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Ketamine induces tau hyperphosphorylation at serine 404 in the hippocampus of neonatal rats***☆

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Abstract

Male Wistar 7-day-old rats were injected with 40 mg/kg ketamine intraperitoneally, followed by three additional injections of 20 mg/kg ketamine each upon restoration of the righting reflex. Neonatal rats injected with equivalent volumes of saline served as controls. Hippocampal samples were collected at 1, 7 or 14 days following administration. Electron microscopy showed that neuronal structure changed noticeably following ketamine treatment. Specifically, microtubular structure became irregular and disorganized. Quantitative real time-PCR revealed that phosphorylated tau mRNA was upregulated after ketamine. Western blot analysis demonstrated that phosphorylated tau levels at serine 396 initially decreased at 1 day after ketamine injection, and then gradually returned to control values. At 14 days after injection, levels of phosphorylated tau were higher in the ketamine group than in the control group. Tau protein phosphorylated at serine 404 significantly increased after ketamine injection, and then gradually decreased with time. However, the levels of tau protein at serine 404 were significantly greater in the ketamine group than in the control group until 14 days. The present results indicate that ketamine induces an increase of phosphorylated tau mRNA and excessive phosphorylation of tau protein at serine 404, causing disruption of microtubules in the neonatal rat hippocampus and potentially resulting in damage to hippocampal neurons.

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Key Words

neural regeneration; tau protein; ketamine; phosophorylation; neonatal rats; hippocampus; neurons; anesthetics; cognition; grants-supported paper; neuroregeneration

Research Highlights

(1) Commonly used inhalation anesthetics such as isoflurane and sevoflurane can induce apoptosis, tau protein hyperphosphorylation and memory disorders.
(2) Tau protein hyperphosphorylation at serine 396/serine 404 is found in intraneuronal neurofibrillary tangles during neurodegeneration.
(3) Hyperphosphorylated tau protein at serine 396 has been found in adult monkey brain tissue following chronic ketamine administration.
(4) We show that hyperphosphorylation of tau protein at serine 404, but not serine 396, occurs in neonatal rat hippocampus following a large dose of ketamine.
(5) Our results suggest that ketamine induces tau hyperphosphorylation at serine 404, resulting in damage to microtubule and axonal transport. Such damage may cause neurotoxicity and neuronal death in neonatal rats, consistent with previous studies demonstrating ketamine-induced neuronal apoptosis.

INTRODUCTION

Recent studies[1-5] have suggested that anesthesia may be a significant risk factor for later development of a learning disability and deviant behavior. However, it is not known whether the anesthesia itself contributes to the learning disability and deviant behavior or whether the need for anesthesia is a marker for other unidentified factors that contribute to the development of learning disabilities and deviant behavior. Moreover, a twin study[6] has suggested that there is no evidence for a causal relationship between anesthesia administration and later learning-related outcomes. Therefore, there is a need to study the effects of anesthetics, such as ketamine, on biochemical changes associated with potential neurotoxicity.

Tau proteins belong to the family of microtubule-associated proteins. They play an important role in the assembly of microtubules, contributing to axonal integrity of the normal mature neuron, but also playing a role at the dendritic and nuclear levels[7]. Hyperphosphorylated and abnormally phosphorylated forms of tau are the major constituents of intraneuronal paired helical filaments observed in Alzheimer's disease, and also of other filaments seen in several neurodegenerative disorders referred to as "tauopathies"[8]. Several other studies[9-10] have shown that the commonly used inhalation anesthetics, for example, isoflurane and sevoflurane, may induce apoptosis in brain tissues of neonatal mice, and that sevoflurane exposure is associated with spatial memory deficits and increased tau phosphorylation through the activation of specific kinases. These data support a correlation between exposure to this anesthetic agent and cognitive decline.

In the present study, we examined tau hyperphosphorylation in neonatal rat hippocampus after administration of ketamine, the most commonly used general anesthetic in pediatric practice. We measured the level of total phosphorylated tau mRNA, and levels of tau phosphorylation at serine residues 396 (serine 396) and 404 (serine 404), principal sites of aberrant tau phosphorylation.

RESULTS

Quantitative analysis of experimental animals

Thirty-six male 7-day-old rats were randomly assigned to the ketamine group and the control group, and injected with ketamine or saline intraperitoneally. Hippocampi were harvested at 1, 7 or 14 days after injection. All rats were included in the final analysis.

Effects of ketamine on the hippocampal structure of neonatal rats

Under an electron microscope, the microtubule cytoskeletons were arranged regularly and parallel to each other in the control group, but appeared disrupted and disorderly in the ketamine group (day 7) (Figure 1).

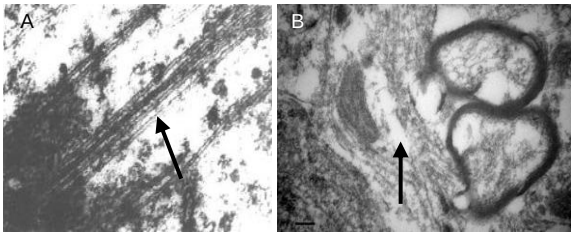

Figure 1 Change of microtubule structure in the neonatal rat hippocampus at 7 days after ketamine treatment (electron microscopy, × 3 500). Arrows represent microtubule arrangement.

(A) Control group: the microtubule cytoskeletons were arranged in orderly rows, parallel to each other.

(B) Ketamine group: the microtubules were arranged in a disorganized manner and not parallel to each other.

Effects of ketamine on the expression of phosphorylated tau mRNA in neonatal rat hippocampal tissues

Quantitative real time-PCR revealed that the levels of phosphorylated tau mRNA in the ketamine group were significantly higher than those in the control group at 1 and 7 days after ketamine administration ($P < 0.05$). This difference disappeared by 14 days after injection ($P > 0.05$; Figure 2).

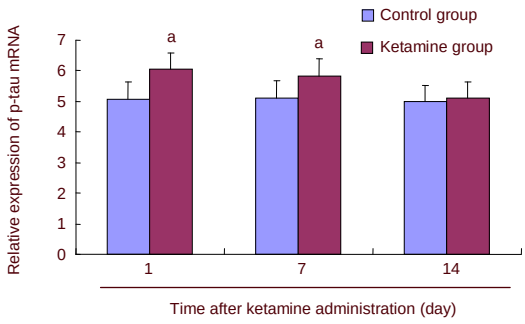

Figure 2 Expression of phosphorylated tau (p-tau) mRNA in neonatal rat hippocampal tissue after ketamine administration (quantitative real time-PCR).

The data are expressed as mean ± SD. There are six rats from each group at each time point. $^aP < 0.05$, *vs*. control group (one-way analysis of variance followed by Duncan's *post-hoc* test).

Effects of ketamine on the expression of phosphorylated tau protein at serine 396 and serine 404 in neonatal rat hippocampus

Western blot analysis showed that the levels of tau protein phosphorylated at serine 396 were significantly lower in the ketamine group than in the control group at 1 day after ketamine administration ($P < 0.05$). A gradual increase was observed at days 7 and 14, during which the difference between tau phosphorylation at serine 396 in the ketamine and control groups was not significant ($P > 0.05$; Figure 3). Hippocampal tau phosphorylation at serine 404 site was significantly higher than controls at 1 day after ketamine injection ($P < 0.05$). A slight reduction could be observed over time, but levels remained significantly higher than those in the control group ($P < 0.05$) on all three days measured. The levels of phosphorylation tau protein at serine 404 in the ketamine group were significantly higher than those in the control group at 1, 7 and 14 days after ketamine administration ($P < 0.05$; Figure 4).

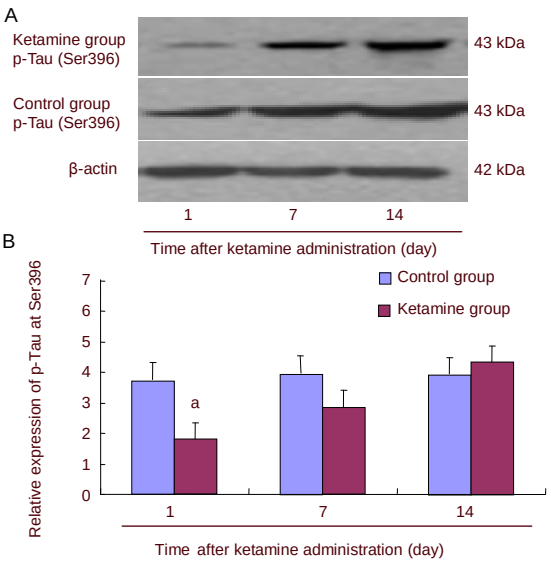

Figure 3 Expression of tau protein phosphorylation level at serine 396 (Ser396) in neonatal rat hippocampal tissue after ketamine administration.

(A) The expression of phosphorylated-tau protein (p-Tau) at Ser396 detected by western blot analysis.

(B) Quantitative expression of p-Tau at Ser396. The data are expressed as mean ± SD (absorbance ratio to β-actin). There are six rats from each group at each time point. $^aP < 0.05$, *vs*. control group (one-way analysis of variance followed by Duncan's *post-hoc* test).

DISCUSSION

Results from this study confirm the presence of hyperphosphorylated tau in the hippocampus of neonatal rats up to 2 weeks after high-dose ketamine administration. We have further shown that ketamine induces excessive phosphorylation of tau protein at serine 404 in neonatal rat hippocampus at 1, 7, and 14 days after administration. Phosphorylated tau mRNA expression was particularly high at 1 and 7 days after injection, consistent with the expression of tau protein phosphorylated at serine 404, and decreased gradually until day 14.

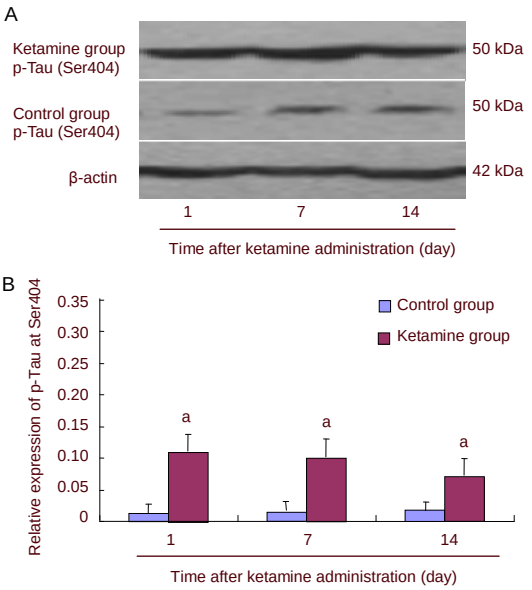

Figure 4 Expression of tau protein phosphorylated at serine 404 (Ser404) in neonatal rat hippocampal tissue after ketamine administration.

(A) The expression of phosphorylated tau protein (p-Tau) detected by western blot analysis at Ser404.

(B) Quantitative expression of p-Tau at Ser404. The data are expressed as mean ± SD (absorbance ratio to β-actin). There are six rats from each group at each time point. $^aP < 0.05$, *vs*. control group (one-way analysis of variance followed by Duncan's *post-hoc* test).

Tau is a microtubule-associated protein that is abundant in the central nervous system and expressed mainly in axons. In humans, tau is encoded by a single gene of 16 exons[11-12] (located on chromosome 17), generating six isoforms of 352–441 amino acids[13]. The amino-terminal region, which is characterized by the presence or absence of one or two amino acid inserts, has been found to interact with the plasma membrane and is essential for determining axonal diameter[14]. The carboxy-terminal region is characterized by the presence of three or four repeats that mediate the microtubule binding properties of tau and promote microtubule stabilization and polymerization[15]. These functions are negatively regulated by phosphorylation at multiple sites in and around the microtubule binding domain[16].

Spatiotemporal progression of tau aggregation from entorhinal cortex and hippocampus to isocortical areas has been shown to correlate with cognitive deficits[17], suggesting a pivotal role of tau pathology in Alzheimer's disease-related memory impairments. In line with this, accumulation of hyperphosphorylated tau proteins was also found to correlate with memory impairments in several animal models[18]. A recent study has shown that activation of a mutant tau gene in mice results in neuronal loss, brain atrophy and memory impairment; suppression of the gene halted neuronal loss, decreased atrophy of the brain and improved memory function[19]. In the present study, electron microscopy revealed significantly altered neuronal structure in the hippocampus, with microtubules appearing disorganized after administration of a large dose of ketamine.

The development of neurofibrillary tangles that results from hyperphosphorylation of tau proceeds in three stages: (1) preneuro fibrillary tangle; (2) intraneuronal neurofibrillary tangle; and (3) extraneuronal neurofibrillary tangle. In the pre-tangle stage, tau is hyperphosphorylated at serines 199, 202 and 409, whereas in the intraneuronal neurofibrillary tangle stage, hyperphosphorylation occurs at serine 396/serine 404 and threonine 231[20-21]. In the present study, excessive phosphorylation of tau protein at serine 404 in the neonatal rat hippocampus was observed at 1, 7 and 14 days after ketamine anesthesia, but not at serine 396 as revealed by western blot analysis. In contrast, Yeung *et al* [22] reported that tau was primarily hyperphosphorylated at serine 396 following chronic administration of ketamine. The difference in results may arise from the expression instability of serine 396 itself and interference from external experimental factors. However, both results provide evidence of neurons in the extraneuronal neurofibrillary stage of tangle formation. Dai *et al* [23] reported that learning and memory were impaired, and expression of tau phosphorylated at threonine 231 was elevated, 1 and 7 days after juvenile rats were injected with ketamine 100 mg/kg per day intraperitoneally However, expression of tau phosphorylated at serine 404 has never previously been shown to alter significantly.

Here, we used neonatal rats to determine phosphorylation sites in developing hippocampal tissue after ketamine administration, and found that serine 404 was altered significantly. While ketamine dose and administration protocols differed between studies, our results nevertheless demonstrate that ketamine is neurotoxic during neuronal development, suggesting that ketamine might affect cognitive function, particularly when given at an early age. Previous studies have indicated that the threonine 231 and serine 404 sites of tau phosphorylation play important roles in the pathophysiology of Alzheimer's disease, and are strongly associated with spatial memory and cognitive dysfunction in patients with the disease[24-25].

Our results indicate that ketamine may induce neurotoxicity in neonatal mice through excessive phosphorylation of tau at serine 404 in the hippocampus, as well as disrupting the stability of microtubules, damaging axonal transport, and ultimately leading to neuronal death. This is consistent with previous studies showing that ketamine can cause neuronal apoptosis[26-28].

In conclusion, ketamine induces an increase in phosphorylated tau mRNA and results in excessive phosphorylation of tau protein at serine 404 in the neonatal rat hippocampus. These findings suggest that ketamine may cause neurotoxicity in neonatal rats. Given the widespread use of ketamine as an anesthetic in pediatric medicine, the findings that anesthesia could be a risk factor for the development of a learning disability in children should lead to further studies to determine the potential neurotoxicity of the drug, including confirmation studies in humans.

MATERIALS AND METHODS

Design

A randomized controlled preclinical study in neonatal rats.

Time and setting

The experiment was performed at the Experimental Animal Center and Key Laboratory of Reproductive Genetics, Ministry of Education, Children's Hospital, School of Medicine, Zhejiang University, China from October 2010 to October 2011.

Materials

Thirty-six neonatal male Wistar rats, aged 7 days, were purchased from Zhe Jiang Acadamy of Medical Science (license No. SYXK (Zhe) 2005-0072). All animals were kept in standard animal cages in 12-hour light/dark cycles at 22°C, with free access to food and water. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of*

Laboratory Animals, formulated by the Ministry of Science and Technology of China[29].

Methods

Anesthesia procedure

Rats in the ketamine group were injected with ketamine 40 mg/kg intraperitoneally, in a solution of 10 mg/mL (Shuanghe Pharmaceutical Co., Ltd., Beijing, China). Half the initial dose (20 mg/kg) of ketamine was subsequently given three more times upon restoration of righting reflex, to make a total dose of 100 mg/kg. All anesthetized rats breathed spontaneously, and the body temperature was monitored with a rectal probe and maintained between 36.0°C and 37.0°C using a heat pad. To determine adequacy of ventilation, arterial blood was sampled at the end of anesthesia by obtaining a single sample (100 μL) from the left cardiac ventricle using a 32-gauge hypodermic needle. Bicarbonate concentration (mmol/L), oxygen saturation (%), pH, $PaCO_2$ (partial pressure of carbon dioxide), and PaO_2 (partial pressure of oxygen) were measured immediately after blood collection, using an ABL700 blood gas analyzer (Radiometer, Copenhagen, Denmark) (supplementary Table 1 online). Heart rate was monitored during the experiments. Equivalent volumes of normal saline were administered intraperitoneally to rats in the control group.

Preparation of hippocampal specimens

The rats were sacrificed at 1, 7 or 14 days after the anesthesia (n = 6 per group). Their brains were removed immediately and part of the hippocampus was frozen in dry ice and stored at –70°C till use for western blot analysis and real-time PCR. The remaining hippocampal tissue was used for morphological studies.

Observation of rat hippocampal ultrastructure using electron microscopy

The hippocampus was cut into 1 mm × 1 mm × 1 mm blocks and fixed in 2.5% glutaraldehyde fixative for 2 hours, followed by 1% osmium tetroxide fixative for 1–2 hours, pH 7.3–7.4. Specimens were rinsed with buffer for 20 minutes, dehydrated, soaked and embedded. An ultra-thin slicer was used to cut the specimens into slices of 1–10 μm thickness. After the sections were stained, perspective electron microscopy (model CM10; Philips, Eindhoven, the Netherlands) was used to observe microtubules and cell ultrastructures. Images were captured through a CCD camera (model C4742-95; Hamamatsu, Bridgewater, NJ, USA) and Advantage CCD Camera System software (Advanced Microscopy Techniques Corporation, Danvers, USA).

Quantitative real-time PCR for total phosphorylated tau mRNA expression

Total RNA was isolated from ketamine-treated and saline-treated hippocampal neurons using the RNeasy mini kit (Takara, Dalian, Liaoning Province, China) according to the manufacture's instructions. First-strand cDNA was synthesized from 5 μg of total RNA using the Super Script III first-strand synthesis kit (Takara) and random hexamer system (Roche, Shanghai, China). Quantification of the target genes was performed with Power SYBR Green PCR master mix kit (ABI, Carlsbad, CA, USA) in the MX3000P real-time PCR system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Triplicate quantitative reverse transcription PCRs were done for each sample. The PCR amplification cycles were as follows: initial denaturation at 95°C for 15 minutes, followed by 40 cycles with denaturation at 95°C for 20 seconds, and annealing-extension at 60°C for 35 seconds. The specificity of the SYBR Green PCR signal was confirmed by melting curve analysis. Acquired data were analyzed by LightCycle 2000 software 3.5 (Roche). The threshold cycle value of each gene was normalized against that of GAPDH. Total phosphorylated tau primer sequences were as follows: phosphorylated tau-sense 5′-ACC CCG CCA GGA GTT TGA C-3′, phosphorylated-tau-antisense 5′-GAT CTT CGC CCC CGT TTG-3′ 244 bp, GAPDH-sense 5′-CTA CAA TGA GCT GCG TGT GGC-3′, GAPDH-antisense 5′-CAG GTC CAG ACG CAG GAT GGC-3′ 207 bp.

Western blot analysis for phosphorylated tau protein at serine 396 and serine 404

For the western blot analysis, samples (80 μg protein) were prepared from hippocampal tissues of the neonatal rats, and were mixed with sample buffer, separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto a nitro cellulose membrane. The membrane was blocked for 1 hour at room temperature with blocking solution (5% nonfat milk in Tris buffered saline with Tween 20). Blots were then incubated overnight at 4°C with mouse monoclonal anti-rat phosphorylated tau protein at serine 396 and serine 404 antibody (5%; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-actin antibody (Santa Cruz Biotechnology), followed by three washes and incubation with a horseradish peroxidase-labeled rabbit anti-mouse IgG (5%; GE Healthcare, Shanghai, China) for 1 hour at room temperature and visualized with chemiluminescence detection (SuperSignal West Pico Chemiluminent Substrates; Pierce Biotechnology, Rockford, IL, USA).

Densitometric techniques were performed to quantify the protein band absorbance (Gel-Pro Analyzer software; Bio-Rad) and expressed as relative densitometric units of corresponding control.

Statistical analysis

All data were expressed as mean ± SD. The statistical analysis was performed using SPSS 11.0 software (SPSS, Chicago, IL, USA). The differences among the different groups at different time points were analyzed by one-way analysis of variance followed by Duncan's *post-hoc* test. *P* values less than 0.05 were considered statistically significant.

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Author contributions: Zhiyong Hu and Haiyan Jin conceived and designed the experiments. Haiyan Jin performed the experiments. Mengjie Dong performed western blot test. Yidong Wu performed real time-PCR gene primer sequences design. Haiyan Jin drafted manuscript. Zhirui Zhu analyzed the data. Lili Xu participated in manuscript revision and analyzed the data. All authors participated in critical revision of the manuscript and approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: The animal protocol was approved by the Standing Committee on Animals at School of Medicine, Zhejiang University (Hangzhou, China).

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.

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