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Alzheimer-like phosphorylation of tau and neurofilament induced by cocaine *in vivo*[1]

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KEY WORDS cocaine; Alzheimer disease; cyclin-dependent kinases; cytoskeleton; tau proteins; neurofilament proteins; rats; hippocampus; cerebral cortex; caudatoputamen

ABSTRACT

AIM: To explore the relationship between cocaine-induced cyclin-dependent kinase-5 (CDK5) overexpression or overactivation and Alzheimer-like hyperphosphorylation of cytoskeletal protein. **METHODS:** Cocaine was injected (ip, 20 $mg \cdot kg^{-1} \cdot d^{-1}$) into rats and the phosphorylation of neuronal cytoskeletal proteins was measured by Western blotting. **RESULTS:** The levels of phosphorylated tau at PHF-1 epitope and phosphorylated neurofilament determined by SMI31 were elevated in rat brain hippocampus, cortex, and caudatoputamen on d 8 and d 16 after the injection of cocaine, when compared with saline control rat at the same brain regions. On the other hand, the levels of tau non-phosphorylated at tau-1 site and non-phosphorylated neurofilament determined by SMI32 were decreased in same brain regions at the same time points examined. No significant difference of phosphorylated tau and neurofilament at those epitopes was seen on d 4. Although cocaine injection could induce significant hyperphosphorylation of neuronal cytoskeletal proteins, the overexpression of CDK5 and p35 was not detected. **CONCLUSION:** Peritoneal injection of cocaine induces Alzheimer-like hyperphosphorylation of tau and neurofilament in rat brain, and the effect may be not relevant to an increase in overexpression or overactivation of CDK5.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence of two histopathological hallmarks called senile plaques and neurofibrillary tangles. The formers are deposits of β-amyloid peptide (Aβ), whereas the latters are consisted of hyperphosphorylated tau protein assembled in paired helical filaments (PHF)[1]. AD-abnormally phosphorylated tau (AD p-tau) is phosphorylated at more sites than tau from adult brain and, for a given site, a higher than normal percentage of tau molecules is phosphorylated[2]. Although the precise mechanism for tau hyperphosphorylation in AD brain is not known, it is widely accepted that hyperphosphorylation of cytoskeletal proteins found in AD brain is due to an imbalance in protein phosphorylation and dephosphorylation systems.

Many protein kinases, such as mitogen-activated protein kinases (MAPKs)[3], cyclin-dependent kinases (CDKs), protein kinase A (PKA)[4], and glycogen synthase kinase-3 (GSK-3)[5] are found to transform nor-

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mal tau into an AD-like state *in vitro*. It is also found in AD brains that (1) the immunoreactivity for GSK-3 and CDK5 is associated with hyperphosphorylated tau[6,7]; (2) CDK5 is over-activated and p25, an activator of CDK5, is increased and accumulated in AD brain[8]; (3) transgenic mice overexpressing human p25 show hyperphosphorylation of tau and neurofilament and cytoskeletal disruptions[9]. These data strongly suggest that overactivation of CDK5 is relevant to tau hyperphosphorylation. However, it is not known which factors contribute to active CDK5 in AD brain.

Cocaine is a major drug of abuse in modern society. It is reported that cocaine enhances dopamine-mediated neurotransmission by blocking dopamine re-uptake at axonal terminals. Chronic exposure to cocaine up-regulates transcription factors, one of such transcription factors is FosB, an upstream regulator of CDK5[10]; it also causes overexpression of CDK5 and p35 in rat brain[10]. Dopamine receptors exist in hippocampus, cortex, and neostriatum[11]. Taking together the above information, it is speculated that chronic exposure to cocaine may lead to overactivation of CDK5 and thus hyperphosphorylation of cytoskeletal protein found in AD brain. In the present study, this hypothesis was tested in rats.

MATERIALS AND METHODS

Chemicals Cocaine, goat anti-mouse and goat anti-rabbit alkaline phosphatase-conjugated secondary antibodies were purchased from Sigma Chemical Co (St Louis, MO, USA). Bicinchoninic acid (BCA) protein detection kit and 5-bromo-4-chloro-3-indole phosphate (BCIP)/nitrobluetetrazolium (NBT) kit were from Pierce Chemical Company (Rockford, IL, USA). Anti-CDK5 and anti-p35 were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Monoclonal antibody (mAb) PHF-1 recognizing Ser396/404 and Tau-1 recognizing Se199/202 of tau were gifts from Drs Davies (Albert Einstein College of Medicine, Bronx, NY) and Binder (North Western University, Chicago, Illinois). mAb SMI31 and SMI32 detecting phosphorylated and nophosphorylated neurofilament were from Sternberger Monoclonals, Inc (Baltimore, MD, USA).

Animal Adult male Sprague-Dawley rats (Grade II) were supplied by Experimental Animal Center of Tongji Medical College. The rats initially weighing 160 g to 240 g were injected with cocaine 20 $mg \cdot kg^{-1} \cdot d^{-1}$ or 0.9 % NaCl intraperitonealy at the same time each day for 4 d, 8 d, and 16 d. All analyses were initiated 12 h after the final dose.

Western blot Cortex, hippocampus, and caudatoputamen were removed from brain and homogenized in buffer containing Tris·Cl (pH 7.6) 10 mmol/L, NaF 50 mmol/L, Na_3VO_4 1 mmol/L, edetic acid 1 mmol/L, benzamidine 1 mmol/L, PMSF 1 mmol/L, and protease inhibitor mixture (2 mg/L each of aprotinin, leupeptin and pepstain A). Three volumes of the homogenized tissue was dissolved in one volume of lysis buffer containing Tris·Cl (pH 7.6) 200 mmol/L, 8 % SDS, 40 % glycerol, and was boiled at water bath for 10 min. The lysates were centrifuged at 12 000×*g* for 30 min. After measurement of protein concentration in the supernatant, dithiothreitol (DTT) was added to attain a final concentration 100 mmol/L. The proteins in the supernatant were separated by SDS-polyacrylamide gel electrophoresis (10 % gel for tau, CDK5 and p35, and 7.5 % gel for neurofilament), transferred to polyvinylidene difluoride (PVDF) membrane. The blot was developed by alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG, using BCIP/NBT as substrates. The protein bands were quantitatively analyzed by Kodak Digital Science 1D software (Eastman Kodak Company, New Haven, CT, USA), and the amount of protein bands was expressed as sum optical density[12,13].

Statistical analysis Data were expressed as mean±SD. Statistical analysis was performed with a one-way ANOVA, followed by LSD's *post hoc* tests, which was provided by SPSS 10.0 statistical software. Statistical significance was accepted at the level of $P<0.05$.

RESULTS

Injection of cocaine induces hyperphosphorylation of tau in rat brain Compared with saline injected controls, the immunoreactivity to PHF-1 was approximately 3-fold, 2-fold, and 2-fold higher in cortex, hippocampus, and caudatoputamen of rats treated with cocaine both for 8 d and 16 d, respectively (Fig 1A, C, E). The immunoreactivity to Tau-1 was decreased to 69 %, 47 %, and 49 % of vehicle injection on d 8, and to 53 %, 47 %, and 65 % on d 16 in above mentioned brain regions, respectively (Fig 1B, D, F). No significant difference was seen in binding activity to PHF-1 and Tau-1 between 8 d and 16 d. Additionally, cocaine injection did not change the basal level of PHF-1 and tau-1 staining on d 4, compared with control (Fig 1). The data suggest that injection of cocaine leads to hyperpho-sphorylation of tau at Ser199/202 and Ser396/

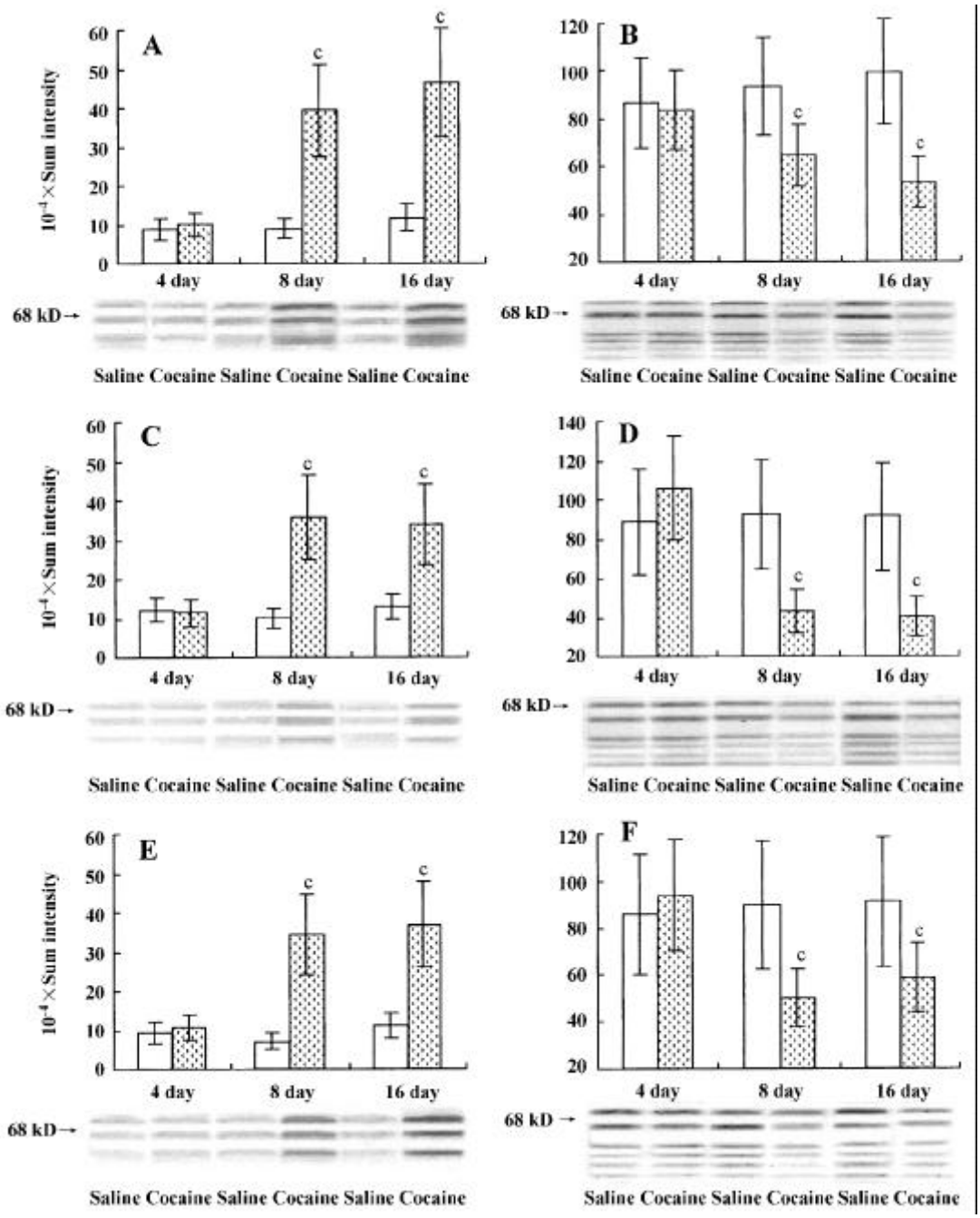

Fig 1. Western blots showing hyperphosphorylation of tau. Immunoreactivity toward PHF-1 (A, C, E) and Tau-1 (B, D, F) in cortex (A, B), hippocampus (C, D), and caudatoputamen (E, F). *n*=7. Mean±SD. $^{c}P<0.01$ *vs* saline. Open column: saline, slashed column: cocaine.

404 in rat brain on d 8 and d 16, but not d 4.

Injection of cocaine induces hyperphosphorylation of neurofilament in rat brain Compared with saline controls, cocaine treatment enhanced SMI31 binding to 2.0-fold, 1.8-fold, and 2.9-fold on d 8, and 2.1-fold, 1.7-fold, and 2.9-fold on d 16 in cortex, hippocampus, and caudatoputamen, respectively (Fig 2A, C, E). Consistent with the above data, cocaine administration caused an obvious decrease in reaction to SMI32, only 39 %, 56 %, and 38 % of binding activity to saline controls in cortex, hippocampus, and caudatoputamen on d 8, and 46 %, 60 %, and 39 % on d 16, respectively (Fig 2B, D, F). Similarly, no significant difference was seen in binding activity to SMI31 and SMI32 between 8 d and 16 d (Fig 2). In addition, cocaine injection did not change the basal level of SMI31

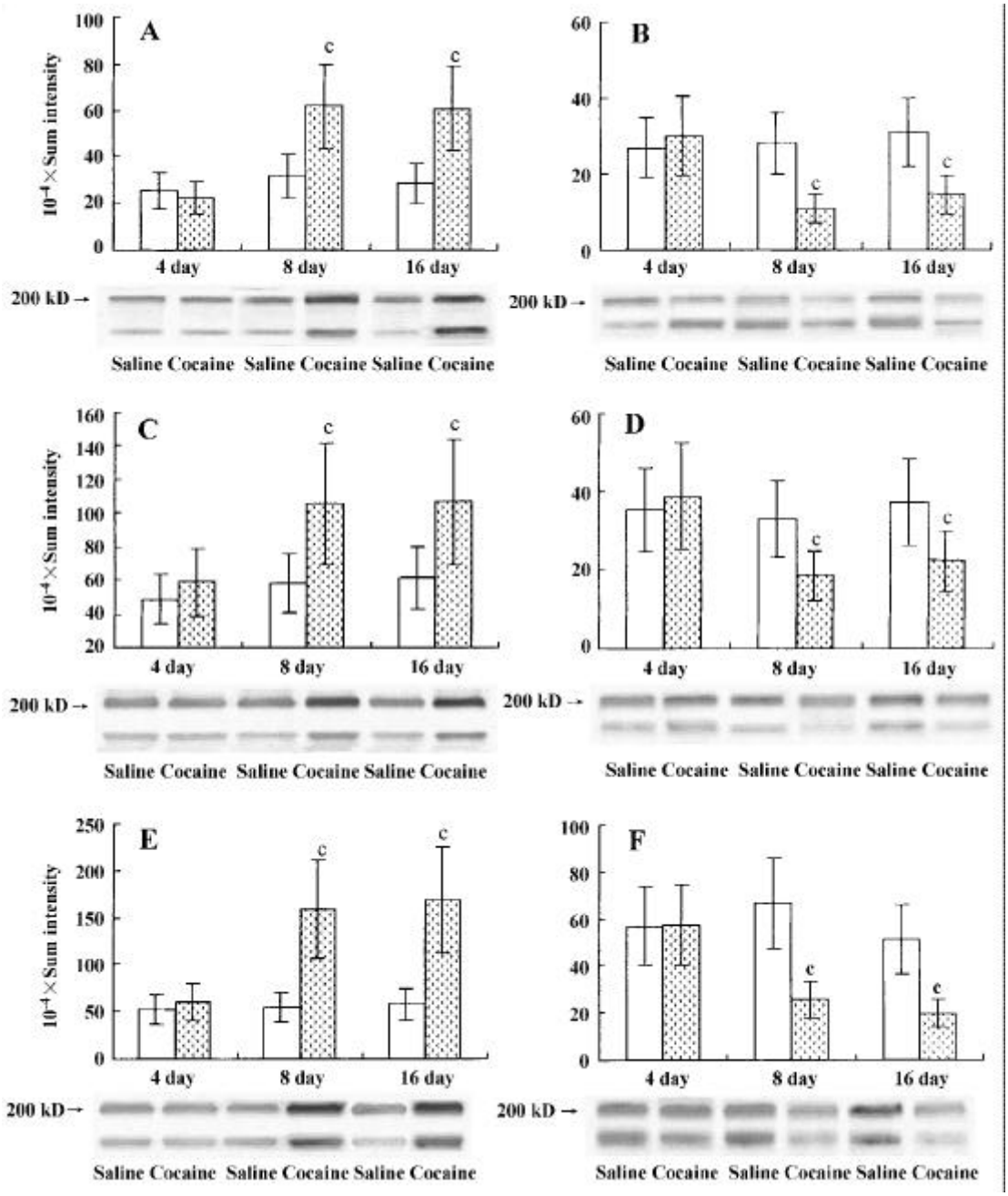

Fig 2. Western blots showing hyperphosphorylation of neurofilament. Immunoreactivity toward SMI31 (A, C, E) and SMI32 (B, D, F) in cortex (A, B), hippocampus (C, D), and caudatoputamen (E, F). *n*=7. Mean±SD. $^{c}P<0.01$ *vs* saline. Open column: saline, slashed column: cocaine.

and SMI32 staining on d 4 (Fig 2). It is suggested that injection of cocaine leads to hyperphosphorylation of neurofilament in rat brain on d 8 and d 16, but not d 4.

Injection of cocaine does not change the expression of CDK5 and p35 Compared with saline control, no significant change in CDK5 and p35 staining was detected in cocaine-injected rats at above mentioned time points and brain regions (Fig 3), suggesting that cocaine-induced hyperphosphorylation of tau and neurofilament is not relevant to overexpression of CDK5 or p35.

DISCUSSION

Alzheimer disease (AD) is the most prevalent dementia affecting the quality of life to a fast growing senile population. One of the pathological hallmarks found in AD brain is the presence of numerous neu-

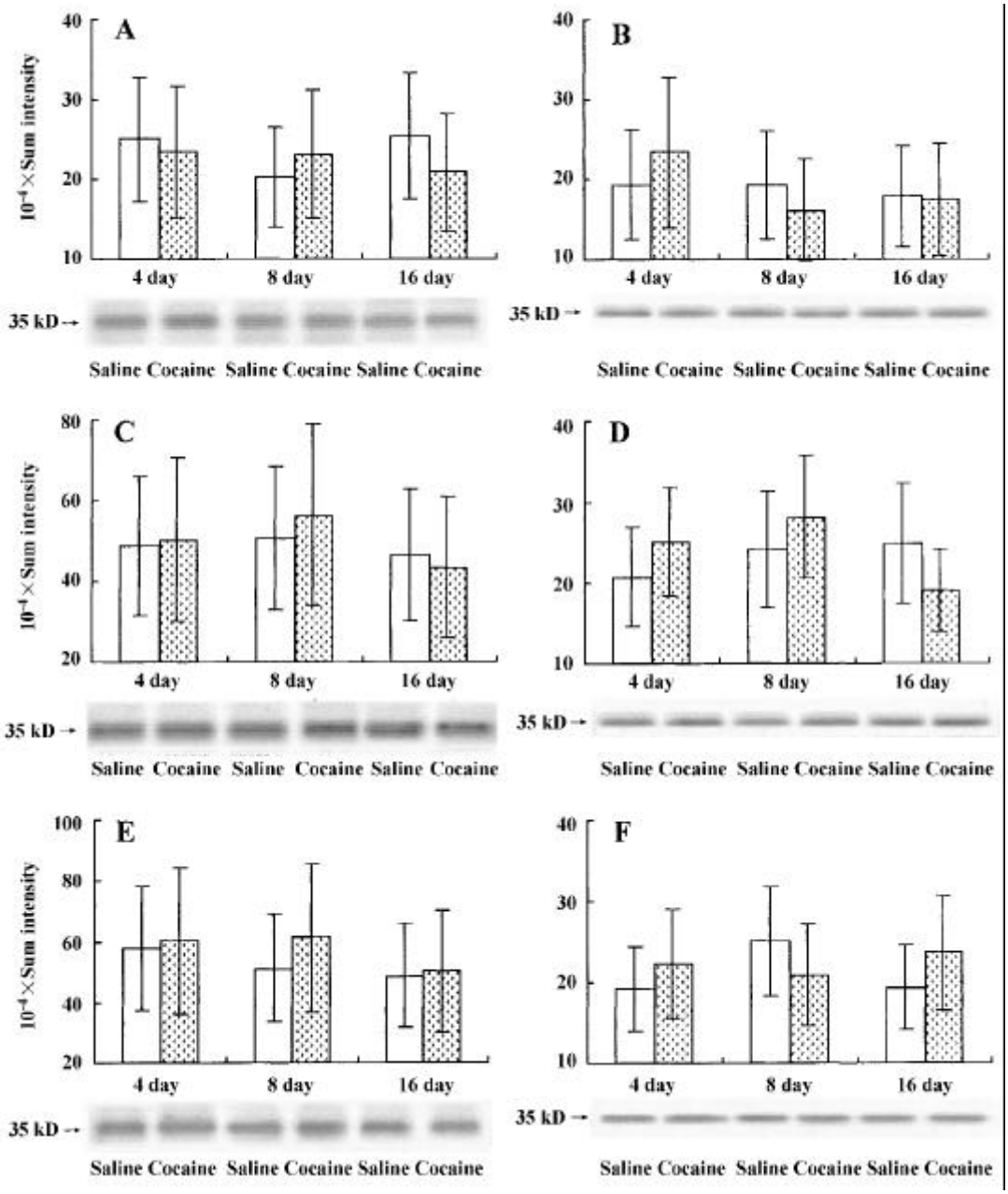

Fig 3. Western blots showing levels of CDK5 and p35. Immunoreactivity toward CDK5 (A, C, E) and p35 (B, D, F) in cortex (A, B), hippocampus (C, D), and caudatoputamen (E, F). *n*=7. Mean±SD. *P*>0.05 at all time points studied. Open column: saline, slashed column: cocaine.

rofibrillary tangles mainly composed of hyperphosphorylated tau[2]. Although it is well accepted that an imbalanced regulation in phosphorylation is involved in this pathological process, it is still not clarified what are the critical kinases and/or phosphatases leading to tau hyperphosphorylation and accumulation. From all the possible target kinases reported recently, CDK5 is one of very few kinases found to be over activated and accumulated in AD neuronal cell[7,8]. Injection of cocaine leads to over expression of CDK5 and its regulatory subunit p35 in corpus striatum in rat brain[10]. Therefore, it was investigated in the present study that the correlation of tau hyperphosphorylation and tentative cocaine-induced CDK5 activation. It was found that peritoneal injection of cocaine for 8 d and 16 d stimulated hyperphosphorylation of tau and as well as neurofilament in brain regions of cortex, hippocampus and caudato-putamen in rats. On the other hand, 4-d injections did not cause the above changes. As hyper-phosphorylation of tau is considered as one of the early events in AD

brain pathology and cocaine stimulates this pathological process as seen in this study, we speculate that cocaine addiction may be one of the simulative factors for AD neurofibrillary degeneration.

Phosphorylation of cytoskeletal proteins is regulated by protein phosphatases and protein kinases. As CDK5 is an immediate downstream target of cocaine-regulated transcription factor[10], and it may be one of the key kinases responsible for the hyperphosphorylation in AD brain[7-9], we detected the expression of CDK5 and its activator of p35 to see the relevance of this kinase with hyperphosphorylation of tau and neurofilament caused by cocaine. We found that there was no alteration in expression of CDK5 and p35 at all time points of cocaine injection, suggesting that hyperphosphorylation of tau and neurofilament caused by cocaine may not correlate to CDK5 or p35. Cocaine, an enhancer of dopamine-mediated neurotransmission, in addition to activating CDK5[10], stimulates the activation of PKA via increasing the level of cAMP in neurons[14,15]. The activated PKA in turn can phosphorylate inhibitor-1 in hippocampus and cortex or dopamine- and cAMP-regulated Phosphorprotein of 32 kDa (DARPP-32) in neostriatum, and phosphorylated inhibitor-1 or DARPP-32 inhibits PP-1 activity. Cocaine inhibits activation of PP-2A[16,17]. And inhibition of PP-2A and PP-1[12,13], activation of GSK-3[18] as well as PKA[19] could induce AD-like hyperphosphorylation of tau. Taking together all the data, we speculated that cocaine might induce imbalance of multiple protein kinase and protein phosphatases, and thus led to hyperphos-phorylation of tau and neurofilament observed in this study. And it is also suggested that enhancement of dopaminergic system, such as seen in cocaine addiction, might be served as one of the most upstream causative factors for the imbalanced regulation in phosphorylation system seen in AD brain.

Until most recently, there is no epidemiological information shown correlation between cocaine addiction and AD. Considering that cocaine addiction is becoming a serious issue both to society as well as to medical science in modern world, and according to the preliminary data observed in this study, we believe that epidemiological investigation on the particular issue might be an interest topic for our future study.

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