Methamphetamine exposure upregulates the amyloid precursor protein and hyperphosphorylated tau expression: The roles of insulin signaling in SH-SY5Y cell line

Lingling Chen1,3,*, Li Zhou1,*, Pengfei Yu3, Fangfang Fang3,5, Lei Jiang2,3, Jian Fei1, Hang Xiao3 and Jun Wang3,4

1Children’s Hospital of Nanjing Medical University, 72 Guangzhou Road, Nanjing, Jiangsu 210029, China
2Department of Emergency Medicine, the First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing, Jiangsu 210029, China
3Key Lab of Modern Toxicology (NJMU), Ministry of Education; Department of Toxicology, School of Public Health, Nanjing Medical University, 818 Tianyuan East Road, Nanjing, Jiangsu 211166, China
4China International Cooperation Center for Environment and Human Health
5Community Health Service Center of Rong Xiang Street, Wuxi City 214000, China

(Received January 15, 2019; Accepted April 26, 2019)

ABSTRACT — Methamphetamine (METH) is a potent and highly addictive central nervous system stimulant. The association between METH exposure and Alzheimer’s disease (AD) has gained more attention, but, the mechanisms behind METH-induced neuron-related adverse outcomes remain poorly understood. With the western blot assay, our results revealed that METH exposure significantly increased the expression of AD-associated pathological proteins, including the amyloid precursor protein (APP) and the phosphorylated tau protein (p-tau). Meanwhile, the insulin signaling was disturbed after the administration of METH, since the key insulin signaling proteins, such as p-AKT, p-GSK3α, p-GSK3β and p-ERK, were reduced. Additionally, the linking between the pathological proteins and the insulin signaling mediated by METH in the present work was verified by the treatment with the insulin signaling enhancer rosiglitazone, which was shown to improve the insulin signaling and decrease APP and p-tau expression. Thus, targeting insulin signaling may provide novel insights into potential therapeutic intervention for METH-mediated AD-like neurodegeneration.

Key words: Methamphetamine, Alzheimer’s disease, Insulin signaling pathway, APP, p-tau

INTRODUCTION

Methamphetamine (METH), a well-known psychostimulant drug, has caused a serious worldwide public health issue. Acute or chronic METH exposure contributes to a spectrum of toxic effects on the central nervous system (CNS) (Prakash et al., 2017). Long-term exposure to METH causes a range of cognitive deficits (Beirami et al., 2018), which involve several mechanisms. For example, METH exposure has been shown to be associated with neuroinflammation in several brain areas, due to its addictive effect (Wongprayoon and Govitrapong, 2015). In recent years, insulin resistance in the brain has been demonstrated to be closely associated with the cognitive changes (Lee et al., 2009; Liu et al., 2011; Moloney et al., 2010; Steen et al., 2005). Thus in the current work, we investigated whether the insulin signaling pathway was involved in AD-associated pathological proteins regulation.

Secreted from the pancreas, hippocampus, prefrontal cortex and other regions in the brain, insulin crosses the blood-brain barrier (BBB) with a saturable transporter, affecting feeding and cognition through CNS mechanisms largely independent of glucose utilization (Banks et al., 2012; Coloma et al., 2000). Insulin receptors (IR) are densely spread in the areas of olfactory bulb, hippocampus and hypothalamus, and autophosphorylation of its activities initiates its downstream substrate, the insulin receptor substrate (IRS) protein, which subsequently binds to phosphatidylinositol 3-kinase (PI3K) and...
activates protein kinase B/Akt. Therefore, the PI3K/Akt pathway in the brain is a critical insulin signaling that is responsible for neuronal protection, learning and memory functions (Zhao et al., 2004). The activation of Akt (phosphorylates at the serine 9 residues) then inactivates GSK3β, one of the key molecules in downstream of the PI3K/Akt signaling pathway and is implicated in several pathological conditions including Alzheimer’s disease (AD) related cognitive deficits (Feinberg et al., 1986; Wang et al., 2010). In addition, another important insulin signaling, the MAPK/ERK pathway, also plays a critical role in the AD formation (Pei et al., 2002), reflecting the complexity of insulin pathway in regulating AD-like pathological changes.

A recent report with the repeated METH exposure in rats showed obvious cognitive deficits in the Y-maze task, accompanying by the insulin signaling (IR/IRS2/PI3K/Akt/GSK3β) impairment (Beirami et al., 2018). However, the effects of METH on the insulin signaling pathway and the expression of AD-associated pathological proteins, including both of the amyloid precursor protein (APP) and p-tau, are not well known. Therefore, the aim of the present study was to determine whether METH exposure causes cognitive impairments concomitant with insulin signaling impairment.

Impaired glucose metabolism in the brains of AD individuals is a widely recognized early feature of the disease. It has been shown that intranasal insulin administration bypasses the blood brain barrier, delivers insulin directly into the special brain areas such as the hippocampus, and obviously improves memory in individuals with mild cognitive impairment and AD without alteration in plasma glucose or insulin levels (Adzovic and Domenici, 2014; Andersen et al., 2017; Lochhead and Thorne, 2012; Renner et al., 2012). Therefore, we hypothesized that the insulin signaling disturbance induced by METH might contribute to the upregulation of APP and p-tau expression, which might finalize cognitive impairments.

MATERIALS AND METHODS

Materials
METH (purity > 99%) was purchased from the National Institutes for Food and Drug Control (Beijing, China). Protease inhibitor cocktail and radio immunoprecipitation assay (RIPA) lysis buffer were obtained from Sigma (St. Louis, MO, USA). The BCA Protein Assay Kit were purchased from Thermo Fisher (Rockford, IL, USA). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Thermo Fisher (Rockford, IL, USA). The BCA Protein Assay Kit were purchased from Thermo Fisher (Rockford, IL, USA). All data were analyzed with SPSS 20.0 software. To test the statistical significance of the differences, one-way analysis of variance (ANOVA) and Dunnett multiple comparison procedures were used, as appropriate, for

Cell culture and sample treatment
SH-SY5Y cells (ATCC#ACS-4004) was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in Dulbecco’s modified eagle’s medium (DMEM) supplemented with high glucose, penicillin 80 units/mL, streptomycin 80 μg/mL, and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO2. SH-SY5Y cells were incubated with various concentration of METH at 0, 100, 300 and 1000 μM for 12 hr.

Western blot
The cells were harvested in an ice-cold RIPA lysis buffer (Sigma) containing protease and phosphatase inhibitor cocktail. The harvested cells were maintained on ice for 10 min and then the lysates were collected and centrifuged at 12,000 rpm for 20 min at 4°C to remove the debris. The protein content of samples was determined by Pierce BCA protein assay reagent (Thermo Scientific Rockford, IL, USA). Equal amounts of protein (40 μg/well) in the lysates were separated by 10% SDS-PAGE and subsequently transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with TBST containing 5% non-fat milk at room temperature for 2 hr, the membranes were incubated with the primary antibodies overnight at 4°C on a shaker. Next to the removal of the primary antibodies, the membranes were incubated with secondary antibodies for 2 hr at room temperature. Finally, antibody-binding bands were revealed with Chemiluminescent HRP Substrate (Millipore Corporation) and normalized to GAPDH. All experiments were repeated at least three times.

Statistical Analysis
All data were analyzed with SPSS 20.0 software. To test the statistical significance of the differences, one-way analysis of variance (ANOVA) and Dunnett multiple comparison procedures were used, as appropriate, for
comparisons. A value of $P < 0.05$ was considered to be statistically significant. A value of $P > 0.10$ was required to assess the homogeneity of variance across the groups.

**RESULTS**

**Effects of METH on the insulin signaling**

The insulin resistance has been identified as a risk factor for developing sporadic AD recently. Therefore, we examined whether METH exposure affects the insulin signaling. The SH-SY5Y cells were incubated with different concentrations of METH for 12 hr, then the p-AKT Ser473 level was detected by western blot. As shown in Fig. 1A and B, a gradual decrease in the phosphorylation levels of AKT Ser473 after treatment of varied concentrations (0, 100, 300, 1000 μM) of METH was observed. Because 1-2 mM METH concentrations have been normally used to investigate amphetamine-type drugs in SY5Y cell line (Chen et al., 2013; Shukla et al., 2019; Wang et al., 2008), we chose the concentration range 0-1000 μM in this study, consistent with our previous research (Chen et al., 2019; Xu et al., 2018). In addition, pS9-GSK3β, a critical insulin resistance protein marker, with a phosphorylation site that is negatively associated with the GSK3β activity, was significantly decreased after the treatment of METH at the dose of 300 and 1000 μM ($P < 0.05$). And similar results were identified for pS21-GSK3α, which is demonstrated to negatively regulate GSK3α activation. As both 300 and 1000 μM METH affected all insulin signaling components assessed, 300 μM was selected for the evaluation of METH-induced insulin signaling disturbances at various time points (0, 0.5, 1, 3, 6, 12, and 24 hr) to avoid METH abusing. For the time course of METH action, the expression of pS473-AKT, pS9-GSK3β and pS21-GSK3α were significantly increased at the early stage of METH exposure, then the levels were obviously decreased at the time points of 3, 6, 12, 24 hr, with a peak response at 6 hr and then gradually decreased to a steady level (Fig. 3C and D). Meanwhile, p-tau, another key neuropathological protein level, was also detected. It was found that METH administration markedly increased the expression of pT205-tau above 100 μM, and the effect was further enhanced to a peak response at 300 μM (Fig. 3E and F). Meanwhile, with the treatment of METH (300 μM), the level of pT205-tau was dramatically increased after 6 hr, with the maximal effect at the time point of 12 hr (Fig. 3G and H).

**Rosiglitazone attenuated METH-induced disturbance of the insulin signaling**

Having determined that METH exposure significantly disturbed the insulin signaling, we then further examined whether enhancing the insulin signaling could attenuate METH-mediated insulin signaling disturbance; therefore, the insulin signaling enhancer rosiglitazone was used in the present work.

After co-incubation with METH (300 μM) in SH-SY5Y cells for 12 hr, it was found that the levels of pY1355-IR and the key downstream signaling molecule pY896-IRS-1 were obviously decreased; noteworthy, these effects were partially rescued by rosiglitazone (Fig. 4A and B). In addition, as a positive control, we confirmed that rosiglitazone treatment elicited a pronounced elevation of pY1355-IR and pY896-IRS-1. In addition to the upstream signaling, the critical downstream molecules were also assessed. Our results showed that p-AKT, the key signal protein regulating insulin resistance, was decreased after the administration of METH. Moreover, pS9-GSK3β and pS21-GSK3α, two glycogen synthase kinases downstream of p-AKT, were investigated. Rosiglitazone substantially retarded METH-mediated down-
Fig. 1. Effects of METH on AKT, GSK3α/β signaling pathway in SY5Y cells. (A) Cells were treated with METH at different concentrations (0, 100, 300, 1000 μM) for 12 hr. (C) Cells were treated with 300 μM METH for different time points (0, 0.5, 1, 3, 6, 12, 24 hr). (B, D) GAPDH levels were assessed in parallel and served as controls. Data are presented as the mean ± SD from three independent experiments. Significant differences between the treatment groups and the control group were determined via one-way ANOVA and the Dunnett multiple comparison procedure. (*P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group).
regulation of p-AKT, p-ERK and ameliorated METH-induced GSK3β and GSK3α activation (Fig. 4C and D, Fig. 5). These results suggest that METH exposure contributes to the disturbance of the insulin signaling.

METH upregulates APP and p-tau expression through disturbing the insulin signaling

Combining the effects of GSK3α and GSK3β on regulation of APP and p-tau respectively, together with the insulin signaling disturbance mediated by METH aforementioned, we then sought to examine the associations between APP and p-tau expression and the insulin signaling. METH-induced upregulation of APP was substantially decreased when SH-SY5Y cells were co-incubated with 10 or 50 μM rosiglitazone (Fig. 6A and B). Similarly, METH administration induced a striking increase in the expression of pT205-tau and this effect was markedly decreased when SH-SY5Y cells were co-incubated with rosiglitazone treatment (Fig. 6C and D). These findings demonstrated the involvement of insulin signaling in METH-induced pathological protein regulation.

DISCUSSION

Accumulating studies have shown that METH exposure causes severe degenerative-like damage to the CNS (Brooks et al., 2016; Liu et al., 2017), demonstrating that METH exposure might contribute to AD-like pathological changes. To identify the potential pathological proteins and mechanisms, we extended our research on METH-induced neural toxicity by further investigating the effects of insulin signaling in this process.

The brain insulin released from the hippocampus, frontal lobe and other brain regions, which are particularly important for learning and memory (De Felice, 2013; De Felice and Benedict, 2015; Gerozissis, 2008; Huang et al., 2010). Therefore, the association between insulin signaling pathway and neurodegenerative disease in the brain has gained more attention. AKT-S473, a key phosphorylation site for insulin signaling transduction (Summers and Birnbaum, 1997), has been shown to exert an important upstream signaling component that blocks GSK-3β activation. Moreover, abnormal expression of PI3K/AKT

Fig. 2. Effect of METH on ERK signaling pathway in SY5Y cells. Cells were treated with various concentrations of METH (0, 100, 300, 1000 μM) for 12 hr or 300 μM METH for different time points (0, 0.5, 1, 3, 6, 12, 24 hr), respectively. Then, the cell lysates were prepared and western blot was performed to analyze dose-response (A) or time course (C) of phosphorylated ERK and total ERK. (B, D) GAPDH levels were assessed in parallel and served as controls. Data are presented as the mean ± SD from three independent experiments. Significant differences between the treatment groups and the control group were determined via one-way ANOVA and the Dunnett multiple comparison procedure. (*P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group).
in the neurons is one of the early features of AD (O’Neill, 2013). Ryder demonstrated that selective down-regulation of AKT Ser phosphorylation had been observed in the lymphoblast cells of familial AD (Ryder et al., 2004). Coincidently, the protein of AKT, GSK3β and p-tau co-localize in some neurofibrillary tangles via immunohistochemistry analysis (Yarchoan et al., 2014). In accordance with the study aforementioned, our results demonstrated a significant decrease in the phosphorylation level of AKT at serine 473 after METH administration. The substrates of GSK3 include a series of metabolic, signaling and structural proteins and transcription factors, among

Fig. 3. Effects of METH on APP and p-tau expression in SY5Y cells. (A, E) Cells were treated with METH at different concentrations (0, 100, 300, 1000 μM) for 12 hr. (C, G) Cells were treated with 300 μM METH for different time points (0, 0.5, 1, 3, 6, 12, 24 hr). (B, D, F, H) GAPDH levels were assessed in parallel and served as controls. The results are presented as the mean ± SD from three independent experiments. Significant differences between the treatment groups and the control group were determined via one-way ANOVA and the Dunnett multiple comparison procedure. (*P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group).
which are highly pertinent to the nervous system (King et al., 2015). The decrease in the phosphorylation of GSK-3α and β are responsible for APP generation and the hyper-phosphorylation of tau, respectively (Zhao et al., 2011). In the present work, METH significantly reduced phosphorylated pS9-GSK3β, which negatively reflects increased GSK-3β activity. Meanwhile, the GSK-3α was also activated since the level of pS9- GSK3α was significantly decreased. Besides, the present study showed that repeated METH administration impaired cognitive per-

Fig. 4. Effect of rosiglitazone on METH-induced disturbance of IR/IRS/AKT/GSK3α/β signaling pathway in SY5Y cells. (A, C) Cells were incubated in the presence or absence of rosiglitazone (10, 50 μM) and METH (300 μM) for 12 hr. Then, the cell lysates were prepared and western blot was performed to analyze phosphorylated and total expression of IR/IRS1/ AKT/GSK3α/β. (B, D) GAPDH levels performed in parallel served as controls. The experiments were repeated three times, and similar results were obtained. Statistical significance was determined by one-way ANOVA and the Dunnett multiple comparison procedure. (*P < 0.05, **P < 0.01 compared to normal control, #P < 0.05, ##P < 0.01 compared to METH treatment).
Fig. 5. Effect of rosiglitazone on METH-induced disturbance of ERK signaling pathway in SY5Y cells. (A) Cells were incubated in the presence or absence of rosiglitazone (10, 50 μM) and METH (300 μM) for 12 hr. Then, the cell lysates were prepared and western blot was performed to analyze phosphorylated and total expression of ERK. (B) GAPDH levels performed in parallel served as controls. The experiments were repeated three times, and similar results were obtained. Statistical significance was determined by one-way ANOVA and the Dunnett multiple comparison procedure. (*P < 0.05, **P < 0.01 compared to normal control, #P < 0.05, ##P < 0.01 compared to METH treatment).

Fig. 6. Effect of rosiglitazone on METH-induced overexpression of APP and p-tau in SY5Y cells. (A, C) Cells were incubated in the presence or absence of rosiglitazone (10, 50 μM) and METH (300 μM) for 12 hr. Then, the cell lysates were prepared and western blot was performed to analyze APP and p-tau expression. (B, D) GAPDH levels performed in parallel served as controls. The experiments were repeated three times, and similar results were obtained. Statistical significance was determined by one-way ANOVA and the Dunnett multiple comparison procedure. (*P < 0.05, **P < 0.01 compared to normal control, +P < 0.05, ++P < 0.01 compared to METH treatment).
formance through the ERK pathway and decreased the phosphorylation of ERK1/2 in the prefrontal cortex of rats (Saeed et al., 2018). Thus, these findings prompted us to infer that METH exposure results in the enhancement of APP and p-tau expression.

To illuminate the specific linkage between insulin signaling disturbance and the enhancement of APP/p-tau mediated by METH, rosiglitazone, a selective ligand of peroxisome proliferator-activated receptor-γ (PPAR-γ), was applied in this study. Rosiglitazone is an insulin sensitizer which is used in a number of insulin-resistant conditions. A spectrum of evidence shows that rosiglitazone ameliorates insulin resistance in the liver and skeletal muscle, and preserves pancreatic β-cell function, which makes it one of the most important drugs for the treatment of diabetes (DeFronzo et al., 2010; Kim et al., 2003). Notably, the increased expression of pathological protein mediated by METH, including APP and p-tau, was markedly attenuated by rosiglitazone, suggesting the pivotal effects of insulin signaling pathways in METH-induced neural damage, showing that insulin effectively relieved METH-induced anxiety-like behavior and neuroinflammation in animals (Beirami et al., 2017). These studies suggest that the insulin signaling pathways can be considered useful targets for METH-induced neural damage.

In summary, the present study provides a novel molecular mechanism of METH-mediated neural damage involving the disturbance of AKT signaling transduction and downstream GSK3α/β and ERK activation, which might in turn contribute to the increased expressions of APP and p-tau (Fig. 7). However, as the in vitro results with Alzheimer Disease-like effects are speculative, more studies are needed to explore the links between insulin signaling disturbance and pathological protein formation.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (81673213, 81202230), the Natural Science Foundation of Jiangsu Province (BK20151557), the Key disease of Jiangsu Province Science and Technology Department (BL2014088)

Conflict of interest---- The authors declare that there is no conflict of interest.
Insulin signaling in METH-induced AD changes


