Stress-induced hyperphosphorylation of tau in the mouse brain

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Abstract **We previously showed that starvation causes reversible hyperphosphorylation of tau in the mouse brain. To explore possible involvement of stress in tau hyperphosphorylation quantitative analysis of phosphorylated tau in four brain regions of mice subjected to cold water stress (CWS) was made by immunoblot analyses using phosphorylation-dependent antibodies directed to eight sites on tau known to be hyperphosphorylated in the brain of Alzheimer's disease (AD) patients. Ser199, Ser202/Thr205, Thr231/Ser235 were hyperphosphorylated 20 and 40 min after CWS. The response was pronounced in the hippocampus and cerebral hemisphere, but weak in the cerebellum in parallel with the regional vulnerability in AD. Among the regulatory phosphorylation of protein kinases studied, a transient phosphorylation of tau protein kinase I/glycogen synthase kinase 3β at Ser9 was most conspicuous.**

Key words: Tau phosphorylation; Protein kinase; Hippocampus; Mouse; Cold water stress; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is characterized by accumulation of senile plaques and neurofibrillary tangles (NFT) in the brain. NFT mainly consist of highly phosphorylated tau proteins [1]. In AD brains tau is phosphorylated at a number of sites [2,3] resulting in a reduced ability to bind microtubules [4]. Discovery of mutations in the tau gene as causes of a form of dementia (FTDP-17) has directed increasing attention to tauopathy as a possibly central pathogenetic process in a number of neurodegenerative diseases such as AD [5].

We recently discovered that food deprivation causes progressive phosphorylation of tau in the normal mouse brain at six sites that are all characteristically highly phosphorylated in AD brain [6]. The enhancement of tau phosphorylation was most prominent in the hippocampus, followed by the cerebral cortex, and minimal in the cerebellum, in parallel with the known rank order of vulnerability to NFT pathology of brain regions in AD patients [7,8]. The discovery raised not only the question of the physiological significance of the reversible tau phosphorylation in the normal brain, but also the possibility of involvement of a common mechanism in early stages of AD pathogenesis. We therefore set out to explore whether other stressful stimuli will cause in vivo phosphorylation with characteristics possibly relevant to AD.

Forced swimming of rodents in cold water is one of the established experimental systems employed in the studies of stress. Korneyev observed an increased tau phosphorylation caused by cold water stress (CWS) in the rat brain [9]. Only soluble tau fractions were analyzed by enzyme-linked immunosorbent assay with two phosphorylation-dependent antibodies. Phosphorylation of tau at one site was reported in the *obese* strain of mouse after CWS [10]. These pioneering studies, however, were limited in the number of phosphorylation sites examined, and the wild-type mice remained to be studied. We have now made a quantitative immunoblot analysis of phosphorylation levels of SDS-solubilized tau at seven independent sites for four regions of the normal mouse brain after CWS. The time course and the extent of phosphorylation were found to depend on the site on tau protein, and also on the brain region. Changes in the levels of regulatory phosphorylation of tau protein kinase I/glycogen synthase kinase 3β (TPKI/GSK3β), Erk2 and p46 JNK (c-Jun N-terminal kinase) protein kinases were found.

2. Materials and methods

2.1. Animals

Male C57BL/6Njcl mice of 10–13 weeks of age (Clea Japan, Tokyo, Japan) weighing 25.0 ± 3.0 g were housed in groups of four and allowed free access to food and water. A total of 59 mice maintained at 23°C and under the light period of 08:00–20:00 were used in compliance with the protocols approved by the Animal Care and Use Committee of Mitsubishi Kagaku Institute of Life Sciences.

2.2. CWS

CWS was given between 13:00 and 18:00. Mice were immersed up to the neck in ice-cold water of 5 cm depth in a 16 cm diameter ceramic container for 5 min, after which they were gently wiped dry and returned to the cages. Mice were killed by cervical dislocation at 20, 40 and 90 min after CWS.

2.3. Western blotting

The brains were removed immediately after sacrifice and placed in ice-chilled saline. Desired brain regions were quickly removed by manual direction: hippocampus, ventroposterior quadrant of the cerebral hemisphere (VP), the rest of the cerebral hemisphere (RH) and cerebellum. The tissues were immediately homogenized using a Physcotron Microhomogenizer (Microtech Nichion, NS-310E) in 10 times volume/tissue weight of O+ buffer (62.5 mM Tris–HCl, pH 6.8, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 5 mM NaF, 100 μM orthovanadate, 1 μM okadaic acid, 1 mM

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Abbreviations: AD, Alzheimer's disease; CWS, cold water stress; Erk, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PHF, paired helical filaments; RH, rest of cerebral hemisphere; TPKI/GSK3β, tau protein kinase/glycogen synthase kinase 3β; VP, ventroposterior quadrant of cerebral hemisphere

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PMSF, 1 mM EDTA, 1 mM EGTA). The homogenates were immediately placed in boiling water for 3 min. Protein concentration was determined after 20 times dilution with saline by the Bio-Rad Protein Assay with bovine serum albumin (BSA) in O+ buffer as standard. Appropriate amounts of samples (4 μg protein/lane for tau antibodies except for PS422, and 18 μg/lane for PS422 and all kinase antibodies) were run on 10% SDS–PAGE and electrophoretically transferred to nitrocellulose membrane (Protran BA 85, Schleicher and Schuell) for 60 min at 8 V in a semi-dry blotting apparatus (Bio-Rad, Trans-Blot SD Cell). Membrane was blocked in phosphate-buffered saline (PBS) containing 10% (w/v) skim milk and 0.5% Tween 20 (Sigma) for 1 h, followed by incubation with primary antibodies for 2 h, washing in PBS with 0.1% Tween 20, and then by incubation with secondary antibodies conjugated to horseradish peroxidase (Pierce, ImmunoPure Goat Anti-Rabbit IgG (H+L) Peroxidase Conjugated, or ImmunoPure Goat Anti-Mouse IgG (H+L) Peroxidase Conjugated, each at 1:10 000) for 1 h at room temperature. For immunoblotting with anti-kinase antibodies, the blots were blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST). Primary and secondary antibodies were diluted in TBST containing 5% BSA and TBST containing 5% skim milk, respectively. After washing, the blots were soaked in SuperSignal West Pico Chemiluminescent Substrate (Pierce), and the signals were quantified with a luminescence image analyzer (LAS-1000plus, Fujifilm) and Image Gauge 3.0 software (Fujifilm), followed by statistical evaluation by analysis of variance. In separate experiments analyzing various amounts of a standard sample we confirmed approximate linear correspondence between the integrated band intensity values and the input protein under the conditions here employed.

2.4. Antibodies

Rabbit affinity-purified antibodies PS199, PT231/PS235, PS262, PS396, and PS422 are specific to tau phosphorylated at the residue indicated, and Tau-C recognizes the C-terminal epitopes of tau [11,12]. Mouse monoclonal antibody AT8 (Innogenetics) recognizes tau dually phosphorylated at Ser202 and Thr205. Rabbit antibody PS9 recognizes TPKI/GSK3β phosphorylated at Ser9, and antibody PY216 recognizes TPKI/GSK3β phosphorylated at Tyr216 and GSK3α phosphorylated at Tyr279 [13]. Anti-TPKI mouse monoclonal antibody T1.7 was produced by immunizing a BALB/c mouse against a C-terminal peptide of rat tau [14]. Anti-cdk5 and anti-p35 C-terminus antisera were described [15,16]. Other rabbit antibodies used to analyze various protein kinases were commercially obtained: anti-ACTIVE JNK pAb (pTGpY) (Promega), JNK1 (Santa Cruz), anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204) antibody (NEB), anti-Erk1/2 pAb (Promega).

3. Results

3.1. Phosphorylation patterns of tau in the mouse hippocampus after CWS

In AD brains tau is hyperphosphorylated, and a recent study has shown that PHF-tau is phosphorylated in at least 25 sites [3]. Changes in phosphorylation levels of tau in the mouse brain after CWS were studied with respect to seven of such sites for which specific antibody probes were available. Mice were put in ice-chilled water for 5 min, and after various periods of recovery, four brain regions were quickly taken: the hippocampus (HC), the VP (containing entorhinal cortex and amygdala), the RH (containing striatum), and the cerebellum (CB). Aliquots containing equal amounts of protein were analyzed by quantitative immunoblotting using phosphorylation-dependent site-specific anti-tau antibodies (Fig. 1B–H). Changes in the phosphorylation state of tau are reflected in altered signal intensities of tau bands, and also by discrete mobility shifts characteristic of phosphorylated tau [17].

In immunoblots of Fig. 1, each lane shows bands obtained for one representative mouse out of four analyzed for each time point. Tau in the hippocampus of unstressed control mice occurred mostly in the fastest migrating band 1 as revealed by the phosphorylation-independent antibody, Tau-C (Fig. 1A, leftmost lane). After 20–40 min following CWS, a large portion of tau was found in the retarded bands 2–4, accompanied by a diminished intensity of band 1. The band 1 regained intensity, with diminution of bands 2–4, after 90 min. These results suggest, by virtue of known phosphorylation-dependent mobility shift, a transient rise in tau phosphorylation level after CWS.

Direct phosphorylation signals obtained with seven antibodies recognizing distinct phosphorylated sites are displayed in Fig. 1B–H, the first column for the hippocampus (HC). For antibodies PS199, AT8, and PT231/PS235 (Fig. 1B–D) the signals were barely detected in the control mice, whereas a robust staining mostly in band 2 appeared at 20 and 40 min after CWS, which declined toward 90 min. For three other antibodies, PS396, PS404, and PS422 (Fig. 1F–H), the phosphorylation signals were detected in the basal band 1 in the controls. After CWS the signals for the antibodies PS396 and PS404 showed transient massive mobility shifts to band 2 (Fig. 1F,G), that largely reverted toward 90 min. Quantitative results obtained by integrating band intensities for four mice at each time point are shown in Fig. 1I–P. No significant changes in integrated band intensity were observed at Ser262 in the hippocampus (Fig. 1M), while a transient decrease was seen for Ser422 (Fig. 1P).

These results show that rapid and reversible phosphorylation of tau at multiple phosphorylation sites takes place in the hippocampus of mice during recovery from stress of CWS. The overall pattern of phosphorylation at the height of response to CWS was broadly similar to that attained in the hippocampus after 2 days of starvation [6], and also to PHF-derived tau from AD brains [2,3].

3.2. Phosphorylation patterns of tau in the cerebral cortex and cerebellum after CWS

In our previous study of starved mice, changes in tau phosphorylation were robust in the hippocampus but minimal in the cerebellum [6]. Analysis of tau phosphorylation after CWS was made also of the cerebral and neocortical tissues of the same set of mice as used for the hippocampal study. The cerebral hemisphere was divided into a ventroposterior quadrant (VP) and the rest (RH) because of particular interest in the entorhinal and amygdalar tissues with strong connectivity to the hippocampus.

When compared on the basis of total extracted protein, the

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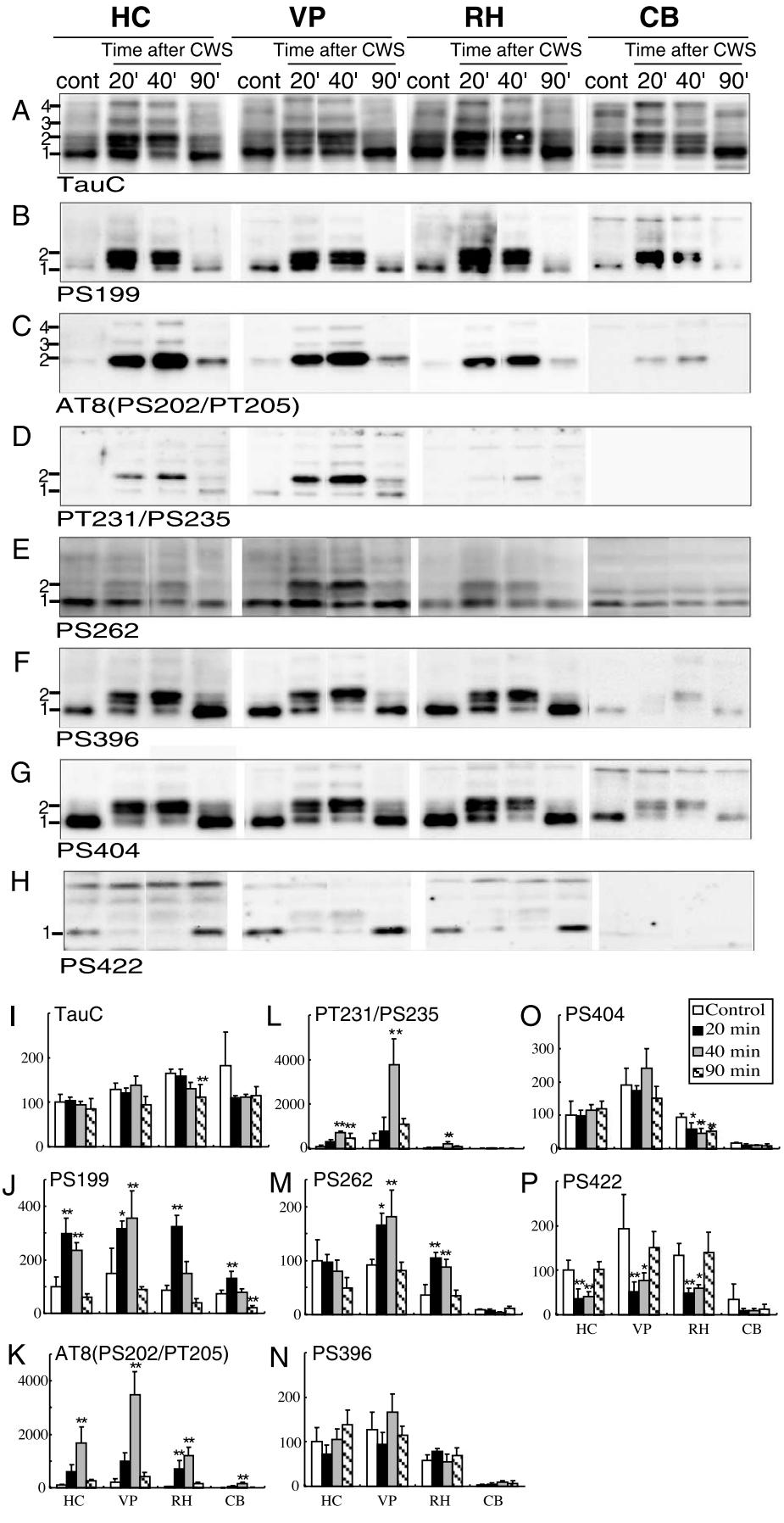
Fig. 1. Increased phosphorylation and electrophoretic mobility shifts of tau in four regions of the mouse brain after CWS. Mice underwent 5 min of CWS and recovered for 20, 40, or 90 min before sacrifice and removal of brain. Control mice received no CWS (cont). Panels A–H show immunoblots of extracts from one representative mouse out of four analyzed for each time point. Panel A was probed with Tau-C antibody specific to tau and independent of phosphorylation. Panels B–H are immunoblots obtained with site-specific phosphorylation-dependent tau antibodies as indicated. Bands 1–4 of tau (apparent molecular weights: 56, 60, 64, and 70 kDa) are indicated at the left. Panels I–P are a quantitative presentation of band intensities as functions of time and brain region at respective epitopes. Significant difference from control at $P < 0.01$ and $P < 0.05$ are denoted by ** and *, respectively, and likewise in Figs. 2 and 3.


HC
VP
RH
CB
Time after CWS
cont 20' 40' 90'
A
TauC
B
PS199
C
AT8(PS202/PT205)
D
PT231/PS235
E
PS262
F
PS396
G
PS404
H
PS422
I TauC
L PT231/PS235
O PS404
Control
20 min
40 min
90 min
J PS199
M PS262
P PS422
K AT8(PS202/PT205)
N PS396
HC VP RH CB

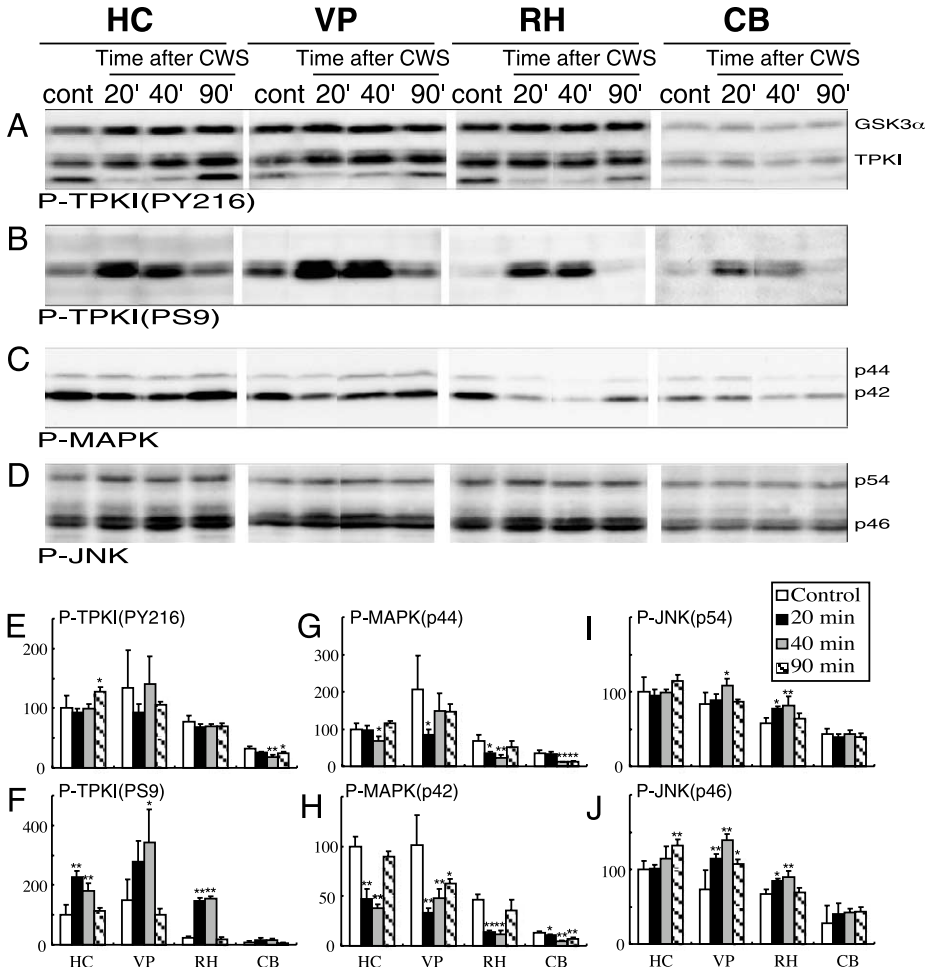


Fig. 2. Changes in the phosphorylation levels of several protein kinases after CWS. The same brain extracts as in Fig. 1 were analyzed by quantitative immunoblotting with phosphorylation-dependent anti-protein kinase antibodies as indicated.

cerebellum had a comparable content of tau protein as the hippocampus (Fig. 1A,I). The phosphorylation level of tau, however, was much lower, and hyperphosphorylation was not observed after CWS apart from weak changes at Ser199 and AT8.

In the hemispheric regions VP and RH, the temporal changes in the phosphorylation levels at respective sites were qualitatively similar to that observed in the hippocampus, with the exception of Ser262, where clear hyperphosphorylation was seen at 20 and 40 min in contrast to the hippocampus. The level of phosphorylation on the basis of equal total protein attained at 40 min was higher in VP than in RH for the epitopes of PS199, AT8, PT231/PS235, and PS262.

These results show that CWS-induced tau phosphorylation takes place in the cerebral hemispheres in a pattern broadly similar to that in the hippocampus, and that the phosphorylation level of tau in the cerebellum is basically unresponsive to CWS.

3.3. Protein kinases after CWS

Previous studies showed that tau can be phosphorylated in vitro by a number of protein kinases [18,19], including TPKI/GSK3β, tau protein kinase II (TPKII) consisting of cdk5 and p25, Erk1 and 2, JNK, and p38 kinase. Cotransfection experiments have shown phosphorylation of tau in cells by TPKI/GSK3β and by TPKII [20–22]. The kinases responsible for phosphorylating tau in the brain, however, remain to be established. As a first step to clarifying the mechanisms of CWS-induced tau phosphorylation, the same set of tissue homogenates as used for Fig. 1 were analyzed with available antibodies specific to the phosphorylated state of several protein kinases (Fig. 2).

Ser199, Thr231, and Ser396 have been shown to be phosphorylated by TPKI/GSK3β [23]. Two regulatory phosphorylation sites are known in this kinase. Phosphorylation of Tyr216 is considered essential for TPKI/GSK3β activity [24], and phosphorylation of Ser9 is associated with partial inhibition [25]. No significant change was observed in the phosphorylation level of Tyr216 after CWS (Fig. 2A,E). On the other hand, dynamic changes were observed at Ser9, whose phosphorylation level sharply rose after CWS and then declined (Fig. 2A,G). During these experiments the protein level of TPKI/GSK3β did not undergo significant changes (Fig. 3A,G).

TPKII phosphorylates tau in vitro at Ser202, Thr205, Ser235 and Ser404 [26], and these sites were all highly phosphorylated in the mouse brain after CWS (Fig. 1). Protein levels of cdk5, the catalytic subunit of TPKII, did not change after CWS (Fig. 3I), but a mobility shift was observed with p35, an activator of cdk5 (Fig. 3C, uppermost bands), indicat-

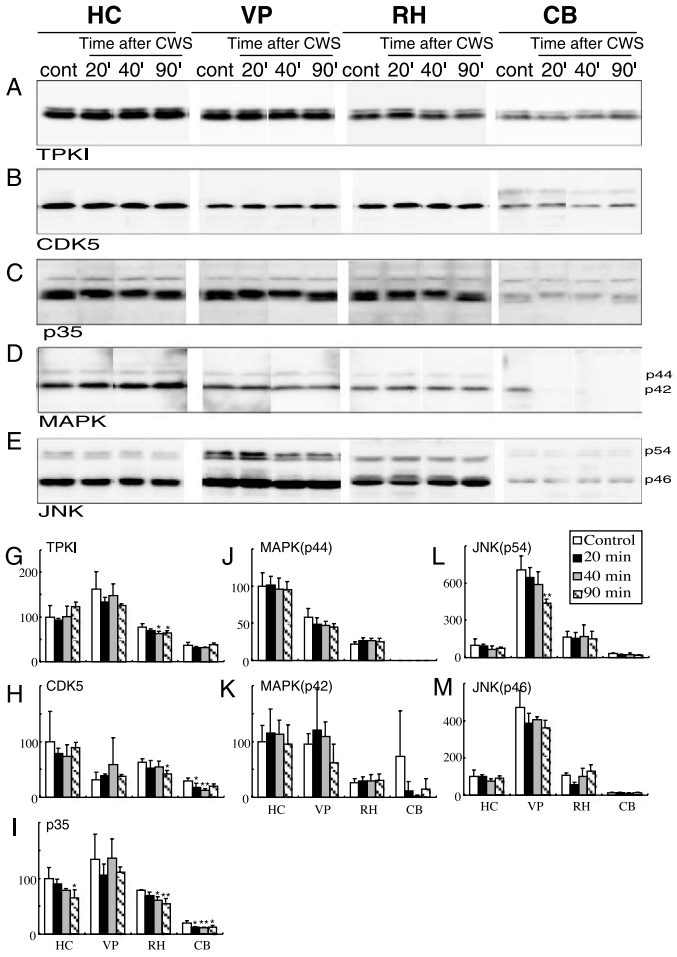


Fig. 3. Relative invariance of protein levels of several protein kinases in the hippocampus after CWS. Quantitative immunoblotting made use of phosphorylation-independent kinase-specific antibodies as indicated.

ing possible activation of cdk5. The antibody used was directed to the common carboxyl-terminus of p35 and p25, a truncated form of p35 [16,27], yet no p25 band was detected in our samples of mouse brain tissues (data not shown).

Ser199, Ser202, Ser235, Ser396 and Ser404 have been shown to be phosphorylated by MAPK (Erk1 and 2) in vitro [28,29], while Ser202 and Thr205 were reported to be the sites for JNK action [30], and Ser202, Thr205, Thr231, Ser235, Ser396 and Ser404 the sites for p38 kinase [29]. The phosphorylation level of p42 MAPK (Erk2) underwent a transient decrease (Fig. 2H), while that of p46 JNK showed an increase (Fig. 2J) after CWS. Protein levels of these isoforms did not show significant changes up to 90 min after CWS (Fig. 3J–M). No significant changes were observed in the phosphorylation of p38 kinase (data not shown).

4. Discussion

Our quantitative immunoblot analysis found that CWS causes a rapid and reversible enhancement of tau phosphorylation in the mouse brain at seven hydroxyl residues studied that are highly phosphorylated in AD brains. This phenomenon was pronounced in the hippocampus and in other parts of the cerebral hemisphere (the hemispheric regions VP and RH), but weak in the cerebellum.

4.1. Significance of tau phosphorylation

We previously showed that food deprivation of mice leads to increased levels of tau phosphorylation, most notably in the hippocampus. The patterns of tau phosphorylation with respect to the six phosphorylation sites and of immunoblot band shifts observed after 2 days of starvation [6] were similar to those found in this study after CWS. Starvation is a form of stress [31]. Papasozomenos studied tau phosphorylation in the brain of heat-shocked rats using Tau-1 and PHF-1 monoclonal antibodies, and found elevated phosphorylation levels only in female rats [32–34]. Korneyev showed a transient increase in tau phosphorylation at PHF-1 and Tau-1 sites in the rat hippocampus and other brain regions after CWS [9], and an enhanced response at AT8 site in fat *ob*/*ob* mice compared to lean *ob*/+ mice [10]. In this context, our finding that forced swimming in cold water causes in wild-type mice rapid and robust hyperphosphorylation of brain tau at seven sites raises a possibility that tau phosphorylation is an integral part of the neural response to stressors in general.

Hippocampus is a nodal tissue in the control of neural reactions to stress [35] as well as of declarative memory which is affected in early stages of AD [36]. In fact the AD neuropathologies have been strongly associated with the hippocampus, entorhinal cortex, and amygdala, and AD has been thus recognized as limbic dementia [37]. Repeated restraint stress

in the rat has been shown to exacerbate neuronal damage in the hippocampus caused by a variety of excitotoxic and metabolic insults to the brain leading to memory loss [38]. It is thus tempting to speculate that phosphorylation of tau is involved in certain aspects of central processing of stress stimuli.

Certain differences in tau phosphorylation and dephosphorylation pattern were found among the phosphorylation sites on tau examined above. The sites N-terminally flanking the microtubule binding domain of tau (roughly amino acids 244–367) [39] exhibited relatively crisp temporal changes in phosphorylation state compared to the C-terminally flanking sites. Thus Ser199, Ser202, Thr205, Thr231, and Ser235 underwent dramatic phosphorylation and dephosphorylation during 90 min after CWS, while Ser396 and Ser404 did not exhibit appreciable changes in integrated phosphorylation levels as detected by immunoblotting. Phosphorylation of Ser199 and Thr231 by TPKI/GSK3β is facilitated by prior phosphorylation by TPKII of Ser202 and Ser235, respectively [11], and phosphorylation of Ser202 and Thr205 by TPKII is stimulated by microtubules [40]. These findings together suggest that the phosphorylation events at sites in the two flanking regions may subserve distinct roles in the function of hyperphosphorylated tau.

4.2. Involvement of protein kinases

In an initial effort to probe into the molecular mechanisms of regulation of tau phosphorylation after CWS, we studied the phosphorylation levels of protein kinases implicated in tau phosphorylation and for which phosphorylated form-specific antibodies were available (Fig. 2). Immunoblot analyses with phosphorylation-independent anti-protein kinase antibodies indicated that protein levels of the protein kinases studied remained largely invariant during the period after CWS studied (Fig. 3).

TPKI/GSK3β has been shown to phosphorylate tau producing a characteristic mobility shift [20,41–43] and is considered to be a most plausible protein kinase to confer PHF-like changes on tau in the human brain [18,44–46]. It is therefore intriguing that the most dynamic change in kinase phosphorylation after CWS was observed at Ser9 of TPKI/GSK3β (Fig. 2). As phosphorylation at this site is reported to lead to a decreased kinase activity (as assayed using substrates such as inhibitor 2, c-Jun, and a peptide from glycogen synthase, but not tau, [25]), increased Ser9 phosphorylation needs to be reconciled with the observed increased phosphorylation at Ser199, Thr231, and Ser396 of tau (Fig. 1), the sites shown to be phosphorylated by TPKI/GSK3β in vitro [23]. The decreased kinase activity of TPKI/GSK3β may be offset by an even larger decrease in protein phosphatase(s) responsible for dephosphorylation of tau at these sites. Alternatively, phosphorylation of Ser9 may entail an altered substrate specificity of the protein kinase or an altered interaction with other proteins such as scaffold proteins, bringing about tau hyperphosphorylation in vivo. A third intriguing possibility is that an increase in Ser9 phosphorylation may be a secondary consequence of increased tau phosphorylation through some negative feedback regulation impinging upon TPKI/GSK3β. Each of these possibilities warrants careful examination.

Phosphorylation of tau by TPKII/cdk5+p25 facilitates subsequent phosphorylation by TPKI/GSK3β [11], and cdk5 is another strong candidate kinase implicated in tau phosphorylation leading to neuronal dysfunction [18,22,47]. In our analysis p25 was not detected, but p35, a cdk5 activator, underwent a mobility shift after CWS, likely due to phosphorylation (Fig. 3C). Recent studies suggest that p35 is phosphorylated by cdk5 leading to proteolytic cleavage by proteasome [48,49]. This may indicate a transient activation of cdk5, followed by regulatory down regulation through p35 degradation, in the mouse brain after CWS.

Activation after CWS was also seen with p46 JNK (Fig. 2J). Interestingly, JNK activation in the mouse brain after restraint stress and novel environmental stimulation has been described [50]. JNK may be involved in phosphorylation at the AT8 epitope (PS202/PT205). A transient decrease in the phosphorylation level of p42 MAPK/Erk2 was found (Fig. 2C,H). This kinase is involved in a complex network of regulatory phosphorylation signaling in neurons [51], and the significance of its transient dephosphorylation after CWS remains to be clarified. Our findings with the various protein kinases indicate that the dynamic changes of tau phosphorylation we observed after CWS are likely to be the result of a complex regulatory network of protein kinases and phosphatases, and not to be ascribed to a single signal cascade [52]. Such a line of thinking encourages us to envisage a possibility that tau phosphorylation may represent an integral part of the normal molecular signal processing machinery, such as transcriptional regulation, beyond simply effecting a decrease in its affinity to microtubules [4].

Further studies are needed to understand fully the physiological significance of tau phosphorylation in the brain upon stress. Pharmacological analyses targeting protein kinases and phosphatases as well as stress hormones will be necessary to delineate the molecular mechanisms involved. It is hoped that further insights into the molecular consequences of stress in the brain will shed important light on the intriguing correspondence in regional susceptibility of tau pathology in AD on the one hand and stress-related physiological hyperphosphorylation on the other.

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