Ether stress-induced Alzheimer-like tau phosphorylation in the normal mouse brain

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Abstract  Tau is reversibly hyperphosphorylated in the mouse brain by starvation or cold water swimming. Here, we report tau phosphorylation in the hippocampus of normal mouse after ether anesthesia, known to trigger typical stress reactions. Robust phosphorylation of tau was observed immediately and 10 min after ether vapor exposure at Ser202/Thr205 and Thr231/Ser235, sites typically phosphorylated in Alzheimer brains. The phosphorylation levels returned to baseline by 1 h. The most conspicuous and consistent change in the protein kinases studied was the inactivating phosphorylation of Ser9 of TPKI/GSK3β in close correspondence with tau phosphorylation. These findings show that tau phosphorylation is a rapid physiological process integral to stress response system, and suggest involvement therein of TPKI/GSK3β.

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1. Introduction

Protein tau was discovered more than 30 years ago [1], but its physiological role has not been delineated beyond a broad and static role of stabilizing neuronal microtubules [2,3]. It is the main constituent of neurofibrillary tangles, one of the two defining histopathological features of Alzheimer’s disease (AD) [4], where it is phosphorylated to higher degrees and at more serine/threonine residues (hyperphosphorylation) [5] than in normal brains [6,7]. Causes of tau phosphorylation and the nature of its postulated toxicity in AD remain to be determined [8].

Our previous works demonstrated that tau in the brain undergoes rapid and reversible hyperphosphorylation when a normal mouse is starved for 2 days [9,10] or forced to swim in cold water stress (CWS) [11], posing a possible clue to the physiological significance of tau phosphorylation. The increases in the phosphorylation level were observed at many of the sites on tau known to be highly phosphorylated in AD brains. The response was most prominent in the hippocampus and its immediately neighboring cortical region but weak in the cerebellum, in correspondence with the known regional difference in susceptibility to AD pathology [12].

Starvation and CWS are both known to cause elevation of plasma corticosterone levels in rodents [13,14]. This led us to postulate that the dynamic physiological tau hyperphosphorylation is an integral part of the neural stress reaction [11]. Close observations of the animals under the above treatments, however, raised an alternative possibility that the phenomenon may simply reflect the intense motor exertion of searching for food or attempted escape from water. It was thus necessary to test a different stressful treatment that will not entail increased motor activity.

Ether is a convenient anesthetic long in use [15]. Inhalation of ether vapor elicits rapid release of ACTH into the circulation followed by corticosterone release from the adrenal cortex [16]. Ether-induced sharp rise in plasma corticosterone concentration attains a peak in about 30 min and reverts to basal levels by about 2 h after ether exposure in rodents [16,17]. Ether anesthesia is thus considered to elicit a typical stress response, and has been employed in numerous neuroendocrinological studies of stress (e.g., [18–20]). Here, we take advantage of ether anesthesia to examine whether and how tau phosphorylation takes place in this peculiar situation of stress without motor activity. Responses elicited in phosphorylation levels of multiple sites on tau and of protein kinases are then compared with those caused by CWS or starvation.

2. Materials and methods

2.1. Animals

Male C57BL/6Njcl mice of 10–11 weeks of age (Clea Japan, Tokyo, Japan) were housed in groups of 4 and allowed free access to food and water. The mice were maintained at 23 °C and under the light period of 0800–2000 and used in compliance with the protocols approved by the Animal Care and Use Committee of Mitsubishi Kagaku Institute of Life Sciences.

2.2. Stress treatments

Ether and CWS experiments were done between 1300 and 1800. For ether exposure, mice were placed in a covered cylindrical container (18 cm diameter, 12 cm height) in which paper towel wetted with diethyl ether was placed and equilibrated for >5 min at room temperature (23–27 °C). Mice were given 30 s of exposure three times, separated by 15 min of recovery periods. At various times of recovery in home cages after the third exposure, the mice were killed by cervical

Abbreviations: AD, Alzheimer’s disease; CWS, cold water stress; TPKI/GSK3β, tau protein kinase I/glycogen synthase kinase 3β

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dislocation. Under these conditions the mice generally recovered from immobility in 5–10 min. CWS treatment was administered as described [11]. Some mice were sacrificed after 2 days of food depriva-
tion [9].

2.3. Immunoblotting

The brains were removed immediately after sacrifice and placed in ice-chilled saline. Hippocampi were quickly removed by manual dis-
section. The tissues were immediately homogenized using a Physco-
tron NS-310E microhomogenizer (Mitsotech Nichion, Tokyo, Japan) in 300 μl of O+ sample buffer [11]. The homogenates were immediately boiled for 3 min. Protein concentration was determined using the Bio-Rad Protein Assay with BSA as standard. Appropriate amounts of samples (6 μg protein/lane for TauC antibody, and 18 μg/ lane for all other antibodies) were run on 10% SDS-PAGE. Protein bands were electrothermally transferred to Protran BA 85 nitro-
cellulose membrane (Schleicher and Schuell) for 60 min at 8V in Trans-Blot SD Cell blotting apparatus (BioRad). The blots were
immunoreacted, developed by ECL, and bands quantified as reported

2.4. Primary antibodies

Rabbit affinity purified antibodies PS199, PT231, PT231/PS235, PS262, PS396, and PS404 are specific to tau phosphorylated at the res-
idue indicated, and Tau-C recognizes a C-terminal epitope of tau inde-
pendent of its phosphorylation [21,22]. Amino acid residue number
refers to human longest tau [23]. Mouse monoclonal antibody AT8
(Innogenetics) recognizes tau dually phosphorylated at Ser202 and
Thr205 [24]. Rabbit antibody PS9 recognizes TPKI/GSKβ3 phosphory-
lated at Ser9, and antibody PY216 recognizes TPKI/GSKβ3 phos-
phorylated at Tyr216 and GSKβ3 phosphorylated at Tyr279 [25].
Anti-TPKI/GSKβ3 mouse monoclonal antibody T1.7 was produced
against a C-terminal peptide of rat TPKI/GSKβ3 [26]. Phospho-Akt
(Ser473) Antibody (#9271) and Akt Antibody (#9272) were from Cell
Signaling Technology (Beverly, MA, USA).

3. Results

3.1. Ether-induced tau phosphorylation

A 30 s exposure to ether vapor was found to be the practical limit with young adult male mice of C57BL/6N strain, as long-
er exposure times entailed risk of fatality. Our initial analysis with mice subjected to single 30 s ether exposure did show in-
creased levels of phosphorylated tau, but a large individual variability was observed in the extent of the response. We
therefore adopted three sessions of 30 s ether exposure separa-
ated by 15 min each of recovery periods in home cages [27]. To confirm that this three-session exposure does cause a stress reaction, plasma corticosterone concentration was assessed 10 min after the end of the third ether exposure. A robust ele-
vation in the steroid hormone was observed (32.8 μg/dl com-
pared to 8.3 μg/dl for untreated controls, n = 4 each) as
assayed by a commercial EIA immunoassay, indicating that
our treatment paradigm also causes a typical stress reaction
established for single-exposure procedures [16–20].

Phosphorylation level of tau was studied by quantitative
immunoblot analysis of hippocampal extracts from individual
mice (as numbered above the lanes) sacrificed at various times
after ether exposure (Fig. 1). As our previous analyses showed
that tau phosphorylation is most robust in the hippocampus
[9,11], and the initial AD neuropathology is associated with this
brain structure, we here focused on analyzing the hippo-
campus. The first blot was probed with antibody TauC to as-
sess possible changes in the protein level of tau (Fig. 1A). Tau
bands of decreased mobility (bands 2–4), characteristic of
hyperphosphorylated tau species [8], were evident for each
mouse sacrificed immediately (0) or 10 min after ether expo-
sure (Fig. 1A, mice 5–12). Integration of band density across
the tau sub-bands 1–4 and statistical evaluation across mouse
groups showed that there was no significant change in the tau
protein level up to 120 min after ether exposure (Fig. 2A, graph).

The same set of hippocampal extracts were further analyzed
with eight antibodies, each specific to different phosphoryla-
tion-dependent epitopes on tau. This set of sites represents a
subset of about two dozen sites shown to be phosphorylated in PHF-tau of AD brains [6,7]. Antibody Tau-1 recognizes
non-phosphorylated stretch of amino acids from 192 to 204
[28]. Decreases in Tau-1 binding observed at 0–20 min after ether (Fig. 1B, mice 5–16) indicate a transient phosphorylation
of this region of tau. Robust increases were observed during
this time frame in the binding of phosphorylation-dependent
antibodies PS202/PT205(AT8), PT231, and PT231/PS235
(Fig. 1D–F) to the respective epitopes straddling the amino
cids referred to. Antibodies PS202/PT205(AT8) and PT231/
PS235 are specific to dually phosphorylated epitopes. Smaller
increases in phosphorylation were indicated at Ser199
(Fig. 1C) and Ser262 (Fig. 1G), but these changes did not
reach statistical significance. Binding of PS404 was not affected
(Fig. 1I). Large transient increases at Ser202/Thr205 and
Thr231/Ser235, and near absence of changes at Ser404 are
qualitatively similar to what were previously observed in the
hippocampus of mice after CWS [11].

A large body of mostly in vitro studies has been devoted to
identifying protein kinases responsible for the many phophor-
ylation sites on tau [29]. Possible changes in the phosphoryla-
tion states of protein kinases TPKI/GSKβ3, Akt, Erk1/2,
JNK, p38, CaMKIIα were probed in the hippocampal extracts
with a panel of phosphorylation-specific as well as phosphory-
lation-independent anti-kinase antibodies (Fig. 2). A dramatic
increase in the phosphorylation level of Ser9 of TPKI/GSKβ3
was observed immediately and 10 min after ether, followed by
rapid virtual reversion to baseline by 20 min (Fig. 2B). This oc-
curred in the absence of significant changes in the protein level
(Fig. 2D) or Tyr216 phosphorylation level (Fig. 2A) of the en-
zyme. Interestingly, a smaller significant increase in the phos-
phorylation level was observed for Ser473 of Akt (Fig. 2C),
considered to be responsible for phosphorylation of Ser9 of
TPKI/GSKβ3 [30]. Protein level of Akt showed no significant
change (Fig. 2E). Changes were significant neither for the
phosphorylation levels or protein levels of other protein ki-
nases studied: Erk1/2 (Thr202/Tyr204), JNK (Thr183/
Tyr185), p38 (Thr180/Tyr182) or CaMKIIα (Thr286) (data
not shown). As phosphorylation of Ser9 of TPKI/GSKβ3
has been shown to cause partial inactivation of the enzyme
[31], the transient increase in this phosphorylation here ob-
served cannot on its own explain the transient increase in tau
phosphorylation at Ser202/Thr205 or Thr231/Ser235 (dis-
cussed below). Further analysis is required to delineate the
complex enzymatic mechanism of ether-induced tau phos-
phorylation.

The characteristic robust, rapid and transient increase in
Ser9 phosphorylation of TPKI/GSKβ3, together with the rela-
tive absence of changes in other protein kinases studied, is
closely reminiscent of the changes observed in the hippocampus
after CWS [11] and suggests that a common signaling pathway
involving tau is engaged in response to the two different types
of stress stimuli.
Fig. 1. Tau phosphorylation levels in the hippocampus after ether exposure. Mice were sacrificed at various times (min) after ether exposure as shown at the top. Each lane represents a single mouse. Immunoblot panels were probed with anti-tau (A) or phosphorylation-dependent anti-tau (B-I) antibodies as indicated. Chemiluminescence signals from relevant bands were quantified and statistically treated, and are shown in graphs to the right to indicate relative changes (Figs. 1–4). Vertical bars denote standard deviation. Difference from control values were significant at $P < 0.01$ (**) or $P < 0.05$ (*) as indicated, and likewise in Figs. 2–4.

Fig. 2. Changes in hippocampal protein kinases after ether exposure. Same set of mice as in Fig. 1. Phosphorylation dependent anti-TPKI/GSK3β (A,B) or anti-Akt (C) antibodies were used. Phosphorylation-independent anti-TPKI/GSK3β (D) or anti-Akt (E) antibodies monitored the protein levels of the kinases.
We previously reported tau phosphorylation in starved mice \[9,10\] and mice subjected to CWS treatment \[11\]. We here made a direct quantitative comparison of the reactions in a single set of analysis. As phosphorylation/dephosphorylation reactions in the three treatment paradigms do not follow the same time course, we chose to compare the extent of Ser/Thr phosphorylation at time points where the overall response empirically reaches a maximum in respective paradigms. Thus, new sets of mice were sacrificed at 10 min after the end of ether exposure, 20 min after 5-min CWS, or after 48 h of food deprivation.

Fig. 3 shows the immunoblots of hippocampal homogenates from five sets of four mice each subjected to five different conditions and respective quantitative evaluations in graphs. After both ether anesthesia and CWS, phosphorylation level was elevated across seven epitopes (Fig. 3B–H), mostly with statistical significance, and in every case the extent of hyperphosphorylation was larger after CWS than after ether. Notably most conspicuous changes were the hyperphosphorylation at AT8 (Fig. 3D) and Thr231/Ser235 (Fig. 3F) sites.

The changes after starvation were less dramatic than after ether or CWS. They were less than in our previous results \[9\], presumably because the starved mice in the current set had larger body weights. Nonetheless the direction of changes was the same as in ether and CWS experiments across the phosphorylation sites. Most robust increases were again at AT8 and Thr231/Ser235 sites. Changes at Ser404 were nonsignificant across all three stress paradigms (Fig. 3I).

The hippocampal homogenates from the three stress experiments were also examined for protein kinases and their phosphorylation states. Dramatic and transient increases in TPKI/GSK3β phosphorylation at Ser9 accompanied by Akt phosphorylation were observed after ether or CWS (Fig. 4B and C). Changes in phosphorylation levels of protein kinases Erk1/2, JNK, p38, and CaMKIIα did not reach 1% significance (data not shown).

The starvation experiments involved a longer time scale of days compared with minutes in the other two stresses, while CWS entailed appreciable hypothermia and ether caused immobility. Despite the disparate nature of the stress stimuli here tested, the overall pattern of tau hyperphosphorylation was common to the three stress paradigms, indicating that a
convergent mechanism was engaged to effect the tau phosphorylation observed.

4. Discussion

Previously we demonstrated that starvation and forced cold water swimming cause reversible hyperphosphorylation of tau in the mouse brain [9–11]. Above we showed that a similar rapid tau phosphorylation occurs in the mouse hippocampus also after ether exposure, a treatment known to cause typical acute stress reactions. These findings together show that tau phosphorylation is a rapid physiological process integral to neural stress response system. It is of particular interest that phosphorylation is a rapid physiological process integral to acute stress reactions. These findings together show that tau phosphorylation after ether exposure as well as CWS. Mechanistic relationship between the two phenomena appears more likely given a high degree of mouse by mouse correspondence between the two phosphorylation reactions among the individually variable mouse sets. No protein kinases studied other than TPKI/GSK3β and Akt exhibited changes in their phosphorylation that bore such a close relationship to tau hyperphosphorylation.

Interestingly, phosphorylation of Ser9 is generally associated with reduced kinase activity of TPKI/GSK3β [31], that takes place in many cellular systems upon stimulation with insulin, IGF-1, endothelin-1, EGF, and isoproterenol among others [32,33]. It is necessary, therefore, to postulate an indirect pathway from the less active GSK3β to increased tau phosphorylation, such as involving tau phosphatase activity, if Ser9 phosphorylation is to be causally reconciled with tau hyperphosphorylation.

Akt kinase also showed an elevation in its phosphorylation level of Ser473 (Fig. 2C). This phosphorylation activates Akt, and is in agreement with its proposed role in phosphorylation of TPKI/GSK3β-Ser9 [32]. A caution, however, is necessary here in interpreting quantitative data on protein kinases assessed in brain tissue homogenates. Unlike tau, which is a neuronal protein, protein kinases are generally ubiquitous with respect to cell types. As for TPKI/GSK3β, we have observed its primarily neuronal localization in the rat brain [34].

Our kinase findings point to the possibility that regulation of protein phosphatase activity may be important in ether-induced tau phosphorylation. Our previous work presented data suggesting involvement of PP2A in starvation-induced tau phosphorylation [10], but the precise identity of phosphatases involved in other stress responses remains to be studied. Unlike protein kinases, for which relatively straightforward inferences on activity levels can be made through their phosphorylation levels, most protein phosphatases comprise catalytic and heterogeneous regulatory subunits, and are elusive with respect to their activation states to be inferred from immunoblots. Moreover, they occur in different cell types in the brain, such that phosphatase activity measurements of brain tissue homogenates cannot escape interpretational ambiguity as to what fraction thereof comes from neurons and what from glia, or as to axonal as opposed to somatodendritic localization of the enzymes. For these reasons this important subject will require careful and extensive studies, including those making use of uniform cells in culture. Tau phosphatases have been studied in AD brains (e.g. [35]).

The neural mechanism of activation of stress responses by ether is complex and not fully understood. Immobilization stress causes glutamate release in the hippocampus [36,37], while cultured primary neurons showed increased tau phosphorylation when stimulated with glutamate [38,39]. The stress-induced tau phosphorylation we observed may reflect mobilization of a neural stress response circuits converging onto the hippocampus.

Arendt et al. [40] described PHF-like tau hyperphosphorylation in the brain of squirrel during torpor phase of hibernation. Hibernation is an elaborate form of adaptation to low ambient temperature and food scarcity, and its control involves the brain stem, hypothalamus and hippocampus [41]. Stress reactions are mechanisms for adaptation to altered environments and restoration of homeostasis including the management of energy balance at the organism level through hypothalamus-pituitary-adrenal axis [42]. To what extent hibernation control and stress response systems share common neural mechanisms remains to be explored.

Tau hyperphosphorylation was also reported in the brains of mice injected with insulin or deoxyglucose [43]. The authors...
ascribed the response to a direct effect of hypothermia on the catalytic activity of kinases and phosphatases. In our previous study forced swimming in cold water triggered a robust tau phosphorylation in the mouse hippocampus but the reaction was marginal in the cerebellum despite its comparable tau content [11]. It is unlikely that the cerebellum escaped hypothermia under the experiment. On the other hand increased phosphorylation of tau has been reported in neurons or neuroblastoma cells in culture after treatment with glutamate [38]. NMDA [44], or insulin [45] among others in the absence of temperature change. Heat shock to 45 °C actually produced reversible phosphorylation of tau in PC12 cells [46]. Considering also that we observed qualitatively similar patterns of tau phosphorylation and concurrent TPKI/GSK3β-Ser9 phosphorylation with our three very different stress stimuli, it seems more plausible to view in vivo tau phosphorylation we observed as a molecular event associated with the activation of stress response neural system.

The rapid nature of tau phosphorylation and dephosphorylation taking place in minutes, as demonstrated here and in cold water stress experiments [11], compels us to suspect a dynamic physiological role of tau phosphorylation in some aspect of signal transduction within neurons, such as neuronal plasticity. It is interesting in this context that tau-deficient mice with largely intact brain anatomy have shown an impairment in aversive learning [47]. We have indeed observed increased phosphorylation of AT8 and Thr231/Ser235 sites of tau together with TPKI/GSK3β-Ser9 phosphorylation in the hippocampus during fear conditioning (Fujio et al., submitted for publication). It is possible that tau phosphorylation is an integral component of the machinery of neural plasticity [40]. It appears then plausible that a stress-related dysregulation of tau phosphorylation in the hippocampus can manifest as memory impairment in the development of diseases such as Alzheimer’s.

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References


