Nicotine exacerbates tau phosphorylation and cognitive impairment induced by amyloid-beta 25–35 in rats

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Nicotine was reported to reduce the plaque burden and could be used as a possible anti-Alzheimer’s disease agent. However, the effect of nicotine on memory and tau pathology in Alzheimer’s disease has been less studied. The present study investigated the effect of nicotine on tau phosphorylation and cognitive impairment induced by hippocampus injections of amyloid-beta (Aβ) 25–35. Rats were treated with nicotine hydrogel tartrate salt dissolved in normal saline by subcutaneous injection twice per day for 14 days. The age and gender matched rats treated with same amount of normal saline were used as the control. Morris water maze was used to detect the cognitive impairment induced by Aβ25–35. Compared to the sham- operated rats, Aβ25–35 injection significantly prolonged the mean escape latency in vehicle-treated rats in the Morris water maze test and increased the number of tau(pS202) and tau(pT231) immunoreactive cells. The data show that nicotine (1 mg/kg in base weight) treatment significantly exacerbates cognitive impairment and tau phosphorylation at Ser-202 and Thr-231 in the hippocampus compared with Aβ25–35 injection groups in the Aβ rat model of Alzheimer’s disease. The use of nicotine for treatment of Alzheimer’s disease should be reassessed.

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1. Introduction

Alzheimer’s disease is the most common cause of dementia in the elderly. It is characterized by the presence of two pathological hallmarks: extracellular amyloid plaques, which are mainly formed by a small peptide called amyloid-beta (Aβ), and intracellular neurofibrillary tangles, which are intracellular inclusions composed of aggregates of hyperphosphorylated tau protein (Nordberg, 2003). It has been reported that abundance in neurofilibrillary tangles plays an important role in the pathogenesis of Alzheimer’s disease (Arriagada et al., 1992; Congdon and Duff, 2008). Neurofibrillary tangle correlates well with the clinical severity of dementia and appears to be closely related to the extent of neuronal loss in Alzheimer’s disease brain, which suggests that neurofibrillary tangles plays an important role in the pathogenesis of Alzheimer’s disease (Arriagada et al., 1992; Congdon and Duff, 2008).

Tobacco smoking is a major health problem that is estimated to cause 4 million deaths worldwide per year. Regarding the relationship between cigarette smoking and the risk of developing Alzheimer’s disease, contradictory results have emerged from epidemiological studies. Results from case–control studies show that smoking is associated with decreased risk of Alzheimer’s disease (Brenner et al., 1993; Ferini-Strambi et al., 1990; Graves et al., 1991; Reitz et al., 2007), while cohort studies seem to indicate that smoking increases the risk of Alzheimer’s disease (Ernst et al., 2001; Juan et al., 2004; Lee, 1994; Ott et al., 1998; Peters et al., 2008). Nicotine is a major bioactive constituent of tobacco. Previous studies have shown that nicotine at low doses can improve memory functions and reduce the plaque burden, and could be used as a possible anti-Alzheimer’s disease agent (Lagostena et al., 2008; Mckee et al., 2008; Shim et al., 2008). Nicotine protects hippocampal neurons against the Aβ-induced apoptosis in vitro (Hellstrom-Lindahl et al., 2004). Nicotine treatment increases the expression of Aβ precursor protein and Aβ precursor-like protein 2 in rat brain and SH-SYSV neuroblastoma cells (Hellstrom-Lindahl et al., 2000). Treatment with nicotine reduced insoluble Aβ40 and Aβ42 in the cortex, and Aβ deposits in parenchyma and vessels in the brain of APPSwe transgenic mice (Wang et al., 2003). However, the effects of nicotine treatment on the tau pathology could not be addressed due to the lack of the critical neuropathological hallmark in these animal models.

The effects of nicotine on tau pathology and cognitive impairment remain unclear (Oddo and LaFerla, 2006; Shim et al., 2008; Welsby et al., 2006). Few studies have examined the relationship between nicotine treatment and tau pathology in vitro and in vivo. Treatment of SH-SYSV cells with nicotine increased tau phosphorylation, and...
chronic consumption of nicotine for five months increases the tau phosphorylation in the 3×Tg Alzheimer’s disease mouse model (Hellstrom-Lindahl et al., 2000; Oddo et al., 2005; Wang et al., 2003). In the present study, we investigated the effects of acute nicotine treatment on tau phosphorylation in the hippocampus and cognitive impairment of a rat model induced by hippocampal injections of Aβ25–35.

2. Materials and methods

2.1. Animals

Twenty-four male Sprague–Dawley rats, 3–5 months old, 250–350 g, were used in the present study. Rats were housed in a cage in a group of 8 rats, in a room maintained at around 22 °C with a 12 h light/dark cycle. Food and water were available ad libitum. All animal experiments were performed according to the Policies on the Use of Animals and Humans in Neuroscience Research, revised and approved by the Society for Neuroscience in 1995. The time scheme of the animal experiments was shown in Fig. 1.

2.2. Drug administration

Aβ peptide (25–35) (Sigma) was dissolved in sterile distilled water at a concentration of 5 μg/μl, and the solution was incubated at 37 °C for 5 days for aggregation. Animals were anesthetized by intraperitoneal injection of 5% chloral hydrate and placed in a stereotaxic instrument. Aβ peptide solution (2 μl) was injected into right and left hippocampus with a microsyringe at a rate of 0.5 μl/30 s with the following coordinates: 4.8 mm anterior to posterior (taken from hippocampus with a microsyringe at a rate of 0.5

2.3. Hippocampal injection of Aβ

Animals were randomly assigned to three groups: sham/vehicle, Aβ/vehicle, Aβ/nicotine (n = 8 per group). For Aβ/vehicle or Aβ/nicotine group, after Aβ25–35 injection rats were administered with vehicle (sterile 0.9% NaCl) (n = 8) or nicotine hydrogen tartrate salt (Sigma) (n = 8) via subcutaneous injections twice a day for 14 days (daily dose was 1 mg/kg in base weight). The sham/vehicle group, used as a normal

2.4. Behavioral test

This task represents a classic version of the Morris water maze task and was performed following the protocols reported previously with minor modifications (Yau et al., 2007). In brief, the test was conducted in a pool (200 cm diameter) which was filled with water (24 ± 2 °C), made opaque with milk powder and surrounded by a set of spatial cues (Markowska et al., 1993). The tank was imaginarily subdivided into four quadrants, and four start positions were located at the intersections of the quadrants (Frick and Fernandez, 2003). The time scheme for platform trials and probe trial was shown in Fig. 1.

2.4.1. Platform trials

Each daily session consisted of four platform trials in which a round escape platform (14 cm diameter) was submerged 2 cm under water surface in a quadrant. The rat navigated in the pool to locate the platform and was then able to escape. If the rat failed to locate the platform within 120 s, it was directed to the platform. Once the rat escaped onto the platform, it remained on the platform for 10 s.

2.4.2. Probe trial

All rats were given a single probe trial, where the platform was withdrawn, at one day after the last platform trial in order to assess the final strength of memory traces. The start position was located in the opposite quadrant. The duration for probe trial was 40 s.

Performance in all tasks was recorded and analyzed by a computer-based video tracking system and image analyzing software (Stoelting). In platform trials, latency (the time to reach the platform from the start location, s), and swim speed (average speed during a trial, cm/s) were measured. While in probe trials quadrant time (percentages of time spent in the platform quadrant) and platform crossings (the number of times that the rats crossed the exact location of the platform) were measured. For latency in platform trials, lower numbers indicated a better performance. For quadrant time and platform crossings in probe trials, higher numbers indicated a better performance (i.e. more time spent in the correct quadrant and more crossings over the platform location) (Frick and Fernandez, 2003).

2.5. Tissue sampling

Animals were overdosed with chloral hydrate and perfused intracardially with 100 ml of 0.1% NaNO2 in phosphate buffer. For histological analysis, left hemibrains were fixed in 4% paraformaldehyde (pH 7.4) for 24 h and subsequently incubated for 24 h in 30% sucrose for cryoprotection. Coronal sections of the brain at 40 μm thickness were collected with a cryosectioning microtome and stored at 4 °C in PBS containing 0.1% sodium azide until use. For biochemical analysis, right hemibrains were snap frozen in liquid nitrogen and stored at −80 °C until processing.

2.6. Immunohistochemistry and quantification

The staining for phosphorylated tau in the hippocampus was processed using the previously described method (Wang et al., 2009). Briefly, a series of five equally spaced tissue sections spanning the hippocampus were stained using free-floating immunohistochemistry. Sections were incubated with primary antibodies pT231 (Biosource, 1:1000 dilution) or pS202 (Biosource, 1:1000 dilution) at 4 °C overnight, further developed with biotinylated secondary antibodies and the ABC kit (Vector Lab, Burlingame, CA) using diaminobenzidine and glucose oxidase as substrates.

Sections were observed under the light microscope with constant bulb temperature and exposure. The immuno-staining positive cells in the hippocampus were counted by an independent experienced neuroscientist who was blind to the animal information. The average of the cell count per animal was used to calculate group means and standard errors.

2.7. Western blotting analysis

Hippocampus homogenates were subjected to SDS-PAGE (10% acrylamide). The blots were probed with the following antibodies: anti-tau(pT231) (1:1000), anti-tau(pS202) (1:1000), and anti-β-actin monoclonal antibody (Sigma-Aldrich). All blots were probed with peroxidase-conjugated secondary antibody and developed with DAM...
system. The band density of the tau proteins was normalized to that of β-actin.

2.8. Data analysis

The results are expressed as mean ± SEM. Statistical comparisons among groups were assessed using one-way ANOVA. P values less than 0.05 were considered significant. All these analyses were performed using SPSS for Windows version 13.0.

3. Results

3.1. Administration of nicotine increases tau phosphorylation in Alzheimer’s disease rat model

To determine the difference of tau phosphorylation among sham/vehicle, Aβ/vehicle and Aβ/nicotine-treated groups, we employed two anti-tau antibodies to phosphorylated tau protein, including tau (pS202) and tau (pT231), to detect phosphorylation on tau protein. The number of tau (pS202) and tau (pT231) immunoreactive cells was shown in Fig. 2. The results showed that the immunoreactive cells of tau (pS202) and tau (pT231) were significantly increased in the hippocampus in Aβ/vehicle rats compared with that of sham/vehicle rats, suggesting that tau was hyperphosphorylated at Ser-202 and Thr-231 in response to Aβ/vehicle rats in hippocampus. The hyperphosphorylated tau mainly located in the cytoplasm around the nucleus in hippocampal pyramidal neurons. The results showed that nicotine treatment markedly increased tau phosphorylation at Ser-202 and Thr-231 in the hippocampus compared with Aβ/vehicle rats (P < 0.05).

To confirm the increase in tau hyperphosphorylation observed in immunohistochemical staining, we further examined the phosphorylated tau protein levels in hippocampal homogenates using Western blotting analysis. Consistently, Aβ/vehicle group had higher levels of phosphorylated tau Ser-202 and Thr-231 than sham/vehicle group (P < 0.05), while Aβ/nicotine group had more phosphorylated tau proteins in relative to Aβ/vehicle Alzheimer’s disease group (P < 0.05, Fig. 2).

3.2. Nicotine exacerbates cognitive impairment in Alzheimer’s disease rat model

It has been suggested that injections of Aβ25–35 into hippocampus causes memory deficits and tau phosphorylation in the hippocampus (Sigurdsson et al., 1996; Takashima et al., 1998). Consistently, in the present study, the injection of 10 μg of Aβ25–35 induced cognitive dysfunction as assessed by Morris water maze tests. We found no difference among sham/vehicle, Aβ/vehicle and Aβ/nicotine groups in the hidden platform trial before hippocampus injection of Aβ25–35 (P = 0.05, Fig. 3A). During 12 to 16 days, statistically significant difference was observed between the sham/vehicle and Aβ/vehicle groups in the hidden platform trial. The Aβ/nicotine group showed a significant increase in escape latency compared to the Aβ/vehicle group (P < 0.05, Fig. 3A). These effects were not attributable to the presence of motor deficits, because the three groups exhibited similar swimming speeds (Fig. 3B).

The probe trial was employed to determine memory of the rats after training. On days 17 the number that the animals crossed over the position where the platform located was less in Aβ/vehicle group than sham/vehicle group. The number of crossing over a position on the platform was further decreased in Aβ/nicotine group relative to Aβ/vehicle group (P < 0.05, Fig. 3C). The Aβ/vehicle rats spent less time than sham/vehicle rats in the target quadrant where the platform was previously located, while nicotine treatment (Aβ/nicotine) further shortened the time in target quadrant (P = 0.165, Fig. 3D).

![Fig. 2. Immunohistochemistry and Western blotting of tau phosphorylation. A–C. Representative images of tau (pS202) immunohistochemical staining in hippocampus. D. Comparison of tau (pS202) positive cell number in hippocampus among groups. E–G. Representative images of tau (pT231) immunohistochemical staining in hippocampus. H. Comparison of tau (pT231) positive cell number in hippocampus among groups. I. Western blotting analysis of tau (pS202) and tau (pT231) levels in hippocampus. J. Comparison of tau (pS202) and tau (pT231) levels in hippocampus among groups. * and ** denote P < 0.05 or 0.01 vs. sham/vehicle group, # P < 0.05 vs. Aβ/vehicle group. Bar = 50 μm.](image-url)
4. Discussion

In the present study, we investigated the effects of nicotine and tau phosphorylation and cognition in Alzheimer’s disease rat model injected with Ab25–35, and found that hippocampal injection of Ab25–35 increased the tau phosphorylation in hippocampus and impaired the cognition, while nicotine treatment exacerbated tau phosphorylation and cognitive impairment of the Alzheimer’s disease rats.

Hippocampal injection of Ab25–35 is often employed to prepare Alzheimer’s disease model (Chen et al., 1996; Giovannelli et al., 1995; Maurice et al., 1996; Nitta et al., 1994; Stepanichev et al., 2003; Yamaguchi and Kawashima, 2001; Yamaguchi et al., 2002). The effects of Ab are considered to be localized to amino acid residues 25–35 of the full-length peptide, and Ab fragment 25–35 (Ab25–35) was known as the neurotoxic domain of the parent Ab peptide. Therefore, Ab25–35 was used in this study. Consistent with previous studies (Nitta et al. 1994; Sigurdsson et al., 1996; Stepanichev et al., 2005), we also found that hippocampal injection of Ab25–35 induced significant cognitive impairment and local tau phosphorylation, suggesting the success in the Alzheimer’s disease modeling in the present study.

Microtubule associated protein tau is a phosphoprotein that functions to stabilize microtubules and is minimally phosphorylated in the normal adult brain (Ribe et al., 2005; Sun et al., 2003). Abnormal hyperphosphorylation of tau is a critical event in the pathogenesis of Alzheimer’s disease which is related to tauropathies, a pathological hallmark of Alzheimer’s disease (Echeverria et al., 2004; Lavados et al., 2005; Rankin et al., 2005a). Tau is aberrantly hyperphosphorylated in the Alzheimer’s disease brain, with three to four times of increase in phosphates (Gohar et al., 2009). To date, all the identified 21 phosphorylated sites in the abnormally hyperphosphorylated tau in Alzheimer’s disease are either on serine or threonine residues (Rankin et al., 2005a). Hyperphosphorylation at Thr–231 and Ser–202 of tau protein is much in relation to the memory retention impairment of rats (Chen, 2005). Specifically, Thr–231 has been shown to directly affect tau binding to microtubules, whereas Ser–202 modulates the kinetics of tau binding to microtubules (Chen, 2005). In this regard we examined tau(pSer202) and tau(pThr231) in the present study. Consistent with previous studies (Chambers et al., 2000; Zheng et al., 2002), our data show that injection of Ab25–35 increased tau phosphorylation at Ser–202 and Thr–231 relative to Ab25–35 injection group. Previous studies have been mostly focused on the relationship between nicotine and Ab peptide in Alzheimer’s disease, however, the effects of nicotine on tau phosphorylation are less studied. To our knowledge, only few studies have investigated the effect of nicotine on tau pathology in vitro, while the effects of nicotine on tau are less reported in vivo. In vitro studies show that nicotine treatment increases the tau phosphorylation in SH-SY5Y and human SK-N-MC neuroblastoma cells (Hellstrom-Lindahl et al., 2000; Wang et al., 2003). Important evidence comes from an in vivo study, which suggests that chronic nicotine administration markedly exacerbates tau phosphorylation and tau pathology in the brain of 3×Tg Alzheimer’s disease mice bearing human mutant APP, PS1 and tau genes (Oddo et al. 2005). In the present study, we add further in vivo evidence that acute administration of nicotine also increases the tau phosphorylation and tau pathology in hippocampus of Alzheimer’s disease rat model. Taken together, these studies raise the notion that nicotine consumption is a crucial factor which promotes the tau phosphorylation and may contribute to the development of Alzheimer’s disease, despite the beneficial effects of nicotine consumption on the Ab pathways. Consistently, a retrospective study found a positive correlation between the amount of smoking and the neurofibrillary changes in the brain of unselected consecutive patients aged 65 years or older (Ulrich et al., 1997), although whether smoking is associated with incidence of Alzheimer’s disease is still controversial in epidemiological studies (Hernan et al., 2008; Lee, 1994; Peters et al., 2008).

In the present study, acute nicotine treatment significantly exacerbated the cognitive impairment of Alzheimer’s disease rat model. The effects of nicotine on cognition are controversial. Some studies suggest that nicotine improves memory function (Arendash et al., 1995; French et al., 2006; Levin and Simon, 1998; Socci et al., 1995), while others find that nicotine administration impairs the cognition in human and animal models (Rukull, 2001; Olds et al., 1994; Ott et al., 1998; Sexton et al., 1990). These inconsistencies could be attributed to the dose of nicotine (acute or chronic, continuous or intermittent), animal chosen, behavioral testing protocols and experimental procedures. It seems that a high dose of nicotine could induce neurotoxicity and stimulate oxidative stress, while a low dose of nicotine could improve performance on cognition (Barros et al., 2007).

The exacerbated cognitive impairment by nicotine treatment observed in the present study might due to several reasons. The first possible reason is that exacerbated tau phosphorylation after nicotine administration might contribute to the cognitive decline. Hyperphosphorylation of tau induced by nicotine administration has been...
been shown to dissociate tau from microtubules, leading to the neuronal cytoskeleton and interference with cellular transport mechanisms, then causing cognitive impairment (Lavados et al., 2005; Rankin et al., 2005b). A recent study also finds that tau protein phosphorylated is correlated with cognitive decline in mild cognitive impairment subjects (Chen, 2005). The second possible reason may exist in the detrimental effects on brain of the high dose of nicotine administration. A recent study finds that nicotine administration at 1 mg/kg day can induce neurotoxicity and stimulate oxidative stress (Barros et al., 2007). The dose used in the present study (daily dose was 1 mg/kg in base weight) is comparable to those used in other studies (Barros et al., 2007; Freir and Herron, 2003; Popke et al., 2000; Welsby et al., 2007). It is also possible that high dose of nicotine administration can cause stress which can interfere the cognitive tests (Giovannelli et al., 2003; Guan et al., 2003; Qiao et al., 2005).

In the present study, we did not see obvious abnormal behavior indicating a stress after nicotine administration during the whole course of experiment. Thus it is less possible that the poor performance of cognitive test is caused by the nicotine induced stress responses in the present study.

In conclusion, our studies suggest that acute nicotine administration can exacerbate the tau phosphorylation and cognitive impairment in Alzheimer’s disease rat model. The mechanisms underlying the effects of nicotine on tau phosphorylation or memory need to be studied in the future. Along with other studies (Hollsten-Lindahl, 2000; Hollsten-Lindahl et al., 2000; Oddo et al., 2005), we propose that nicotine may play dualistic roles in the pathogenesis of Alzheimer’s disease with beneficial roles in Aβ metabolism and detrimental roles in tau pathologies, suggesting that simple use of nicotine for treatment of Alzheimer’s disease should be reassessed.

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