.

1. Introduction

The microtubule-associated protein ‘tau’ (MAPT) gene is located on chromosome 17 and consists of 16 exons [1]. Alternative splicing leads to six isoforms of tau, all of which contain an amino-terminal projection domain and carboxy-terminal with microtubule-binding repeats [2]. Tau contains several critical serine and threonine residues, the phosphorylation of which regulates its binding affinity for microtubules [3,4]. It is believed that through this binding, tau has major roles in stabilizing microtubules [5]. During neuronal development, tau expression is increased in response to nerve growth factor [6], and subsequently enriched in axons, a process that is required for maintaining axon morphology [7]. The extent to which tau may have additional functions unrelated to axonal microtubule stabilization, however, is not known.
Tauropathies, such as Alzheimer’s disease (AD), are characterized by widespread accumulation of hyperphosphorylated tau. Once hyperphosphorylated, tau is known to accumulate in somatodendritic compartments and forms the core component of neurofibrillary tangles (NFTs) [8]. It is generally believed that hyperphosphorylation of tau is the critical step in causing it to be missorted from the axon to dendrites, where it interferes with neuronal function [9]. Associated with this accumulation, there is a loss of synapses and eventually neurons [10,11]. However, the mechanism by which this occurs is unknown.

Increasing evidence suggests that in AD, synaptic dysfunction may initiate the cascades that result in cognitive impairment and neurodegeneration. For example, it is well established that oligomeric forms of β-amyloid (Aβ) induce a rapid alteration in synaptic plasticity, the process widely believed to underlie learning and memory in the brain [12]. More specifically, Aβ causes inhibition of long-term potentiation (LTP) and enhancement of long-term depression (LTD) in the hippocampus [13]. LTD involves the removal of AMPA receptors (AMPA Rs) from synapses leading to a reduction in synaptic efficiency, and can also result in the shrinkage and elimination of synapses [14]. Therefore, a shift in favour of LTD may lead to neurodegeneration. That such processes may be causally related to neurodegeneration in AD is suggested by the finding that key molecules that are associated with this disorder, such as glycogen synthase kinase (GSK)-3 and caspase-3, are required for the induction of LTD in the hippocampus [15–18] and mediate the Aβ inhibition of LTP [19]. Interestingly, recent evidence has shown that Aβ inhibition of LTD is absent in the tau knockout (KO) mouse [20]. These data, together with the observation that GSK-3β directly phosphorylates tau [15,18], suggest that tau may be a downstream effector of GSK-3β in LTD. Therefore, we decided to examine the role of tau in LTD in the hippocampus.

In this study, we found that in tau KO mice there is a loss of LTD, whereas LTP is not affected. Furthermore, knockdown of tau in hippocampal slices resulted in a complete loss of LTD in the absence of any direct discernible effects on synaptic transmission. We found that LTD was associated with the phosphorylation of tau by GSK-3β [18]. Collectively, these data suggest that tau phosphorylation is an essential component of LTD.

2. Results

(a) Long-term depression is absent in MAPT+/− and MAPT−/− mice

The physiological role of tau in the hippocampus was initially investigated using tau KO mice. We compared long-term synaptic plasticity in adult (7–11 months old) MAPT+/+-, MAPT+/− and MAPT−/− mice. Because the tau kinase GSK-3β is required for LTD in the hippocampus [17], the primary focus of our investigation was on this form of synaptic plasticity. Field excitatory postsynaptic potentials (fEPSPs) were evoked in area CA1 of anaesthetized mice in response to electrical stimulation of the ipsilateral Schaffer collateral–commissural pathway. We found no differences in synaptic transmission between MAPT+/−, MAPT+/− and MAPT−/− mice, as assessed using input–output curves (figure 1a), and we observed no significant differences in paired-pulse facilitation over a range of inter-stimulus intervals (figure 1b). However, we found that while LTD could be readily induced in adult MAPT+/+ mice, it was completely absent in MAPT+/− and MAPT−/− mice (figure 1c). By contrast, similar levels of LTP were observed in the three genotypes (figure 1d). Therefore, tau is specifically required for LTD in the hippocampus in vivo.

Next, we investigated LTD in acute brain slices from young (14- to 17-day-old) mice. Consistent with the observations in vivo, LTD was absent in slices prepared from MAPT−/− mice but was readily induced in slices obtained from MAPT+/+ mice (figure 2a). We also investigated LTD induced by a brief application of NMDA (25 μM, 3 min) and found a specific deficit in slices from the MAPT−/− mice (figure 2b). These results show that the LTD deficit in MAPT−/− mice is apparent early in development and therefore is not directly associated with ageing.

(b) Knockdown of tau by shRNA prevents long-term depression induction

In these experiments, tau was absent or reduced throughout the life of the animals, potentially leading to developmental complications. Therefore, to investigate more directly whether tau is involved in the LTD process, we used an shRNA probe against rat tau and studied synaptic function in rat hippocampal organotypic slice cultures (figure 3). To study the effects of tau knockdown on synaptic transmission, simultaneous recordings of excitatory postsynaptic currents (EPSCs) were performed from tau-shRNA transfected and neighbouring untransfected neurons. There were no significant differences in AMPAR- and NMDA receptor (NMDAR)-mediated EPSCs (EPSCA and EPSCN, respectively) between tau-shRNA transfected cells and neighbouring untransfected neurons (figure 3a). By contrast, similar levels of LTP were observed in MAPT+/+ and MAPT−/− mice, as assessed using input–output curves (figure 1a), and we observed no significant differences in paired-pulse facilitation over a range of inter-stimulus intervals (figure 1b). However, we found that while LTD could be readily induced in adult MAPT+/+ mice, it was completely absent in MAPT+/− and MAPT−/− mice (figure 1c). By contrast, similar levels of LTP were observed in the three genotypes (figure 1d). Therefore, tau is specifically required for LTD in the hippocampus in vivo.

Next, we investigated LTD in acute brain slices from young (14- to 17-day-old) mice. Consistent with the observations in vivo, LTD was absent in slices prepared from MAPT−/− mice but was readily induced in slices obtained from MAPT+/+ mice (figure 2a). We also investigated LTD induced by a brief application of NMDA (25 μM, 3 min) and found a specific deficit in slices from the MAPT−/− mice (figure 2b). These results show that the LTD deficit in MAPT−/− mice is apparent early in development and therefore is not directly associated with ageing.

(c) Tau is found in the postsynaptic compartment

The finding that tau is required for LTD is surprising since LTD is generally considered to be mediated postsynaptically, via the synaptic removal of AMPARs, whereas tau is present primarily in axons. One possibility is that LTD causes the redistribution of tau to dendritic shafts and/or spines.
alternative possibility is that a small proportion of tau is normally expressed in a postsynaptic compartment and it is specifically this fraction that is involved in LTD. We explored the latter possibility in two ways. First, we used immunogold electron microscopy (EM) and compared the labelling of tissue from MAPT+/+ and MAPT−/− mice (figure 4a). We could detect some immunoreactivity within dendritic spines of the MAPT+/+ but not MAPT−/−, mice. Second, we probed for the presence of tau, and another microtubule-associated protein (MAP2), in microsome/organelle (P3), cytoplasmic (S3) and synaptosomal (LP1) fractions prepared from the hippocampus of MAPT+/+ mice (figure 4b). As expected, tau and MAP2 were recovered in the P3 fraction and to a lesser extent in the S3 fraction. However, tau was additionally detected in the LP1 fraction. Thus, a proportion of tau is localized at a postsynaptic site where it could, in principle, function directly in LTD.

Because GSK-3β is a major tau kinase [15,18,21] and is activated during LTD [17], this seemed a likely candidate to mediate the physiological phosphorylation of tau. We hypothesized that the GSK-3β mediated phosphorylation of tau could be an important regulator of LTD. If this is indeed the case, then a prediction is that the induction of LTD should be associated with an increase in the phosphorylation of tau. To investigate this, we delivered low-frequency stimulation (LFS) and measured the phosphorylation status of tau in the CA1 microdissected dendritic region of rat hippocampal slices (figure 4c). We observed a dramatic increase in the phosphorylation of tau

Figure 1. Tau is required for LTD in vivo in mice. (a) No differences are shown in synaptic transmission between MAPT+/+ , MAPT+/− and MAPT−/− mice. The graph plots the fEPSP amplitude versus the fibre volley (f.v.) amplitude (stimulus intensity range: 10–100 μA, grey dots). Input-output curves show regression (continuous lines) and 95% confidence limits (dashed lines). (b) No differences are shown between MAPT+/+ , MAPT+/− and MAPT−/− mice in paired-pulse facilitation at various inter-stimulus intervals. (c) LTD is absent in MAPT+/+ and MAPT−/− mice. Pooled data from 7- to 11-month-old MAPT+/+ (76 ± 2%; n = 16; MAPT+/− : 96 ± 3%; n = 15; MAPT−/− : 98 ± 2%; n = 11; p < 0.001 in comparison with MAPT+/− mice, Bonferroni’s multiple comparison test). (d) No differences are shown in LTP between genotype. Pooled data from 7- to 11-month-old MAPT+/+ (141 ± 7% of baseline quantified at 60 min after the tetanus, n = 5), MAPT+/− (141 ± 6%, n = 6) and MAPT−/− mice (134 ± 3%, n = 4). HFS, high frequency stimulation. (Online version in colour.)

Figure 2. Tau is required for LTD in vitro in mice. (a) 1 Hz, 900 pulses induces LTD in MAPT+/+ mice (77 ± 3%, n = 6) but is absent in MAPT−/− mice (106 ± 7%, n = 5). Pooled data from postnatal 14- to 17-day-old mice. (b) Bath application of NMDA (25 μM, 3 min) induces LTD in MAPT+/+ mice (71 ± 8%, n = 5) but no LTD in MAPT−/− mice (99 ± 4%, n = 6). Pooled data from postnatal 14- to 17-day-old mice using PHF-1 (p < 0.01, n = 4, figure 4d,e), an antibody that recognizes phosphorylation at residues Ser396 and Ser404 [22], but observed no difference following LFS in the total
levels of tau using Tau-5 (p > 0.05, n = 4, figure 4d,f), a phosphorylation-independent anti-tau antibody [23]. We next tested whether the phosphorylation of tau was due to activation of GSK-3 during LTD by applying a highly selective GSK-3β inhibitor, CT-99021, during LFS (figure 4d). This treatment eliminated the increase in tau phosphorylation (figure 4d,e), while having no effect on the total levels of tau (assessed using Tau-5; figure 4d,f). Collectively, these data demonstrate that LFS leads to the phosphorylation of tau in a GSK-3β-dependent manner, further supporting the idea that this phosphorylation event has a key role in the induction of LTD.

### 3. Discussion

In this study, we have provided several lines of complementary evidence to suggest that tau is important for LTD in the hippocampus. First, we have shown that LTD at CA1 synapses in vivo is not detectable in mice in which tau is absent or its expression levels are reduced. Second, we found that LTD was absent in slices acutely prepared from juvenile hippocampal tissues of MAPT+/− mice. Third, we have demonstrated that knockdown of tau completely blocks LTD in organotypic-cultured slices. Fourth, we have shown that LFS used to elicit physiological LTD leads to enhanced phosphorylation of tau at the PHF-1 epitope, via a GSK-3β-dependent mechanism.

It is widely believed that tau, under normal conditions, is primarily involved in stabilizing microtubules in axons, and that the dysregulation of this function somehow leads to neuronal pathology [24]. The most prevalent form of such dysregulation occurs through the hyperphosphorylation of tau, which is involved in the generation of NFTs and plays a key role in neurodegenerative conditions such as AD [25]. Hyperphosphorylated tau is missorted to somatodendrites instead of axons, where it is known to accumulate [26–28]. Such missorting is assumed to contribute to neuronal pathology, because it positions tau where it can interfere directly with synaptic function. How tau becomes missorted is not known.

An alternative possibility is that some tau is normally present at synapses and it is this tau that is specifically associated with the neuropathology. Indeed, emerging evidence suggests that tau may be present in dendrites even in the absence of tauopathy [29] and that it could regulate interactions between scaffolding proteins and signalling pathways in the postsynaptic density (PSD) [30]. Furthermore, the localization of tau within the postsynaptic complex can be affected by NMDAR activation [31]. This raises an important question as to what the physiological function of tau in dendrites might be. Here, we have found, using both tau KO mice and RNAi in organotypic slices prepared from rats, that tau is required for LTD. This role is likely to be specific, because we found no evidence that tau is required for maintaining normal synaptic transmission or for LTP. Previous work [9,32] has shown that overexpression of tau may lead to inhibition of LTP. Based on the present findings, we propose that this may be because excess activation of tau induces a chronic form of LTD that is manifest as an impairment in LTP.

We also found that LFS, a physiological LTD induction protocol, resulted in the phosphorylation of tau and that this was dependent on GSK-3. Thus, tau is most probably a physiological substrate of GSK-3β during LTD. The next key question concerns the physiological downstream effectors of tau during LTD. At present, we can only speculate on this issue. Because tau is a microtubule-associated protein, and because microtubules may be involved in LTD [33], it is possible that tau is involved in the regulation of LTD-dependent microtubule dynamics.

Tau can be divided into a projection domain (towards the N-terminus, encompassing an acidic region and a proline-rich region) and a microtubule-binding domain (towards the C-terminus, including the microtubule-binding repeat region) [34], each having specific roles in the regulation of tau function [35,36]. Within these domains exist multiple regulatory sites of phosphorylation on serine/threonine residues. Our findings suggest a role in LTD for serine residues within the PHF epitope (Ser396/404). Consistent with our findings, Mondragon-Rodriguez et al. [31] recently reported a facilitation of Ser396/404 phosphorylation following NMDA treatment. Critically, Ser396/404 residues can both be phosphorylated by GSK-3β [37,38], an enzyme that is required for the induction...
of LTD [17]. However, it was unknown which, if any, of the potential GSK-3β phosphorylation sites on tau are phosphorylated during the physiological activation of this kinase. Our finding that phosphorylation of Ser396/404 following LFS is prevented by CT-99021 demonstrates that GSK-3β is upstream of tau in LTD and that this particular phosphorylation event probably has a physiological function.

4. Conclusion
We have shown that tau is required for NMDAR-dependent LTD in the hippocampus. Our data suggest a model whereby during LTD, activation of GSK-3β leads to phosphorylation of tau and this promotes LTD.

5. Methods
(a) In vivo electrophysiology
Male C57/BL6J mice were used for all comparative KO experiments. MAPT+/+ and MAPT+/− mice were maintained by backcrossing with C57/BL6J mice. Mice were individually housed and kept on a 12 h light/dark schedule. All mice had free access to food and water. Each mouse was anaesthetized with 3% isoflurane–air mixture, and fixed in a stereotaxic device (model 900, David Kopf...
Instruments, USA). After exposing the skull, a bipolar-stimulating electrode (two enamel-coated wires with 10 μm diameter and 200 kΩ impedance) was positioned into the stratum radiatum of the left hippocampal CA1 area (~1.7 mm from bregma, 1.65 mm from medial, 1.3 mm depth) and a mono-polar recording electrode (0.5–1 MΩ) was placed 200 μm posterior to the stimulating electrode. The animal was maintained anaesthetised (1.5% isoflurane–air mixture) for at least 3 h (body temperature was kept at 36 °C). For the fEPSP measurements, the electrical signal was amplified 100 times (ER-1, Cygnus Technology, USA), digitized (Digidata 1321A, Axon Instruments, Foster City, CA, USA) and processed on a computer. To induce LTD, 100 pulses at 1 Hz were delivered at a holding potential of 70 mV. NMDAR-mediated EPSC amplitude (EPSCN) was measured by a custom application based on MATLAB (version 8, Mathworks Inc., CA, USA). fEPSPs were analysed only when the latency of the minimum peak from stimulus was shorter than 7 ms. Experiments were performed blindly.

(b) In vitro electrophysiology

For in vitro electrophysiology experiments, acute hippocampal slices were obtained from P24 to P28 male Wistar rats or MAPT+/− and MAPT−/− mice. Animals were sacrificed by decapitation. In some experiments, dual patch clamp recordings were made simultaneously from a pair of neighbouring CA1 pyramidal neurons, one transfected and the other untransfected.

Acknowledgements. We thank Drs P. St. George-Hyslop (University of Cambridge), Eckhard Mandelkow (German Center for Neurodegenerative Disease), M. Vitek (Duke University), O. Almeida (Max Planck Institute of Psychiatry), N. Sausa (University of Minho) and Hana Dawson (Duke University) for critical discussion.

(d) Tau constructs

0N3R and 2N4R human tau cDNAs were framed in pEGFP-C1 host vectors (Clontech, Mountain View, CA, USA), and provided by Drs R. Brandt (University of Ostenbrück, Germany) and S. Lovestone (King’s College, UK).

(e) Immunogold electron microscopy

Under deep pentobarbital anaesthesia, animals were perfused with 4% paraformaldehyde in 0.1 M cacodylate buffer (CB, pH 7.4). After further fixation of the brain at 4 °C overnight, 300-μm-thick hippocampal slices were made. After incubation with blocking solution (5% normal goat serum in 0.1 M CB) for 1 h at room temperature, the slices were incubated with primary anti-tau antibody, JM (rabbit, 1:300), at 4 °C for 2 days, followed by a secondary anti-rabbit IgG conjugated with FITC-gold (goat, Nanophores, NY, USA, 1:100) overnight. The slices were re-fixed with a mixture of 2.5% glutaraldehyde and 1% tannic acid at 4 °C overnight. The gold signal enhancement procedure was performed according to the manufacturer’s instruction (GoldEnhance-EM, Nanophores). After the osmication of slices (1% OsO4–1.5% potassium ferrocyanide in 0.1 M CB) at 4 °C for 10 min, the slices were dehydrated, and embedded in epoxy resin. The stratum radiatum of CA1 region was examined electron microscopically (JEM-1200EX, JEOL, Japan) after metal-staining using uranine acetate and lead citrate.

(f) Subcellular fractionation

Partial subcellular fractionation was performed on mouse hippocampus basically according to a previous report [39]. Postnuclear supernatant was subjected to centrifugation (12 500g), and divided into the crude synaptosomal fraction and synaptosome-depleted fraction. The crude synaptosomal fraction was further purified by hypotonic lysis and centrifugation (25 000g), and the resultant pellet was the PSD-95-rich synaptosome fraction (LP1). The synaptosome-depleted fraction was further subjected to ultracentrifugation (100 000g), and separated to the microsome fraction (P9) and cytoplasm fraction (S3). We used the following antibodies for experiments: NeuN, mouse monoclonal (Millipore; 1:1000) Tau-5, mouse monoclonal (Invitrogen; 1:500); PSD-95 (Millipore; 1:1000) and MAP2 (Millipore; 1:1000).

(g) Low-frequency stimulation, microdissection and western blotting

Rat hippocampal slices from P24 to P28 were subjected to a standard 1 Hz, 900 pulses, LFS protocol (stimulation set at a predetermined intensity; 70% of the maximal fEPSP amplitude voltage stimulation), using a bipolar stimulation electrode, in the presence or absence of CT-99021 (1 μM). The dendritic CA1 region was then immediately dissected and snap frozen. Samples were lysed and SDS-PAGE was performed as previously shown [19]. PHF1 monoclonal antibody (kindly provided by Dr Peter Davies) was used at 1:1000 for western blot. Optical densities of immunoreactive bands were quantified using NIH ImageJ software (downloaded from http://rsb.info.nih.gov/ij/); n indicates the number of independent experiments from different animals.
Neurodegenerative Disease Initiative Programme. K.C. was supported by the Korea–UK Alzheimer’s Disease Research Consortium programme from the Ministry of Health and Welfare (Korea). G.L.C. was supported by the WCU Programme (Korea). I.S. was supported by the British Council. The collaboration between K.C. and A.T. was supported by a Sasakawa Foundation grant awarded to K.C. K.C. was supported by the Wolfson Research Merit Award and the Royal Society, London.

References


3. Lindwall G, Cole RD. 1984 Phosphorylation of endogenous tau: effect of protein kinase C, D.J.W. and G.L.C. were supported by UK Wellcome Trust–MRC Funding statement. A.T. was supported by the research funding for longevity sciences (25–39) from National Center for Geriatrics and Gerontology, and the Strategic Research Programme for Brain Science (‘Integrated Research on Neuropsychiatric Disorders’) and Grant in Aid for Scientific Research on Innovative Areas (‘Brain Environment’) from the Ministry of Education, Science, Sports and Culture of Japan. K.C., D.J.W. and G.L.C. were supported by UK Wellcome Trust–MRC...


38. Liu SJ et al. 2004 Tau becomes a more favorable substrate for GSK-3 when it is prephosphorylated by PKA in rat brain. J. Biol. Chem. 279, 50 078–50 088. (doi:10.1074/jbc.M406109200)