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Short Communication

Phosphorylation of the tau protein sequence 199–205 in the hippocampal CA3 region of Syrian hamsters in adulthood and during aging

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Abstract

Paired helical filaments formed by the abnormally phosphorylated microtubule-associated tau are a main sign of Alzheimer's disease and other neurodegenerative disorders. The hippocampal CA3 region, a brain region with a high degree of synaptic plasticity, is known to be strongly involved in tau hyperphosphorylation in several neurodegenerative diseases. In addition, reversible tau phosphorylation was observed during hibernation in European ground squirrels. The present study provides data on the tau phosphorylation status in the hippocampus of euthermic Syrian hamsters (*Mesocricetus auratus*), laboratory animals potentially prone to hibernation. Mossy fibers in the CA3 region of all investigated hamsters were immunostained using an antiserum detecting phospho-serine 199 of tau. A similar staining pattern was obtained with CP-13 detecting phospho-serine 202. In contrast, the monoclonal antibody AT8, recognizing both phosphorylated serine 202 and threonine 205, stained the CA3 region only in old hamsters. These findings implicate an additional link between aging, tau phosphorylation and synaptic plasticity. Furthermore, the presented data allow analyses whether tau phosphorylation is reversible in these facultative hibernators and versatile laboratory animal as it was recently shown for the hibernation cycle of European ground squirrels.

Theme: Development and regulation
Topic: Aging process

Keywords: Tau; Phospho-epitope; Hippocampus; Hyperphosphorylation; Mossy fiber; Plasticity

Neurofibrillary tangles mainly consisting of abnormally phosphorylated microtubule-associated protein tau are a major pathological hallmark of Alzheimer's disease (AD) [16,23]. Tau hyperphosphorylation most likely results from a disturbed balance between tau kinases and phosphatases, although it is not well understood how the degree of tau phosphorylation influences the formation of paired helical filaments (PHF). Brain regions with a high degree of synaptic plasticity are preferentially affected by tau phosphorylation and PHF formation. Recent studies support the hypothesis that failures in synaptic plasticity might be crucial in the pathomechanism of AD [2–4,24,27,34]. A more direct link between plasticity and tau phosphorylation could be demonstrated in the mossy fiber system of European ground squirrels during hibernation [5]. A decrease of synaptic markers of mossy fiber terminals during torpor was associated with the formation of highly phosphorylated tau in target neurons, i.e., CA3 pyramidal cells. After arousal of hibernating animals, hyperphosphorylated tau disappeared in parallel with the re-establishment of mossy fiber synapses in the stratum lucidum of CA3 pyramidal cells. Interestingly, the Syrian hamster (*Mesocricetus auratus*) is an animal potentially prone to hibernation [29,31] and could be used as a model to further study whether reversible tau phosphorylation might be a more general attribute of hibernation. As

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prerequisite for such investigations, the present work provides data on the tau phosphorylation status in Syrian hamsters. Our study includes the brains from aged animals which nearly reached the maximal life expectancy for hamsters (about 2 years [28]).

Besides AD [10], embryogenesis [32], hibernation [5], starvation [37], mitosis [35] and hypothermia [30], increased degree of tau phosphorylation has been observed in a variety of species during aging, a condition potentially associated with synaptic disconnection and a failure of synaptic plasticity. An age-dependent increase in the phosphorylation of tau and the formation of neurofibrillary tangles has, for example, been observed in aged bears [14], sheep, goats [11], bison [21,22], mouse lemurs [9], baboons [33] and various other mammals [21].

To elucidate the phosphorylation status of hamsters, we used a panel of highly specific antibodies directed against phospho-epitopes in the tau sequence 199–205. The present study was focused on the hippocampus, which is known to be affected very early by tau pathology in humans, as well as during "normal" aging (see, e.g., [15]).

This study comprised male wild-type hamsters at the age of 3 months (n = 4, "young"), 10–11 months (n = 10, "middle-aged") and 22 months (n = 4, "old") from a breeding stock at the Zoological Laboratory, Haren, The Netherlands. All animals were housed individually in 12-h light and 12-h dark lighting condition, at room temperature of 23 ± 1 °C, with food and water supplied ad libitum. The use of the animals was approved by the Animal Experimentation Committee of the University of Groningen (DEC # 3018, DEC # 2954).

Hamsters were deeply anaesthetized with an intraperitoneal (i.p.) injection of 1.5 ml 6% pentobarbital and subsequently transcardially perfused with 4% paraformaldehyde in phosphate buffer. After removing the brains from the skulls, they were post-fixed in the same fixative overnight. Next, brains were equilibrated with 30% sucrose in phosphate buffer, pH 7.6. Thirty-micrometer-thick frontal frozen sections were cut using a freezing microtome and collected in 0.1 M Tris-buffered saline, pH 7.4 (TBS), containing sodium azide. Prior to immunoperoxidase staining, endogenous peroxidase activity in the sections was abolished by the treatment with 0.6% hydrogen peroxide in TBS for 30 min. Next, non-specific binding sites for subsequently applied immunoreagents were blocked with 5% normal goat serum in TBS also containing 0.3% Triton X-100 (NGS-TBS-T) incubated with primary antibodies (diluted in NGS-TBS-T) for 14–18 h at room temperature. Table 1 summarizes the antibodies used for the immunoperoxidase staining of phospho-epitopes of tau focused on the amino acid residues 199–205. Immunoreactivities were then visualized by conventional peroxidase–anti-peroxidase (PAP) or streptavidin–biotin techniques with the chromogen nickel-enhanced diaminobenzidine as previously described [20]. Controls were performed by omitting primary antibodies causing the expected absence of any marked cellular structure. Following all histochemical procedures, the sections were rinsed with TBS, briefly washed with distilled water, mounted on slides, air-dried and coverslipped with Entellan (in toluene; Merck, Heidelberg).

To further testify the specificity of the applied antibodies, Western blot analysis on brain extract of one hamster was performed. The animal was anaesthetized by i.p. injection of 1.5 ml 6% pentobarbital and subsequently decapitated. The brain was removed within 2 min, frozen on dry ice immediately, transferred into liquid nitrogen and stored at −80 °C. One hemisphere of the brain was separated and homogenized in protein extraction buffer (20 mM Tris (HCl), pH7.4; 150 mM NaCl; 2 mM $MgCl_2$; 5 mM NaF; 1 mM Na_3VO_4; 1% NP40; 1 mM DTT; 1 mM PMSF; 1 μg/ml leupeptin; 1 μg/ml aprotinin; 1 μg/ml pepstatin) and subsequently centrifuged at 30.000 × g for 30 min at 4 °C. The clear supernatant was removed from the pellet and transferred into a new tube. The protein concentration of the sample was measured using the Bradford assay. Thirty-microgram protein per lane was separated in a 10% acrylamide gel by SDS–PAGE. After transferring the gel to a PVDF membrane by semi-dry blot the lanes were separated, washed two times in PBS, blocked in PBS containing 2% BSA and probed with primary antibodies (Table 1; diluted in blocking buffer). The blot stripes were washed in PBS containing 0.05% Tween 20 (PBS-T), incubated with biotinylated donkey anti-rabbit or sheep anti-mouse (Amersham Biosciences, Freiburg, Germany; both 1:4000 diluted in blocking buffer), washed again in PBS-T and incubated with ExtrAvidin peroxidase conjugate (Sigma, Taufkirchen, Germany; 1:10,000, diluted in blocking buffer). The peroxidase reactivity was recorded by

Table 1
Antibodies for immunolabeling and Westerrn blot analysis

Antibody	Host	Dilution for immunolabeling (Im) and Western blot (Wb)	Epitope on tau molecule	Source	References
P199	Rabbit	1:500 (Im) 1:500 (Wb)	Phospho-serine 199	N. Sergeant (Lille, France)	[25]
CP-13	Mouse	1:200–1:500 (Im) 1:1000 (Wb)	Phospho-serine 202	P. Davies (New York, USA)	[36]
AT8	Mouse	1:500–1:2000 (Im) 1:2000 (Wb)	Phospho-serine 202 + Phospho-threonine 205	Innogenetics (Gent, Belgium)	[18,26]
P205	Rabbit	1:200–1:500 (Im) 1:1000 (Wb)	Phospho-threonine 205	Biosource (Camarillo, USA)	[1]
AT180	Mouse	1:1000 (Im)	Phospho-threonine 231 + Phospho-serine 235	M. Goedert (Cambridge, UK)	[17]
Anti-human tau	Rabbit	1:1000 (Wb)	Whole tau molecule	DakoCytomation (Hamburg, Germany)	[8]