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

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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.
Tauopathies, 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 (AMPARs) 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)-3β 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 LTP 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 interstimulus 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 (EPSCA in transfected cells, 252 ± 12 pA; EPSCA in untransfected cells, 253 ± 18 pA, n = 15 pairs, p > 0.05; EPSCN in transfected cells, 256 ± 16 pA; EPSCN in untransfected cells, 268 ± 12 pA, n = 15 pairs, p > 0.05; figure 3a). We next investigated whether tau-shRNA had any effect on LTD. Consistent with the LTD experiments in MAPT+/− and MAPT−/− mice, tau-shRNA blocked LTD, whereas LTD was routinely induced in simultaneously recorded, neighbouring untransfected cells (tau-shRNA transfected: 92 ± 3% of baseline; untransfected: 63 ± 7%, n = 5, p < 0.05, tau-shRNA versus control; figure 3b). The block of LTD was a specific consequence of the knockdown of endogenous tau, because expression of a non-effective, scrambled tau-shRNA had no effect on LTD (61 ± 7%, n = 5, p > 0.05, compared with control, figure 3c). Furthermore, the tau-shRNA-mediated LTD deficit was rescued by co-expression of human tau (60 ± 4%, n = 5, p > 0.05, compared with control, figure 3d), which was resistant to knockdown (rat tau-shRNA selectively reduces the expression of rat tau but has no effect on human tau; data not shown). These findings are consistent with our KO studies, confirming that tau is required for LTD induction. Taken together, our data show that tau is required for LTD across two species (rats and mice) and in both juvenile and adult tissue.

(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. An
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-3\( \beta \) 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-3\( \beta \) 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 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

**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 \( \mu \)A, 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 mice (age between 7 and 11 months; \( MAPT^{+/+} \): 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 \( \mu \)M, 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.
levels of tau using Tau-5 (p > 0.05, n = 4, figure 4d), 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, 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, it is assumed to contribute to neuronal pathology, because it pos-
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 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 anesthetized (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-L, Cygnus Technology, USA), digitized (Digitdata 1321A, Axon Instruments, Foster City, CA, USA) and processed on a computer. To induce LTD, 100 pulses at 100 Hz were applied three times (at 180 s intervals), and to induce LTD, 900 pulses at 1 Hz were delivered. The amplitude and slope of each recorded fEPSP was measured using a custom application based on the MATLAB version 8 (Mathworks Inc., CA, USA). fEPSPs were analysed only when the maximal amplitude was over 1 mV, and 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 before being placed in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 124, KCl 3, NaHCO3 26, NaH2PO4 1.25, CaCl2 2, MgSO4 1 and d-glucose 10 bubbled with 95% O2/5% CO2. Transverse hippocampal slices (400 μm thick) were prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Hippocampal slices were stored in aCSF (20–25°C) for 1–2 h before transferring to the recording chamber, in which they were submerged in aCSF (30°C) flowing at 2 ml min−1. Extracellular field potentials were recorded in the CA1 region using glass electrodes containing NaCl (3 M). A stimulating electrode in CA2 was used to evoke field EPSPs (constant voltage, 100 μA, repeated at 30 s intervals). The slope of the evoked fEPSP was measured and expressed relative to the normalized preconditioning baseline. Data were captured and analysed using WinLTP (www.winltp.com). Experiments in which changes in the fibre volley occurred were discarded.

(c) Hippocampal slice culture

Hippocampal slice cultures were prepared from 6- to 7-day-old male Wistar rats, as previously described [19]. Whole-cell patch clamp recordings of CA1 neurons transfected with shRNA plasmids were made 3–4 days following transfection. Using a biolistic Gene Gun (BioRad, USA), neurons were transfected with plasmids expressing shRNA against rat tau protein (OriGene Technologies, MD, USA). A mixture of four different tau (0N/3R, 0N/4R, 1N/4R and 2N/4R) shRNA constructs (1:1:1:1) in pEGFP-V-RS vector) were used for tau silencing. A non-effective scrambled sequence shRNA was used as a negative control against MAPT. EPSCs were recorded using a multi-clamp 700B amplifier (Axon Instruments). Recordings were carried out in a solution containing (in mM): NaCl 119, KCl 2.5, CaCl2 4, MgCl2 4, NaHCO3 26, NaH2PO4 1, glucose 11, picrotoxin 0.02 and 2-chloroadenosine 0.01, gassed with 5% CO2/95% O2, at pH 7.4. To induce LTD, 200 pulses at 1 Hz were delivered at a holding potential of ~40 mV. AMPAR-mediated EPSC amplitude (EPSCa) was determined as the peak EPSC amplitude at a holding potential of ~70 mV. NMDAR-mediated EPSC amplitude (EPSCN) was determined 50–70 ms after the EPSCa peak at a holding potential of +40 mV. In some experiments, dual patch clamp recordings were made simultaneously from a pair of neighbouring CA1 pyramidal neurons, one transfected and the other untransfected. n values indicate number of cells, each obtained from independent slices. Error bars indicate s.e.m.

(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 Osnabrück, 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, Nanoprobe, 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, Nanoprobes). 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 uranum 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 (12500g), and divided into the crude synaptosomal fraction and synaptosome-depleted fraction. The crude synaptosomal fraction was further purified by hypotonic lysis and centrifugation (25000g), and the resultant pellet was the PSD-95-rich synaptosome fraction (LPI). The synaptosome-depleted fraction was further subjected to ultracentrifugation (100000g), and separated to the microsome fraction (P3) and cytoplasm fraction (S3). We used the following antibodies for experiments: NeuN, mouse monoclonal (Millipore; 1:1000) Tau-5, mouse monoclonal (Innogen; 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.

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