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Hyperphosphorylated tau in the brains of mice and monkeys with long-term administration of ketamine

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ABSTRACT

Ketamine, a non-competitive antagonist at the glutamatergic N-methyl-D-aspartate (NMDA) receptor, might impair memory function of the brain. Loss of memory is also a characteristic of aging and Alzheimer's disease. Hyperphosphorylation of tau is an early event in the aging process and Alzheimer's disease. Therefore, we aimed to find out whether long-term ketmaine administration is related to hyperphosphorylation of tau or not in the brains of mice and monkeys. Results showed that after 6 months' administration of ketamine, in the prefrontal and entorhinal cortical sections of mouse and monkey brains, there were significant increases of positive sites for the hyperphosphorylated tau protein as compared to the control animals receiving no ketamine administration. Furthermore, about 15% of hyperphosphorylated tau positive cells were also positively labeled by terminal dUTP nick end labeling (TUNEL) indicating there might be a relationship between hyperphosphorylation of tau and apoptosis. Therefore, the long-term ketamine toxicity might involve neurodegenerative process similar to that of aging and/or Alzheimer's disease.

1. Introduction

Ketamine, a non-competitive antagonist at the glutamatergic N-methyl-D-aspartate (NMDA) receptor, is frequently used in human and veterinary medicine as an anesthetic especially in pediatric patients (White et al., 1982; Haas and Harper, 1992; Strayer and Nelson, 2008; Loxton et al., 2008). Ketamine is also a controlled substance but illegally used as a recreational drug primarily by young adults often at "raves" parties of nightclubs (Gable, 2004; Britt and Cance-Katz, 2005). Ketamine abusers has grown fast in recent years worldwide (Joe Laidler, 2005; Lankenau et al., 2007). Although the use of ketamine as an anesthetic or sedative agent generally is regarded as safe, adverse effects of the airway obstruction had occurred in 10 to 20% of subjects receiving ketamine (Strayer and Nelson, 2008). Furthermore, because of ketamine's anesthetic and hallucinate effects, the abuser might not be aware of injury induced or related physical assaults in addition to weird behavior which could be fatal (Gable, 2004).

NMDA receptor in the brain is associated with cognition; for example, NMDA receptor antagonists disrupt memory function in mice and rats (Lalonde and Joyal, 1993; Uchihashi et al., 1994). Several studies also showed that ketamine administration at sub-anesthetic and anesthetic levels could impair the performance of learning and memory of mice and rats (Lalonde and Joyal, 1993; Uchihashi et al., 1994; Gerlai and McNamara, 2000; Mickley et al., 2000). This effect of NMDA receptor antagonists on cognition was partly due to blockage in the induction of hippocampal long-term potentiation (LTP) that presumably underlies certain forms of learning and memory (Morris et al., 1986; Errington et al., 1987; Collingridge and Bliss, 1995). However, the confirmed mechanism of this memory deficit is still unclear. Recently, however, it had been shown that ketamine can interact with serotonin in the hippocampus of rats (Adams et al., 2009). On the other hand, one of the hallmarks of aging and Alzheimer's disease (AD) is the loss of memory, and the hyperphosphorylated of tau proteins in the brain is regarded as an early event in aging and one of the processes in the development of AD (Goedert et al., 1994; Mandelkow and Mandelkow, 1998; Huang and Jiang, 2009). The question is then would the memory impairment of ketamine be related to the hyperphosphorylation of tau? To answer this, we aimed to test the presence of hyperphosphorylated tau protein in the brains of mice and monkeys after long-term ketamine administration to reveal

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the early possible event of ketamine toxicity on the central nervous system.

2. Materials and methods

Young Cynomolgus Monkeys or Crab-eating Macaques (*Macaca fascicularis*) of 3 years old and ICR (imprinting control region) mice of 2 months old were used to set up the long-term animal abusive models. Six monkeys were given daily intravenous ketamine at dose of 1 mg/kg for 6 months. Eighteen mice in total were divided into three groups of six mice each and each animal in each group received daily intraperitoneal ketamine at dose of 30 mg/kg for either 1, 3 or 6 months. All animals were treated according to National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. Experimental procedures had been approved by the ethical committee of the Chinese University of Hong Kong.

Monkeys were anesthetized with Sumianxin 864 (a mixture of xylidinothiazoline, dihydroetophrine hydrochloride, haloperidol and EDTA) (Veterinary Research Institute, Changchun, PRC 130012) and were sacrificed by exsanguinations and perfusion with 4% paraformaldehyde in phosphate buffered saline (PBS). The skull was opened and the prefrontal and entorhinal cortices dissected out and immersed in 4% paraformaldehyde in PBS for further fixation at 4 °C overnight. Mice were anesthetized with 7% chloral hydrate (1 mg/g body weight) and sacrificed by open-

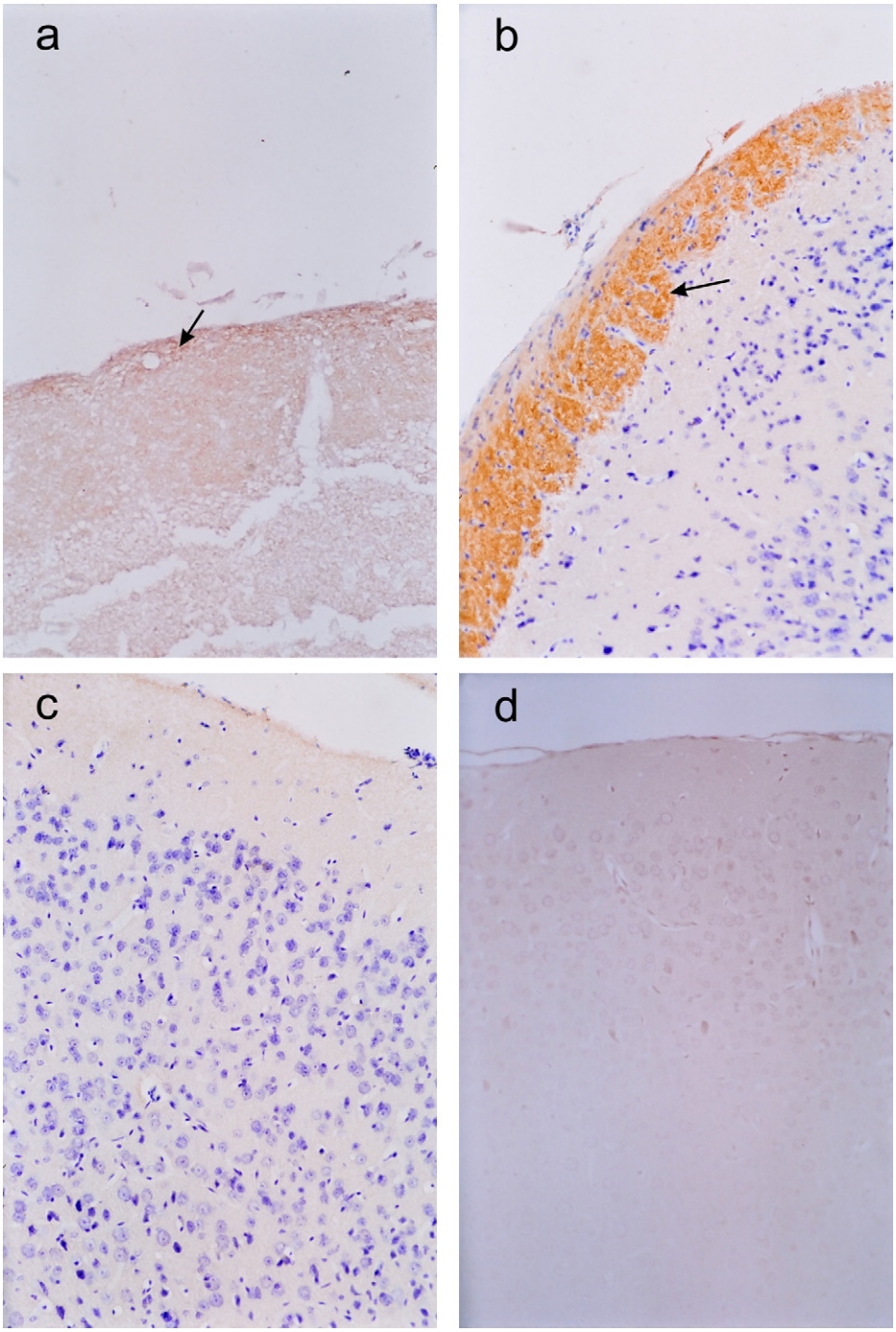

Fig. 1. Hyperphosphorylated tau immunostaining of sections from the prefrontal and entorhinal cortices of mice with administration of ketamine and control mice receiving no ketamine. (a) A section of the prefrontal cortex from a mouse treated with ketamine for 3 months showed there were some positively stained sites (arrow) for hyperphosphorylated tau in layer I of the prefrontal cortex. ×400. (b) From a mouse with ketamine administration for 6 months, extensive hyperphosphorylated tau positive sites (arrow) were present in layer I of the prefrontal cortex of the mouse. ×400. (c) There were no stained positive sites for hyperphosphorylated tau in this section of prefrontal cortex from a control mouse. ×400. (a–c) had counterstaining of Cresyl violet. (d) A section of the entorhinal cortex from a mouse treated with ketamine for 6 months showed no sites stained positive for hyperphosphorylated tau. ×400.

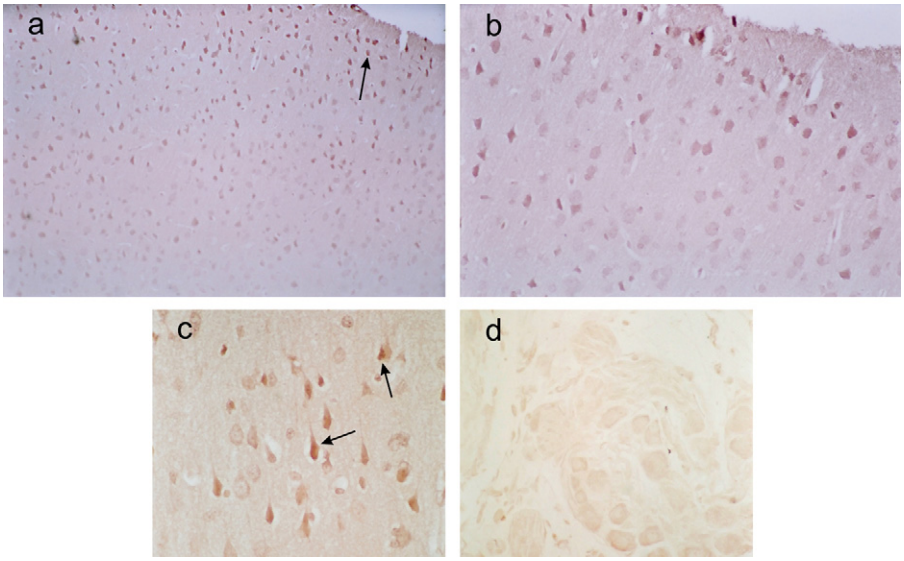

Fig. 2. Hyperphosphorylated tau immunostaining of sections from the prefrontal and entorhinal cortices of monkeys with administration of ketamine for 6 months and control monkeys receiving no ketamine. (a) This section shows hyperphosphorylated tau positively stained cells were in the outer layers of the prefrontal cortex. ×200. (b) Same as (a). ×400. (a and b) Had counterstaining of Cresyl violet. (c) For the entorhinal cortex, there were cells (arrows) stained positive for hyperphosphorylated tau in the deep layer of entorhinal cortex. ×400. (d) A section of the cortex from a control monkey showed no cell stained positive for hyperphosphorylated tau. ×400.

ing of chest, followed by perfusion of 4% paraformaldehyde in PBS. The prefrontal and entorhinal cortices were dissected out and fixed. The cortical samples were dehydrated in alcohol, cleared in xylene and embedded in paraffin. Six micrometer sections were cut for immunohistochemistry of hyperphosphorylated tau (Ser199) and/or terminal dUTP nick end labeling (TUNEL) staining.

Immunohistochemistry was performed according to our previous published method (Wai et al., 2009) with primary antibodies of hyperphosphorylated tau (Ser199)(1:500; BioSource™, California; 44-734G). After the primary and secondary antibody reactions, the sections were subsequently reacted with Vectastain ABC-Peroxidase Kit (Vector Laboratories, Burlingame, California, USA) for visualization of the positive cells. The TUNEL study was performed using the in situ apoptosis detection kit (S7100-KIT, Oncor, Gaithersburg, MD) followed by 3,3-diaminobenzidine (DAB) color development for the specimens of monkeys with 6 months administration of ketamine, as described previously (Li et al., 1997; Wai et al., 2009).

In order to obtain the density of hyperphosphorylated tau positive cells, morphometrical studies were performed using a stereological method (Wai et al., 2009). For hyperphosphorylated tau, three consecutive sections were obtained from each prefrontal and entorhinal cortices sample for counting of positive cells. For each section, a grid of 1500 μm^2 was laid randomly on the specimen at 200X magnification and the grid has 25 squares, only the positive cells in the middle row of 5 squares were counted. For TUNEL, morphometrical procedures were performed in the same way with consecutive sections of prefrontal cortex from monkey specimens. The mean ± standard deviation (SD) was computed for each group and the difference between groups was compared using student's t-test. The difference between groups was considered as statistically significant when p-value was less than 0.05.

3. Results

Hyperphosphorylated tau positive cells were found in the prefrontal and entorhinal cortices of mice and monkeys. In the mice, the hyperphosphorylated tau positive cells were located mainly on the layer I of the prefrontal cortex and were observed initially after 3 months of administration of ketamine (Fig. 1a). By 6 months of administration, the amount of positive hyperphosphorylated tau sites increased (Fig. 1b). There was no hyperphosphorylated tau positive cell in the prefrontal cortex of a control mouse without ketamine treatment (Fig. 1c). In the entorhinal cortex of the mice treated with ketamine for 6 months, however, the positive cells were not significant (Fig. 1d).

In the monkeys, a significant amount of hyperphosphorylated tau positive cells were observed in the prefrontal and entorhinal cortices after 6 months of ketamine administration, while the positive cells were present in the outer layers of the prefrontal cortex (Fig. 2a and b), and the deep layers of the entorhinal cortex (Fig. 2c). The negative control on the other hand showed no hyperphosphorylated tau positive cell in the cortex of a control monkey without ketamine treatment (Fig. 2d). In the brains of ketamine treated monkeys, combined TUNEL and hyperphosphorylated tau immunohistochemistry revealed some cells labeled with both (Fig. 3a and b) while a few other cells had only TUNEL labeled nucleus, like those of the control brains (Fig. 3c). From the prefrontal cortex specimens of ketamine treated monkeys, a count in the percentage of positive hyperphosphorylated tau cells with TUNEL positive nuclei versus total TUNEL positive cells revealed 14.6 ± 6.4% (mean ± SD) of TUNEL positive cells were hyperphosphorylated tau positive. Thus, not all the cells

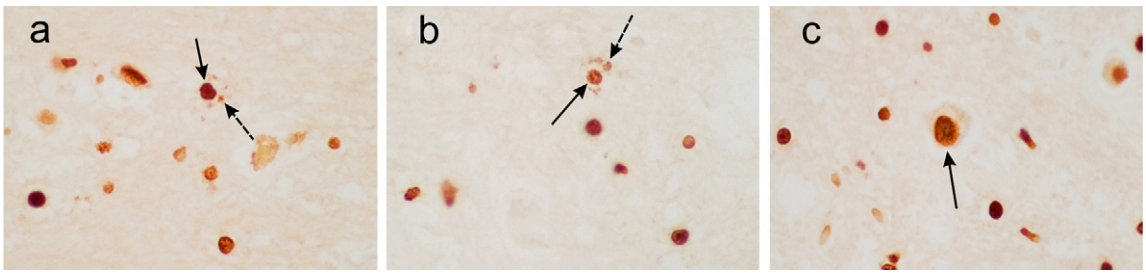

Fig. 3. Double staining of hyperphosphorylated tau and TUNEL was performed with sections from the prefrontal cortex of monkeys after administration of ketamine for 6 months (a and b) and control monkeys receiving no ketamine (c). These sections show cells positive for the double staining of hyperphosphorylated tau and TUNEL. Solid arrows in (a) and (b) point to a positive TUNEL nucleus and the broken arrows point to the cytoplasm of a cell with hyperphosphorylated tau stained positive in the cytoplasm. ×200. (c) The arrow points to a cell with positive TUNEL nucleus occasionally and the surrounding cytoplasm showed no positively stained hyperphosphorylated tau in the control. ×200.

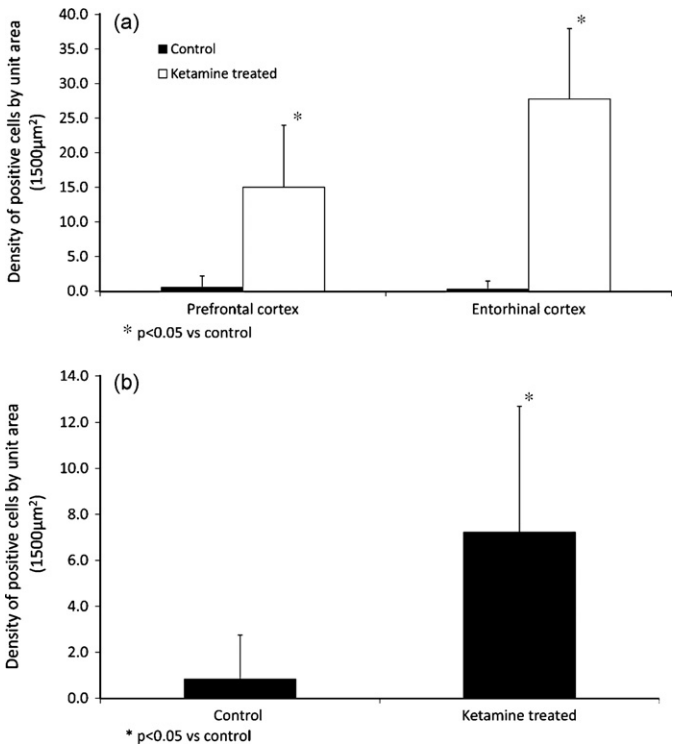

Fig. 4. Densities of the hyperphosphorylated tau positive cells were evaluated using morphometric method with sections from the prefrontal and entorhinal cortices of monkeys after administration of ketamine for 6 months and control monkeys receiving no ketamine. (a) Histogram shows the densities of the hyperphosphorylated tau positive cells were increased significantly ($p < 0.05$) in both prefrontal and entorhinal cortices of ketamine treated monkeys as compared with the control monkeys. (b) The density of TUNEL positive cells was increased significantly ($p < 0.05$) in the prefrontal cortex of ketamine treated monkeys as compared with the control monkeys.

with TUNEL positive cells were hyperphosphorylated tau positive.

Morphometric evaluations using sections from monkey specimens revealed that densities of the hyperphosphorylated tau positive cells increased significantly ($p < 0.05$) in both the prefrontal and entorhinal cortices of ketamine treated monkeys (15.0 ± 9.0 and 27.8 ± 10.2 cells/1500 μm^2 respectively for prefrontal and entorhinal cortices) as compared with those of the control monkeys (0.6 ± 1.6 and 0.2 ± 1.2 cells/1500 μm^2 respectively for prefrontal and entorhinal cortices) (Fig. 4a). Likewise, the density of TUNEL positive cells increased significantly ($p < 0.05$) in the prefrontal cortex of ketamine treated monkeys as compared with those of the control monkeys (7.2 ± 5.5 and 0.8 ± 1.9 respectively for ketamine and control groups) (Fig. 4b).

4. Discussion

Our results showed clearly the presence of hyperphosphorylated tau in the brains of animals (mice and monkeys) with 3 months ketamine administration or above. Although we did not have samples of human ketamine abusers, we have in an unpublished study of cocaine addicts which also revealed that hyperphosphorylated tau did exist in the human brains of addicts. It is therefore likely that abusive uses of different drugs could induce formation of hyperphosphorylated tau in neurons in the brains of animals and human.

Tau is a microtubule associated protein which was abnormally phosphorylated in AD (Augustinack et al., 2002; Huang and Jiang, 2009). The hyperphosphorylation of tau results in aggregation of the paired filaments in the formation of neurofibrillary tangles (NFT). In hyperphosphorylated tau development, three stages had been documented which were (1) preneurofibrillary tangles, (2) intraneurofibrillary tangles, and (3) extraneurofibrillary tangles. In the preneurofibrillary stage, the hyperphosphorylated tau were located at the serine (S) sites S199, S202 and S409, whereas those in the intraneuronal NFT had tau hyperphosphorylation at the S396 and threonine (T) T231 positions, while the extraneuronal NFTs were primarily at the S396 positions (Kimura et al., 1996; Augustinack et al., 2002). In the present study, the punctate forms of hyperphosphorylated tau as revealed by immunochemistry, seemed to align with those reported in the preneurofibrillary stage (Augustinack et al., 2002). The punctate positive sites were either present all over the cell or around one pole of the neuron, but unlike those reported by Li et al. (1995), the positive sites were normally not present in the nucleus, which indicated that they were not related to Huntington disease. The hyperphosphorylated tau antibody that we used was typically against serine site S199, showing neurons in our results were in the prefibrillary stage of the NFT formation. It was also possible that other stages of the NFT formation were not made apparent by this antibody. The stage of preneurofibrillary formation here may be similar to AD Braak and Braak Stage (IV) documented by Augustinack et al. (2002). Together, our novel results indicated that there were hyperphosphorylation of tau in neurons of mice and monkeys with long-term ketamine administration. There was, however, no additional close resemblance of these ketamine treated animals to those of AD patients. In spite of the presence of early hyperphosphorylated tau in cells of the brain, preliminary observation in this laboratory on our specimens failed to reveal any significant amount of β-amyloid plaques by immunohistochemistry using Abeta (1-42 amino acids) antibody (data not shown). Since β-amyloid plaques are also definitive pathological hallmark of Alzheimer's disease (Tremblay et al., 2007; Barrio et al., 2008; Kienlen-Campard et al., 2008; Viola et al., 2008), our specimens did not have all the required diagnostic parameters of this disease. But one could not rule out the possible β-amyloid plaques accumulation which might happen later in these brains of longer term ketamine treatment.

TUNEL, a technique employed to identify nick and breaks in DNA, was originally used as a marker for apoptotic cells in the nervous system of animals including human (Li et al., 1997). Due to the controversy to DNA breaks sometimes can reattach, the TUNEL technique is now used as a degenerative and/or apoptotic marker for cells. In this study, some cells with hyperphosphorylated tau had TUNEL positive nucleus, thus indicated a possible relationship between hyperphosphorylated tau formation and apoptosis. Further still, the injury involved ketamine and thus the possibility of ketamine as a neurodegenerative agent during its abused uses must be carefully pondered. Recent studies in our laboratory also showed a change in the density of nerve fibers (Yeung et al., 2009) in the ketamine addicted mice and in culture neuroblastoma cells (Mak et al., 2009). Further studies of ketamine toxicity should be initiated and be considered along this direction of neurodegeneration.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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