Starvation induces tau hyperphosphorylation in mouse brain: implications for Alzheimer's disease

Masahiro Yanagisawa[1,2], Emmanuel Planel[1], Koichi Ishiguro, Shinobu C. Fujita*

Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194-8511, Japan

Received 18 October 1999

Abstract Hyperphosphorylated tau is the major component of paired helical filaments in neurofibrillary tangles found in Alzheimer's disease brains, and tau hyperphosphorylation is thought to be a critical event in the pathogenesis of this disease. The objective of this study was to reproduce tau hyperphosphorylation in an animal model by inducing hypoglycemia. Food deprivation of mice for 1 to 3 days progressively enhanced tau hyperphosphorylation in the hippocampus, to a lesser extent in the cerebral cortex, but the effect was least in the cerebellum, in correspondence with the regional selectivity of tauopathy in Alzheimer's disease. This hyperphosphorylation was reversible by refeeding for 1 day. We discuss possible mechanisms of this phenomenon, and propose the starved mouse as a simple model to study in vivo tau phosphorylation and dephosphorylation which are altered in Alzheimer's disease.

Key words: Tau phosphorylation; Starvation; Hippocampus; Alzheimer's disease; Glucose; Hypoglycemia

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence in the brain of two histopathological hallmarks called senile plaques and neurofibrillary tangles (NFT) [1,2]. NFT are structures present in the neuronal body and consist of paired helical filaments (PHF), mainly composed of highly phosphorylated tau protein [3]. Tau is a microtubule-associated protein expressed mostly, but not exclusively, in the nervous system, and its normal physiological function is to bind and stabilize microtubules. Tau is 'hyperphosphorylated' in AD brains (PHF-tau) [3], to contain up to four times as much phosphate [4], and also at sites that are not phosphorylated in the brains of normal human adults [5]. This hyperphosphorylation has been shown to reduce the binding affinity of tau for microtubules in vitro [6]. These observations have given rise to the hypothesis that tau hyperphosphorylation would lead to microtubule destabilization, appearance of NFT, and neurodegeneration in AD [7]. Recently, mutations in the tau gene have been shown to be responsible for FTDP-17, and there is a growing recognition that tauopathy may be a common converging point of pathogenic processes of a number of neurodegenerative diseases including AD [8].

AD brains have been widely reported to show regional reductions in glucose transport and utilization [9]. The brain depends on glucose for most of its energy needs and is thus highly vulnerable to diminished availability in glucose [10]. These facts suggest that the decrease in glucose turnover in the brain may play a role in the pathogenetic mechanisms of AD [11], and in vitro studies found that glucose deprivation induces AD-like changes in hippocampal neurons by eliciting NFT-like tau antigenicity [12].

In the present study we tested whether food deprivation of adult mice and consequent hypoglycemia may induce AD-like tau hyperphosphorylation in the brain by examining the levels of tau phosphorylation through immunoblotting with a battery of anti-tau antibodies specific to phosphorylated or non-phosphorylated epitopes [13]. Starvation up to 3 days reversibly induced tau hyperphosphorylation in the hippocampus, to a lesser extent in the cortex, but with only a small effect in the cerebellum. We discuss the possible causative mechanisms of this hyperphosphorylation with regard to glucose and insulin, and consider the relevance of starvation-induced tau hyperphosphorylation as a simple and useful model to investigate the in vivo regulatory mechanisms of tau phosphorylation and dephosphorylation that are altered in AD.

2. Materials and methods

Eight-week-old C57BL/6NJcl male mice (Clea Japan, Tokyo, Japan) weighing 20.5 ± 0.5 g were singly housed in cages with grid floors to deny coprophagy. Food was removed for up to 3 days, but mice were allowed free access to water. Room temperature was 23°C and the light cycle was 12 h light and 12 h dark.

Rabbit antisera PS199, PS396, PS413 and PS422 are specific to tau phosphorylated on Ser-199, Ser-396, Ser-413 and Ser-422 (numbering of amino acid based on the 441 amino acids human tau [14]), respectively, and Tau-C recognizes the C-terminus of tau [13]. Mouse monoclonal antibody AT8 (Innogenetics) recognizes tau phosphorylated on both Ser-202 and Thr-205 [15] and Tau-1 (Boehringer) has an optimal immunoreactivity when tau is dephosphorylated on Ser-195, Ser-198, Ser-199, Ser-202 and Thr-205 [16].

Mice were killed by cervical dislocation, brains immediately removed, and hippocampi, cerebral cortices and cerebella were dissected from brains in ice-chilled PBS. The tissues were quickly weighed and homogenized in 9×volume of buffer O+(62.5 mM Tris-HCl pH 6.8, 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) SDS, 100 μM orthovanadate, 1 μM okadaic acid, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM EGTA and 1 mM EDTA). The samples were then placed in boiling water for 3 min, centrifuged for 10 min at $20\,000 \times g$ at room temperature and the protein contents of the supernatant were determined with the Bio-Rad protein assay with BSA as standard. Samples were separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membrane (Protran, Schleicher and Schuell). The blots were blocked with 3% skim milk in PBS for 30 min at room temperature, washed with PBS and incubated for 2 h at room temperature with primary antibodies. After a second wash, the blots were incubated with biotinylated anti-rabbit IgG or biotinylated anti-mouse IgG (Amersham) secondary antibodies for 1 h

*Corresponding author. Fax: (81) 42 724 6314.
E-mail: fujita@ls.m-kagaku.co.jp

[1] Both authors contributed equally to this work.
[2] Present address: Department of Neurobiology and Anatomy, University of Texas Medical School, Houston, TX 77225, USA.

0014-5793/99/$20.00
PII: S0014-5793(99)01480-5

at room temperature. After a third wash, the blots were incubated with avidin-biotinylated peroxidase complex (Vectastain ABC Kit, Vector Laboratories) for 40 min at room temperature. After a final wash, immunoreactive bands were visualized with 4-chloro-1-naphthol and H_2O_2.

3. Results

3.1. Starvation enhances tau phosphorylation most prominently in the hippocampus

Food deprivation of young adult mice resulted in progressive and severe weight losses (Fig. 1). After 1 day of starvation the mice lost 20% of their initial body weight and 29% after 2 days.

Effect of starvation on the phosphorylation state of tau was first examined in the cerebral cortex, hippocampus and cerebellum. A large mobility shift of tau was observed upon starvation for 2 days in the hippocampal extract, as visualized by a phosphorylation-independent anti-tau antibody, Tau-C (Fig. 2, Tau-C, Hp lanes). The normal adult tau band of 56 kDa was diminished, and the 60 kDa band greatly intensified, with the appearance of 64 kDa and 70 kDa bands. This was accompanied by even more pronounced enhancement of immunostaining by all phosphorylation-dependent anti-tau antibodies tested each specific to five distinct epitopes (PS199, AT8, PS396, PS413 and PS422), indicating a large increase in phosphorylation of tau. This result was corroborated by a greatly diminished staining of tau bands by Tau-1, which has an optimal immunoreactivity when tau is dephosphorylated on Ser-195, Ser-198, Ser-199, Ser-202 and Thr-205 [16].

When the three brain regions were compared, the enhancement of tau phosphorylation was largest in the hippocampus, followed by the cerebral cortex with a similar pattern of changes (Fig. 2). Much smaller effects were observed in the cerebellum, where the changes were relatively more prominent with PS413 and PS396. AD tauopathy is known to manifest earliest in the hippocampal region [17,18]. The rest of the study, therefore, focused on the hippocampus.

3.2. Development of starvation-induced tau phosphorylation in the hippocampus

To control for individual variability in the development of tau phosphorylation, batches of five young adult mice were starved for up to 3 days prior to killing, and the results of the Western blot analysis of hippocampal extracts are shown in Fig. 3. For ease of comparison, individual samples are presented, within each batch, in the order of increasing degree of hyperphosphorylation.

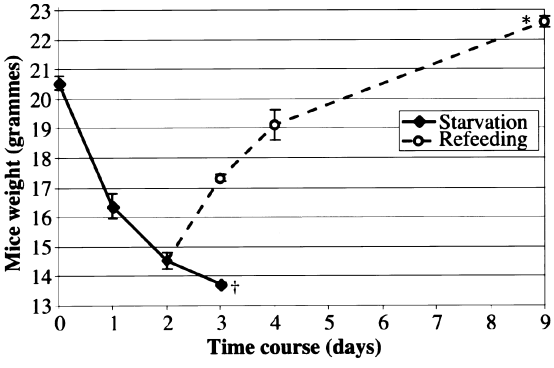

Fig. 1. Body weight changes in mice during starvation and refeeding. Batches of 8 week old B6 male mice were starved for 0, 1, 2 and 3 days ([NOCHAR:lozf, solid lines). After 2 days of starvation, some mice were refed for 1, 2 or 7 days (○, broken lines). For the starved mice, $n=5$; except for day 3 (†, $n=2$). For the refed mice, $n=4$; except for day 9 (*, $n=3$; one mouse excluded, see text).

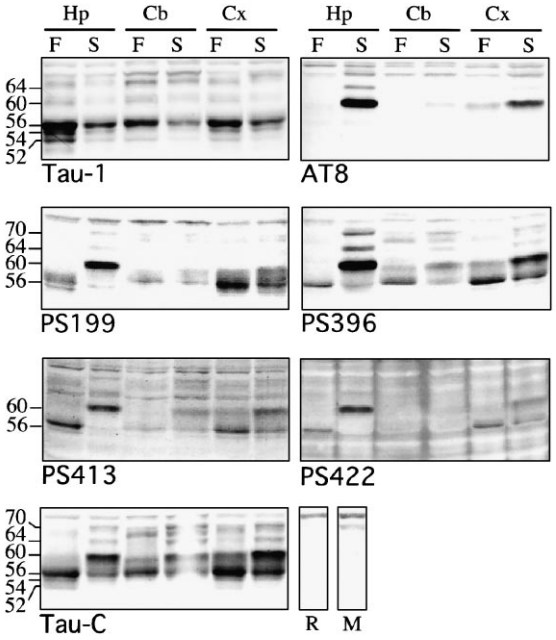

Fig. 2. Phosphorylation of tau protein in the hippocampus (Hp), cerebellum (Cb) and cerebral cortex (Cx), after normal feeding (F) or 2 days of starvation (S). Immunoblots were incubated with anti-tau antibodies directed to specific phosphorylated epitopes (polyclonals PS199, PS396, PS413, PS422 and monoclonal AT8), non-phosphorylated epitope (monoclonal Tau-1), or a phosphorylation-independent epitope (polyclonal Tau-C). R and M correspond to controls (hippocampus; starved 2 days; no primary antibody) incubated with anti-rabbit or anti-mouse secondary antibodies, respectively.

Each antibody showed gradual and progressive changes in the tau hyperphosphorylation and mobility shift over the course of 3 days, confirming and extending the results of Fig. 2. There was recognizable individual variation within batches of 1 or 2 day starvation (cf. lanes 7 vs. 11, or 11 vs. 16), but this was not so large as to overlap with the variability within the neighboring batches (cf., lanes 11 vs. 12), demonstrating reproducibility and reliability of our analysis. Thus phosphorylation of serines at amino acid 199, 396, 413, 422 and at AT8 epitopes become highly enhanced by starvation, with concomitant decrease in the Tau-1 epitope.

3.3. Reversibility of starvation-induced tau hyperphosphorylation

Reversibility of starvation-induced tau hyperphosphorylation was of great interest, as less than complete reversibility would lead to an accumulation of phosphorylated tau and eventual formation of NFT-like deposit. Batches of four mice were starved for 2 days and then allowed access to food ad libitum for up to 7 days before killing and analysis of hippocampal tau protein.

After 2 days of starvation the mice weighed about 70% of their initial weight (Fig. 1) One day of refeeding allowed

them to recover to 85% and 1 week to 110% of their initial weight. In this experiment, there was one exceptional mouse, which attained only 93% of its initial weight after 1 week of refeeding (see below). This mouse was not included in Fig. 1.

In all the refed animals except one, the hippocampal tau had reverted to the original phosphorylation state (Fig. 4). Thus, the starvation-induced hyperphosphorylation is reversible within 1 day. The only but remarkable exception was the mouse represented in lane 12 of Fig. 4, which did not regain its initial body weight after 1 week of refeeding. The hippocampal phosphorylation pattern of this mouse was similar to that of 2 day starved mouse (lane 3), suggesting that some profound change had taken place in the brain of this mouse. Factors that caused this condition are under investigation.

4. Discussion

The objective of this study was to generate tau hyperphosphorylation in an animal model. We speculated that a starvation-induced decrease in brain glucose level might lead to tau hyperphosphorylation. Our results with adult mice clearly show that starvation enhances brain tau phosphorylation at multiple sites, as revealed by immunoblot analysis using phosphorylation-dependent antibodies. After 2 days of starvation, hyperphosphorylation was most pronounced in the hippocampus, with lesser effect in the cerebral cortex, but was nearly undetectable in the cerebellum (Fig. 2), in correspondence with the regional selectivity of tauopathy in AD. This phenomenon was reversible by refeeding. Mechanisms behind this reversible hyperphosphorylation and relevance to AD studies of the tau hyperphosphorylation model need to be considered.

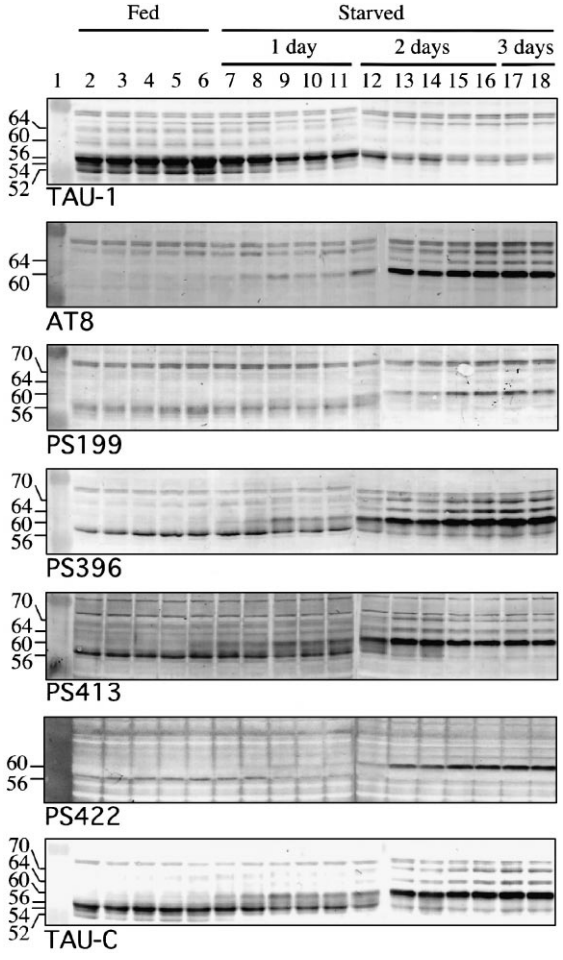

Fig. 3. Phosphorylation of tau protein from hippocampus of 8 week old B6 male mice after normal feeding (lanes 2 to 6), or starved for 1 (lanes 7 to 11), 2 (lanes 12 to 16), or 3 days (17 and 18). Each lane represents an extract of individual mouse. Lane 1 represents the pre-stained molecular weight marker. Immunoblots were developed using various anti-tau antibodies (see legend to Fig. 2).

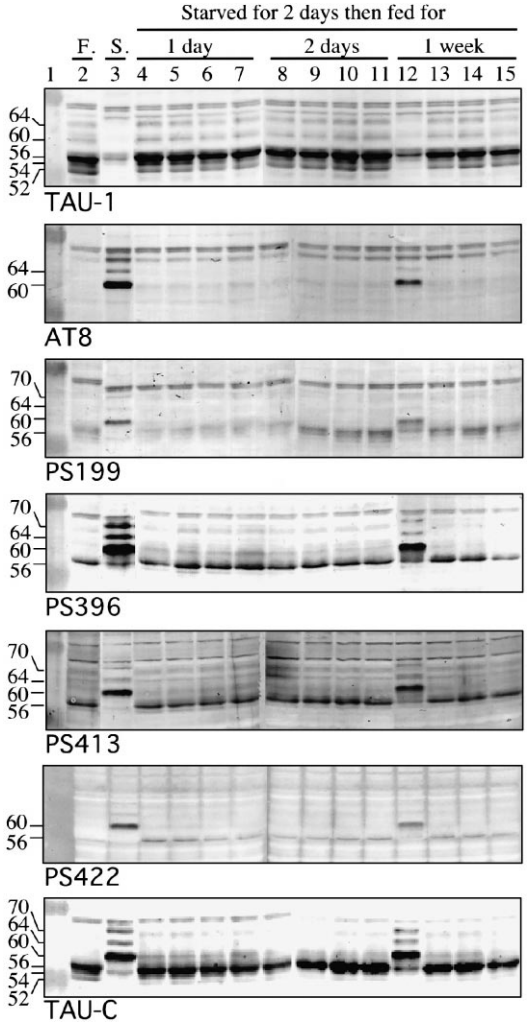

Fig. 4. Phosphorylation of tau protein from hippocampus of 8 week old B6 male mice after normal feeding (F, lane 2), 2 days of starvation (S, lane 3); 2 days of starvation followed by 1 day (lanes 4 to 7), 2 days (lanes 8 to 11) or 1 week (lanes 12 to 15) of refeeding. Each lane represents an extract of individual mouse. Lane 1 contains the pre-stained molecular weight marker. Immunoblots were developed using various anti-tau antibodies (see legend to Fig. 2).

4.1. Possible mechanisms

Tau protein has many serines and threonines that can be phosphorylated. Experiments involving phosphatases as well as phosphorylation of recombinant tau protein by various kinases [13,19–21] have shown that phosphorylation at certain serine and threonine residues cause retardation of electrophoretic migration, called mobility shift, of the tau molecule, although the responsible residues have not been firmly identi-

fied. Since tau occurs in several alternatively spliced forms with distinguishable molecular weights [19], there is a formal possibility that some of the apparent mobility shift may actually be due to changes in isoform composition.

Several of the kinases known to phosphorylate tau have been reported to be specific to sites included among the epitopes investigated in this report [22]. Ser-199 can be phosphorylated by tau protein kinase I (TPKI) [23], also called glycogen synthase kinase 3β (GSK3β), and by extracellular signal-regulated kinases 1 and 2 (ERK1/2) [24]. Ser-202 and Thr-205 (AT8 epitope) have been shown to be phosphorylated by TPKII (cdk5) [13] and other cyclin-dependent kinases, by ERK1/2 [24], and by 5 stress-activated protein kinases (SAPK): Jun N-terminal kinase 1 (JNK1 or SAPK1γ) [20], p38 (SAPK2a) [25], SAPK2b, SAPK3 and SAPK4 [26]. Ser-396 is phosphorylated by TPKI/GSK3β [23], TPKII [27], ERK1/2, and the five SAPKs. Only TPKI/GSK3β is known to phosphorylate Ser-413 [23], while Ser 422 is phosphorylated by ERK1/2 and the five SAPKs [22]. Thus, the mechanisms responsible for tau hyperphosphorylation here reported need to be discussed with respect to possible induction of the mentioned kinases by decreased glucose or insulin availability under starvation.

The effects of hypoglycemia on the mammalian brain have been extensively studied. Deep insulin-induced hypoglycemia provokes decreases in brain glucose levels and energy state, excitatory amino acids (EAA) accumulate in the extracellular space and activate *N*-methyl-D-aspartate (NMDA) receptors, leading to excitotoxic neuronal damages in the cerebral cortex and hippocampus but not in the cerebellum [10]. In a study of excitotoxicity using rats given kainate intraperitoneally, JNK-1 activity in the brain was found to be elevated [28]. Activation of SAPK and ERK was observed upon glutamate application to cultured striatal neurons [29]. Recent immunoblot analysis of increased tau phosphorylation in neuroblastoma cells upon ionophore-mediated calcium stimulation implicated TPKI/GSK3β [30].

These reported findings together with the fact that all the phosphorylation sites of tau examined in this study show enhanced phosphorylation upon starvation suggest that no single protein kinase is solely responsible for the phenomenon, but rather multiple kinases, or possibly a regulated network of kinases, are involved.

Decreased availability of glucose is expected to affect insulin levels, but the functions and regulation of brain parenchymal insulin are largely unknown. After 2 days of starvation, the concentration of blood insulin is reduced by 85% in the rat [31], and it was observed that the brain insulin parallels changes in blood insulin concentration [32]. In studies based on cultured cells, SAPK/JNK, MAPK and p38 were found to be activated by insulin [33], whereas TPKI/GSK3β was down-regulated [34]. In fact, insulin and insulin-like growth factor 1 (IGF1) have been shown to reduce tau phosphorylation by inhibiting TPKI/GSK3β in NT2N neuroblastoma cultures [35].

Thus, reduced levels of both glucose and insulin in the brain can activate a number of protein kinases to cause hyperphosphorylation of tau. Indeed, we have observed a concerted enhancement of phosphorylation of at least five distinct sites on tau that cannot be explained by any single kinase. To clarify the mechanisms of tau hyperphosphorylation observed in our study, it will be important in future to determine the activation states of the protein kinases discussed above during starvation and study additional conditions, such as pharmacological treatments, that affect tau phosphorylation. Our data also showed that starvation-induced tau hyperphosphorylation is reversible. Therefore, the role of protein phosphatases in the regulation of tau phosphorylation levels [36] need to be studied.

4.2. Relevance of the starved mouse model for AD research

Recent finding that mutations in the tau gene cause development of NFT and loss of neurons leading to a type of dementia [8] lends further support to the idea that some dysfunction associated with hyperphosphorylation of tau is cardinal to the pathogenesis of AD as well [7]. Several attempts have been made to induce tau phosphorylation in the brain of animal models by injecting various substances such as aluminum salts, colchicine and okadaic acid [37]. In non-invasive experimental approaches using heat-shocked or cold water stressed rats, transient increase in tau phosphorylation has also been reported [38,39]. Our study raises the possibility of making use of starved mice as an in vivo experimental model to study a particular aspect of AD pathogenesis: tau hyperphosphorylation.

Neuroimaging studies of AD patients [9] pointed out reduced regional glucose metabolism in the regions of the brain that are most prone to be affected histopathologically in AD [17,18]. Reduced energy metabolism may be an aggravating, if not causative, factor in the development of AD. Thus starvation of experimental animals may be considered as a physiologically relevant stimulus.

Phosphorylation sites of tau protein here examined and found to be hyperphosphorylated in the starved mice are all prominently hyperphosphorylated in PHF-tau from AD brains [40]. Thus the pattern of phosphorylation of tau is likely to be analogous between the starved mice and AD patients.

In incipient AD, tau pathology is known to manifest earliest in the hippocampal region, then to spread to neocortical regions [17,18], while neurofibrillary changes are absent in the cerebellum. In our model mouse tau hyperphosphorylation was most prominent in the hippocampus, followed by the neocortex, with the cerebellum showing the least effect. This parallel in regional selectivity suggests the existence of a common or overlapping mechanisms of tau hyperphosphorylation in the starved mice and AD.

These lines of consideration lead us to propose that the starvation-induced tau phosphorylation may provide a relevant and convenient system to study the pathogenetic mechanisms of AD and related neurodegenerative diseases. It will be important in future to establish this correspondence through corroboration by immunohistochemical analyses and identification of the conditions that can lead to accumulation and deposition of hyperphosphorylated tau in an NFT-like form. It is hoped that the starved mice will thus shed light on the critical mechanisms of neurodegeneration.

Acknowledgements: We thank Dr. Hiroki Hamanaka for discussions during the course of the study, Dr. Nao R. Kobayashi and Dr. Mariko Kobayashi for critical reading of the manuscript and Mr. Yasuhiro Okawa for help with the experiments. This work was supported by an European Union Science and Technology Fellowship ERB IC17 CT97 0051.

References

[1] Alzheimer, A., Stelzmann, R.A., Schnitzlein, H.N. and Murtagh, F.R. (1995) Clin. Anat. 8, 429–431.
[2] Selkoe, D.J. (1991) Neuron 6, 487–498.
[3] Lee, V.M., Balin, B.J., Otvos Jr., L. and Trojanowski, J.Q. (1991) Science 251, 675–678.
[4] Kopke, E., Tung, Y.C., Shaikh, S., Alonso, A.C., Iqbal, K. and Grundke-Iqbal, I. (1993) J. Biol. Chem. 268, 24374–24384.
[5] Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K. and Ihara, Y. (1995) J. Biol. Chem. 270, 823–829.
[6] Gustke, N., Steiner, B., Mandelkow, E.M., Biernat, J., Meyer, H.E., Goedert, M. and Mandelkow, E. (1992) FEBS Lett. 307, 199–205.
[7] Trojanowski, J.Q. and Lee, V.M. (1994) Am. J. Pathol. 144, 449–453.
[8] Goedert, M., Crowther, R.A. and Spillantini, M.G. (1998) Neuron 21, 955–958.
[9] Minoshima, S., Frey, K.A., Koeppe, R.A., Foster, N.L. and Kuhl, D.E. (1995) J. Nucl. Med. 36, 1238–1248.
[10] Auer, R.N. and Siesjo, B.K. (1993) Bailliere's Clin. Endocrinol. Metab. 7, 611–625.
[11] Meier-Ruge, W. and Bertoni-Freddari, C. (1996) Rev. Neurosci. 7, 1–19.
[12] Cheng, B. and Mattson, M.P. (1992) Exp. Neurol. 117, 114–123.
[13] Ishiguro, K., Sato, K., Takamatsu, M., Park, J., Uchida, T. and Imahori, K. (1995) Neurosci. Lett. 202, 81–84.
[14] Goedert, M., Spillantini, M.G., Jakes, R., Rutherford, D. and Crowther, R.A. (1989) Neuron 3, 519–526.
[15] Goedert, M., Jakes, R. and Vanmechelen, E. (1995) Neurosci. Lett. 189, 167–169.
[16] Szendrei, G.I., Lee, V.M. and Otvos Jr., L. (1993) J. Neurosci. Res. 34, 243–249.
[17] Braak, H. and Braak, E. (1991) Acta Neuropathol. 82, 239–259.
[18] Delacourte, A., David, J.P., Sergeant, N., Buee, L., Wattez, A., Vermersch, P., Ghozali, F., Fallet-Bianco, C., Pasquier, F., Lebert, F., Petit, H. and Di Menza, C. (1999) Neurology 52, 1158–1165.
[19] Goedert, M., Crowther, R.A. and Garner, C.C. (1991) Trends Neurosci. 14, 193–199.
[20] Reynolds, C.H., Utton, M.A., Gibb, G.M., Yates, A. and Anderton, B.H. (1997) J. Neurochem. 68, 1736–1744.
[21] Jicha, G.A., O'Donnell, A., Weaver, C., Angeletti, R. and Davies, P. (1999) J. Neurochem. 72, 214–224.
[22] Johnson, G.V.W. and Hartigan, J.A. (1998) Alzheimer's Dis. Rev. 3, 125–141.
[23] Ishiguro, K., Omori, A., Takamatsu, M., Sato, K., Arioka, M., Uchida, T. and Imahori, K. (1992) Neurosci. Lett. 148, 202–206.
[24] Illenberger, S., Zheng-Fischhofer, Q., Preuss, U., Stamer, K., Baumann, K., Trinczek, B., Biernat, J., Godemann, R., Mandelkow, E.M. and Mandelkow, E. (1998) Mol. Biol. Cell 9, 1495–1512.
[25] Reynolds, C.H., Nebreda, A.R., Gibb, G.M., Utton, M.A. and Anderton, B.H. (1997) J. Neurochem. 69, 191–198.
[26] Goedert, M., Hasegawa, M., Jakes, R., Lawler, S., Cuenda, A. and Cohen, P. (1997) FEBS Lett. 409, 57–62.
[27] Paudel, H.K., Lew, J., Ali, Z. and Wang, J.H. (1993) J. Biol. Chem. 268, 23512–23518.
[28] Mielke, K., Brecht, S., Dorst, A. and Herdegen, T. (1999) Neuroscience 91, 471–483.
[29] Schwarzschild, M.A., Cole, R.L., Meyers, M.A. and Hyman, S.E. (1999) J. Neurochem. 72, 2248–2255.
[30] Hartigan, J.A. and Johnson, G.V. (1999) J. Biol. Chem. 274, 21395–21401.
[31] Newsholme, E.A. (1976) Clin. Endocrinol. Metab. 5, 543–578.
[32] Yalow, R.S. and Eng, J. (1983) Adv. Metab. Disord. 10, 341–354.
[33] Avruch, J. (1998) Mol. Cell. Biochem. 182, 31–48.
[34] Srivastava, A.K. and Pandey, S.K. (1998) Mol. Cell. Biochem. 182, 135–141.
[35] Hong, M. and Lee, V.M. (1997) J. Biol. Chem. 272, 19547–19553.
[36] Matsuo, E.S., Shin, R.W., Billingsley, M.L., Van deVoorde, A., O'Connor, M., Trojanowski, J.Q. and Lee, V.M. (1994) Neuron 13, 989–1002.
[37] Cole, G.M. and Frautschy, S.A. (1997) Alzheimer's Dis. Rev. 2, 2–10.
[38] Papasozomenos, S.C. (1996) J. Neurochem. 66, 1140–1149.
[39] Korneyev, A., Binder, L. and Bernardis, J. (1995) Neurosci. Lett. 191, 19–22.
[40] Goedert, M. (1996) Ann. N.Y. Acad. Sci. 777, 121–131.