

Final Report

Long-term Ketamine Abuse and Apoptosis in Cynomolgus Monkeys and Mice

Executive Summary

The potential toxicity of ketamine had been studied in the monkeys and mice. The dosage used for the monkeys was 1mg/kg daily, a minimal dosage suggested to produce schizoid behaviour. This dosage is low when compared with the anesthetic dosage of children (about 10mg/kg). In the mice, the anesthetic dosage is at least 10 times higher because these animals have a very fast metabolism (Science 2011) (e.g. the heart rate of a mouse is 10 times that of a human). The anesthetic dose of the mice is therefore documented to be 100-150mg/kg, and our experiment showed that by 60mg/kg, the hind limbs of the mice would have incoordination. Therefore, we maintained our dose at half of 60mg/kg, i.e. 30mg/kg daily. This was calculated to be 1mg for each mice daily. Our preliminary evaluation from reports of addiction from the public security departments in the Southern China demonstrated that the addicts took an average dose of roughly 600mg per day.

From our experimental animals with the dosages used as mentioned above, we have documented the following changes:

- 1) Ketamine was able to kill neuronal cells in cell culture.
- 2) Ketamine injected daily to the animals will cause neuronal apoptosis, at a timing of 3 to 6 months.
- 3) Potentially, it was even more dangerous in that ketamine could cause phosphorylation of tau protein, a marker of Alzheimer degeneration in the brain of these animals.
- 4) fMRI indicated changes in the limbic system, corpus striatum, cerebellum, dorsomedial thalamus, prefrontal cortex (including basal forebrain). Some of these changes appeared as early as one month after ketamine treatment. The limbic system and the prefrontal cortex are important emotional and personality areas. The corpus striatum is an important area for regulation of movement, and any changes will result in deregulation of motion. The cerebellum is interestingly down regulated after ketamine intoxication, resulting in incoordination of muscles. The dorsomedial thalamus, in this case, is an important relay station between the hypothalamus and the prefrontal cortex, and any changes will therefore affect the endocrine and the brain.
- 5) The ketamine toxicity will also affect memory as was shown by our water maze study of the mice.
- 6) Molecular studies by this laboratory indicated the upregulation of GABA_A receptors in the brain after ketamine addiction.
- 7) Not only will the central nervous system be affected, but also other areas. In these

models, we have discovered that the kidneys and bladders are affected after 1 month of ketamine treatment. In both cases, infiltration of lymphocytes and monocytes were seen in the bladder and kidney. Further treatment would result in fibrosis and muscular degeneration of the urinary bladder so that the bladder became contracted. This is what happened in the clinical human situation. We further found that the sperm motility is affected as well by ketamine.

All the results above are either published or in manuscript forms ready to be submitted. There were, to this date, four published papers, one submitted paper and three manuscripts ready for submission. They represent de novo information for scientific studies in ketamine. We thank the Beat Drugs Fund Association for the grant (Ref. No.: BDF080048) awarded for this purpose.

課題總結

我們在猴子和小鼠中對氯胺酮的潛在毒性進行了研究。對猴子使用的藥物劑量為每天 1mg/kg，這是能產生精神分裂行為的最小劑量。該劑量低於兒童的麻醉劑量（大約 10mg/kg）。由於小鼠的新陳代謝率非常高 (Science 2011)（例如：小鼠的心率是人類的十倍），相應的麻醉劑量至少在十倍以上，約為 100-150mg/kg。我們的實驗結果表明，60mg/kg 的劑量能使小鼠後肢運動失調。因此，我們在研究過程中使用該劑量的一半，即每天 30mg/kg，每只小鼠每天大約使用 1mg 氯胺酮。我們對中國南方公安部門關於吸毒報告進行了初步分析，結果表明氯胺酮成癮者的平均使用量大致為每天 600mg。

在對實驗動物使用上述的劑量氯胺酮後，我們觀察到以下的變化：

- 1) 氯胺酮能殺死培養的神經元細胞
- 2) 每天對動物注射氯胺酮，三到六個月後能引起神經元凋亡
- 3) 更為嚴重的是，氯胺酮可使動物的腦中 tau 蛋白高度磷酸化，這是阿爾茨海默氏症的標誌之一
- 4) 功能性磁共振研究發現，在邊緣系統、紋狀體、小腦、背內側丘腦和前額皮層（包括基底前腦）均有所變化。其中一些變化早在給藥一個月之後就出現。邊緣系統和前額皮層是重要的情緒與性格區域。紋狀體是一個重要的運動調節區域，它的任何改變都會導致運動失調。在使用氯胺酮後小腦的改變會導致肌肉不協調。背內側丘腦是下丘腦和前額皮層的中繼站，因此它的改變能影響到內分泌和腦。
- 5) 小鼠水迷宮實驗發現，氯胺酮的毒性會影響記憶功能。
- 6) 分子生物學研究表明，氯胺酮成癮後，腦中 GABA5 受體上調。
- 7) 氯胺酮不僅能影響中樞神經系統，也能對其他區域產生影響。在該模型中，我們在一個月的氯胺酮處理小鼠中觀察到腎臟和膀胱的變化。在兩者中都觀察到了淋巴細胞和單核細胞浸潤。繼續使用氯胺酮會造成膀胱肌肉纖維化，導致攣縮膀胱，這也見於人類臨床症狀。我們進一步的研究發現氯胺酮還能影響精子的運動能力。

上述之結果已經發表或者已經成文即將發表。到目前為止，已經有四篇論文正式發表，一篇已經投稿和三篇準備投稿。它們都是對氯胺酮的原創性科學研究。我們感謝禁毒基金會對研究工作的資助(Ref. No.: BDF080048)。

Final Report on Drug-related Research Projects

(as at 31 October 2010)

Project Title

Long-term Ketamine Abuse and Apoptosis in Cynomologus Monkeys and Mice

Background

Ketamine, a noncompetitive antagonist at the glutamatergic N-methyl-D-aspartate (NMDA) receptor, is currently used in human and animal medicine as an injectable anesthetic. Ketamine is also a controlled substance, illegally used as a recreational drug (“Special K”, “Vitamin K”). It is used primarily by young adults often at all night dance parties called “raves” that take place in nightclubs (Britt and Cance-Katz 2005). The Central Registry of Drug Abuse database (CRDA) showed different drug abuse trends in Hong Kong. The percentage of ketamine users has grown very fast, particularly in the last five years. The first few reported cases appeared in 1999, but by 2002, the percentage of ketamine users under 21-year old reached 70%, a figure comparable to heroin use in the mid-1990s (Joe Laidler 2005). The Government announced that ketamine abusers increased sharply in the past two years and is one of the main abusive drugs in Hong Kong.

The reason for prevalent use of ketamine is that people thought that it would not lead to physical dependence. However, ketamine, as an NMDA antagonist, can inhibit the reuptake of serotonin, dopamine, and norepinephrine, although the mechanism underlying this action is not entirely clear (Britt and Cance-Katz 2005). Acute adverse effects of ketamine abuse include physiological (such as heart rate, hypertension, motor function, nausea, etc.) and psychological (such as anxiety, dissociation, depression, amnesia, symptoms of schizophrenia, etc.). Effects due to chronic misuse include cognitive difficulties in areas such as attention, learning and memory. There are, however, less scientific evidences of the neurobiological or neurochemical alterations of ketamine abuse. No study shows the long-term effects on neurons in young adults with ketamine abuse.

Programmed cell death or apoptosis is a physiologically important process in neurology wherein around 50% of the neurons die by apoptosis during maturation and aging of the nervous system. Premature apoptosis and/or aberrations in apoptosis

control contribute to the pathogenesis of a variety of neurological disorders including acute brain injury such as trauma, spinal cord injury, ischemic stroke, ischemia/reperfusion, and in chronic disease, such as Alzheimer's, Parkinson's and Huntington's (Ekshyyan and Aw 2004). Some of the apoptotic effectors, such as caspase-3 (Blomgren et al. 2001; Ni et al. 1998), apoptotic protease activating factor-1 (APAF-1) (Ota et al. 2002), B-cell leukemia/lymphoma-2 (Bcl-2) (Merry et al. 1994) and Bcl-2-associated X protein (Bax) (Vekrellis et al. 1997), are expressed at higher levels in the developing and aging brain and apoptosis are seen to be more important in the development of brain injury (Hu et al. 2000; Blomgren et al. 2001; Ni et al. 1998; Gill et al. 2002). The exposure of immature animals, during the developmental period of synaptogenesis, to NMDA antagonist can cause neurons in the central nervous system (CNS) to commit suicide (die by apoptosis) (Ikonomidou et al. 1999). Therefore it is interesting to know whether there are any connections between apoptosis in CNS and ketamine abuse in young adults.

With the improvements of imaging technology, structural, functional and neurochemical profiles can be visualized across the entire brain. Functional magnetic resonance imaging (fMRI) has been employed to investigate neuronal activity associated with particular stimuli. The animal studies suggest that repeated exposure to noncompetitive NMDA antagonists leads to sustained impairment of cognitive performance. These deficits have been linked to the reduced function of the prefrontal dopaminergic system. Preliminary study of repeated exposure to ketamine in human is associated with up-regulation of D1 receptors in the dorsolateral prefrontal cortex (Narendran et al. 2005). In addition, what about other receptors which may also be involved? The alterations of cortical and sub-cortical functions, therefore, need to be further explored in acute and long-term ketamine abuse.

In present study, we have documented that apoptosis is a potential mechanism for the ketamine abuse-related loss of function in the frontal cortex. In addition, we reported fMRI changes in the neuronal activities in a number of cortical and subcortical brain areas in the ketamine abuse monkey model. We have discovered that the presence of hyperphosphorlated tau protein (a marker in Alzheimer's disease) was evident in the brains of these animals (both mice and monkeys). We have also found a receptor "GABA5" which is majorly related to deficits in the CNS. Finally, we have deciphered why the kidney and bladders are affected in these animals.

Materials and Methods

Young monkeys were given 1 mg/kg ketamine via intravenous injection for 1, 3, and 6 months to build up long-term ketamine abuse models. For the mice, 1 mg per mouse was injected intraperitoneally for 1, 3, and 6 months as well. The dosage for mice and monkeys was calculated according to literatures and preliminary studies, taking into consideration that mice have a much higher metabolism.

fMRI images were obtained via a T3 MRI machine for monkeys at the conclusion of 1, 3, and 6 months. Pro-apoptotic proteins (caspases and Bax) and anti-apoptotic proteins (Bcl-1) were measured in both brains of monkeys and mice by using Western blotting in long-term ketamine abuse models. Terminal dUTP nick end labeling (TUNEL) for cell death were performed as well for confirmation. Hyperphosphorylated tau was detected by immunohistochemistry. For the studies of receptors, real time PCR techniques and water maze behavior test were employed.

Experiments in our laboratory on cell cultures of a primary human neuronal cell line, SH-SY5Y and PC12 cell lines (with catcholamine) were all performed. The SH-SY5Y cell lines were subdivided into differentiated and undifferentiated categories. Increased amounts of cell death after addition of ketamine were observed in both cases.

Some other specimens e.g. kidneys and urinary bladders were also processed for microscopic histopathology for observation, employing routine staining and immunohistochemistry.

Work done for the last three months (i.e. from 1 Aug – 31 Oct 2010):

- i) All the fMRI results of 1, 3, 6 months of addictions in monkeys were evaluated and compared.
- ii) Cell death of monkeys' brain tissues was evaluated.
- iii) Research papers were written. Some were submitted, others are in the process of submission.

Results obtained during the last 3 months

- i) The overall results of fMRI indicated that the limbic system had already been hyper-activated after one month of ketamine addiction. The most important part is perhaps the activation of prefrontal cortex (orbitofrontal and basal forebrain), the corpus striatum, the fusiform cortex and the “downregulation” of the

cerebellum. In addition, the dorsomedial thalamus is clearly involved while the basal forebrain is important for degeneration in the CNS. The DM thalamus ties very well with prefrontal cortex, indicating scientific evidences of changes in judgement, memory and emotion upon ketamine intoxication. Downregulation of the cerebellum and the involvement of the corpus striatum signified motor irregularities. We have prepared 1-2 papers on this for submission to international journals.

- ii) Meanwhile, the monkeys' brain tissues showed cell death (apoptosis) in 1, 3, and 6 months samples (Fig. 1). This, together with our earlier published report of mutation of tau in the neurons, indicated definitive detrimental effects on the nervous system.
- iii) In the course of these last 3 months, we have also found that the urinary bladder after 3 to 6 months of treatment had muscle loss (Fig. 2) and extensive invasion by fibrous connective tissues. This may be the reason of bladder dysfunction. One paper on this is to be submitted.
- iv) Totally, we expected at least 3 more papers on the way. We have also written up a paper in which the GABA_A receptor had been found upregulated upon ketamine treatment. We shall inform the funding agency when the papers are accepted for publication, and shall let the agency have a copy when they are in print.

**Papers already published during the period of the whole project
(i.e. from 1 Sep 2008 to 31 Oct 2010):**

- i) Yeung LY, Rudd JA, Lam WP, Mak YT, Yew DT (2009) Mice are prone to kidney pathology after prolonged ketamine addiction. *Toxicol Lett.* 191(2-3):275-8. *(details in Attachment 1)*
- ii) Mak YT, Lam WP, Lü L, Wong YW, Yew DT (2010) The toxic effect of ketamine on SH-SY5Y neuroblastoma cell line and human neuron. *Microsc Res Tech.* 73(3):195-201. *(details in Attachment 2)*
- iii) Yeung LY, Wai MS, Fan M, Mak YT, Lam WP, Li Z, Lu G, Yew DT (2010) Hyperphosphorylated tau in the brains of mice and monkeys with long-term administration of ketamine. *Toxicol Lett.* 193(2):189-93. *(details in Attachment 3)*

- iv) Sun L, Lam WP, Wong YW, Lam LH, Tang HC, Wai MS, Mak YT, Pan F, Yew DT (2010) Permanent deficits in brain functions caused by long-term ketamine treatment in mice. Hum Exp Toxicol. (E. Pub.) *(details in Attachment 4)*

Book chapter, conference abstracts, and papers produced:

a. Book chapter published:

- i) Yew D.T. “氯胺酮的動物實驗 – 科研的結論 (Animal Studies of Ketamine)”. In: “香港濫 K (Drug Abuse in Hong Kong)”. Publisher: Mingpao (June 2010) *(details in Attachment 5)*

b. Conference abstracts published:

- i) Yu H., Li Q., Wang D., Shi L., Mak Y.T., Lu G., Sun L., Wang L., Chen M., Pan F., Yew D.T. (2010) Chronic ketamine abuse causes dysfunctions of different brain areas relevant to neurodevelopmental psychiatric disorders: Evidence from fMRI in a primate model. Int. J. Neuro - Psychopharmacol. (Abstract No.: P-01.046). *(details in Attachment 6)*
- ii) Yew D.T. (2010) Toxicity of ketamine on animal models – A review of studies in our laboratory. East Asian Archives of Psychiatry Vol. 20 No. 4 (Supplement) (Abstract No.: S2.2.1). *(details in Attachment 7)*
Conference: “The 2nd Joint International Conference of The Hong Kong College of Psychiatrists and The Royal College of Psychiatrists (UK)” (12 Dec 2010).
A copy of the presentation material is also enclosed *(details in Attachment 8)*

c. Papers submitted recently:

- i) Yu H., Li Q., Wang D., Shi L., Lu G., Sun L., Wang L., Zhu W., Mak Y.T., Pan F., Yew D.T. (2010) Mapping the central effects of chronic ketamine administration in adolescent primate using functional magnetic resonance imaging (fMRI). (Submitted to BMC Neuroscience).

Abstract:

Ketamine, the noncompetitive NMDA-receptor antagonist, leads to an excessive glutamate release and subsequent cortical excitation which may induce

psychosis-like behavior and cognitive anomalies. Most evidences show that an acute use of ketamine induces dose-related positive and negative schizophrenia-like symptoms. An acute effect of ketamine is mainly on the activation of the prefrontal cortex and the limbic structures, in which increased levels of glutamate and dopamine have been detected. However, little is known about the long-term effects of ketamine abuse on brain functions. Recently, the numbers of ketamine abusers are rapidly growing, especially among young people. Therefore, we aim to investigate the chronic effects of ketamine on brain functional integrity using functional magnetic resonance imaging (fMRI) in adolescent cynomolgus monkeys. In addition, we conducted a pilot experiment to explore the alterations of dopamine system in the cortex of monkeys with chronic exposure to ketamine. Motor behavior was measured to assess the chronic ketamine action as well. Repeated exposure to ketamine resulted in a significant decrease of activities in ventral tegmental area, substantia nigra of the midbrain, posterior cingulate cortex, and visual cortex. However, hyperfunction had been found in striatum and entorhinal cortex (Ent) in monkeys with ketamine exposure. Pilot experiment demonstrated a decreased level of dopamine in the cortex of primates models with chronic ketamine exposure. The total movements in monkeys with ketamine exposure were decreased when compared with the control. These pieces of evidence support that mesolimbic, mesocortical and Ent-striatum systems are vulnerable to chronic effects of ketamine. Dysfunctions of these brain pathways have been linked to several psychiatric disorders, such as depression, schizophrenia or attention deficit disorder. From neurochemical and phenotypic aspects, a decreased level of dopamine in prefrontal cortex, together with hypolocomotor activity, demonstrated that repeated ketamine exposure may be a good pharmacological model for exploring central effects for the neuropsychiatric disorders.

- ii) Gene expression changes in alpha GABA receptors in the mice brains after prolonged ketamine administration.

Abstract:

Ketamine is an anesthetic and an abusive drug. Despite its popular use and abuse, little is known about its long-term effects on the brain. The present study was aimed to evaluate working memory changes after such a treatment and the underlying changes in gene expressions. Ketamine dosage of 1mg per animal was given to ICR mice for 1 and 3 months. Behavioral test, namely spatial navigation, was performed according to a morris water maze protocol. Increase

in timing to find the platform in the maze was observed in all treated mice, particularly in those being treated for 3 months. This indicated that the longer the period of treatment, the more severe the memory loss. Gene expression changes were examined by Affymetrix Genechip, and the expression of alpha GABA receptors which mediated tonic inhibition in the CNS was confirmed by quantitative real-time PCR and Western blotting. Compared with the saline control, the behavioral performance of ketamine-treated mice declined. Genechip results showed that 110 genes were up-regulated and 136 genes were down-regulated. GABA receptor signaling was found amongst the most significant changed terms in Gene ontology analysis. In particular, there was a significant up-regulation of Gabra5 in the prefrontal cortex. In conclusion, chronic exposure to ketamine impaired the working memory in mice. Ketamine mediated memory impairments may be explained, at least partly, by up-regulation of Gabra5 in the prefrontal cortex. This is a new and exciting finding as everybody was looking at a different receptor - the NMDA receptor.

- iii) Ketamine effects on the urogenital system – changes in the urinary bladder and sperm motility.

Abstract:

Different doses of ketamine were injected intraperitoneally (I.P.) respectively to male ICR mice to determine the optimal dosage for chronic administration. At and above 40mg/kg I.P. injection, mice had decreased hindlimbs movement. Subsequently, 30mg/kg was used for ketamine administration on urinary bladder and sperm motility. The treatment group were sub-divided into two (n = 10 each group); one received daily ketamine treatment for three months and another group for six months. Mice in control groups (n = 5 each group) received saline. Terminal dUTP nick end labeling (TUNEL) study and Sirius red staining were carried out on the slides of the urinary bladders. Apoptosis in the bladder epithelium was observed initially in the 3-month ketamine treated mice and the number of apoptotic cells was significantly different ($p < 0.05$) between the 6-month ketamine treated mice and the control. The relative thickness of muscular layers in the bladder decreased significantly ($p < 0.05$) between the 6-month treated mice and the control. Sirius red staining revealed an increase of collagen in the urinary bladder of the treated mice. The ketamine treated animals also had a decreased percentage of mobile sperms than those of the control ($p < 0.05$).

- iv) Effects of chronic ketamine administration on brain function and increase the expression of apoptosis-related proteins in the brain: Evidence from fMRI in a 3-month and 6-month primate models of ketamine use.

Abstract:

Recreational use of ketamine is spreading rapidly especially among youngsters. An acute use of the N-methyl D-aspartate (NMDA) receptor antagonist ketamine induces marker psychosis-like effects included negative and positive symptoms, as well as cognitive impairments. According to our previous study, repeated exposure of ketamine for 6 months in the young primate induced deficits of mesolimbic, mesocortical and Ent-striatum systems. However, little is known about the effects of different exposure time of ketamine on brain functions or the biochemical mechanism involved in the alteration of brain functions. Therefore, we compared the effects of ketamine exposed to 3 and 6 months on brain functional integrity using functional magnetic resonance imaging (fMRI) in adolescent Cynomologus monkeys. In addition, motor behavior has been measured. Apoptosis-regulated protein bcl-2 was measured to investigate the role of apoptosis in chronic ketamine administration model in the young primates. Repeated exposure to ketamine (1mg/kg) for only 3 months showed significant increase of activities on the left side of medial prefrontal cortex, frontal cortex, and bilateral caudate nucleus when compared with the control. Enhanced activities were observed in thalamus, splenium, and caudate nucleus of the young primates with 3-month ketamine exposure when compared to those with 6-month, using immunohistochemistry and Western blotting. There was a significant decrease in expression of bcl-2 protein in 6-month ketamine groups when compared with the control. Movement was decreased in both 3- and 6-month ketamine administered groups after ketamine injection. These findings implied that the longer the use of ketamine, the more severe the deficit of brain function in chronic ketamine administration in the young primates.

Press conferences and interviews

- i) Press conference: “Important reactive and addictive sites in the central nervous system: examples of ketamine and heroin (氯胺酮及海洛英於中央神經系統的重要反應區及成癮區)”. (8 January 2010)
- ii) Press conference: “Long-term ketamine abuse and apoptosis in cynomologus

monkeys and mice”. (Radio Television Hong Kong – 10 August 2009)

- iii) T.V. interview on “Ketamine addiction”. (Broadcasted on Tuesday Report 星期二檔案, *TVB Jade* – 30 March 2010)
- iv) T.V. interview (by RTHK on 17 December 2010) for a TV series to be broadcasted in 2011.

Final summary of what we have achieved in the whole project:

To summarize, there are a few salient points established by this project. These de novo findings are either published or will be published in the near future:

- i) Ketamine causes cell death in the nervous system – both in vivo and in vitro. It is important to note that undifferentiated cells are easier to be damaged, which points to the susceptibility of the embryonic cells in development (*Attachment 2*).
- ii) Cell deaths are found primarily in the prefrontal cortex and the hippocampus. In addition, degenerative marker (hypertau) was seen in neurons (*Attachments 3 & 4*).
- iii) fMRI indicated upregulation of activity in the limbic system (entorhinal cortex); in particular, the fusiform cortex, corpus striatum, dorsomedial thalamic nucleus, prefrontal cortex, and basal forebrain (*Attachments 6, 7 & 8*).
- iv) Animal models of both monkeys and mice are established. We have established the dosage of toxicity for both monkeys (1mg/kg) and mice (30mg/kg).
- v) An upregulation of one group of α GABA receptors has been identified, which is α GABA5, as revealed by molecular techniques.
- vi) Urinary system damages have been clarified. Kidney had infiltration of mononuclear cells and bladder – loss of muscles with extensive fibrosis, subsequent to losing of neuromuscular junctions (*Attachment 1*).
- vii) Neuronal cells already with transmitters also die from ketamine treatment, e.g. PC12 cells (phaeochromocytoma) containing catecholamines could be killed by treatment of ketamine with an equivalent those applied to other culture cells (*Fig.*

4).

The P. I., on behalf of all Associates and Assistants, would like to thank the Beat Drugs Fund Association for the support of the project.

The Way Forward – what needs to be done in future to promote scientific research to combat drug abuse and to promote Hong Kong as a major ketamine combat centre

In the past two years, we have accomplished all the proposed targets in this grant (please refer to the summary above). With the light of these results, more questions have also arisen:

- 1) How does ketamine interact with alcohol?
- 2) How would the other organs react to ketamine treatment?
- 3) We have shown that nervous cells are susceptible cells, ketamine induced cell death and degeneration (hypertau formation). What could be the mechanism – superoxide formation, chemical inflammation, etc?
- *4) We have shown that in culture, undifferentiated cells are more susceptible to cell death induced by ketamine. What would happen to pregnancy, embryo, and also the developing organs?
- *5) What happened to the organs damaged after withdrawal?

Items 1, 2, and 3 can be dealt with in the new awarded grant (BDF100052) started in November 2010.

* For items 4 and 5, they are important issues which require longer time, manpower, and resources, and therefore could only be dealt with in other new projects.

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Fig. 1

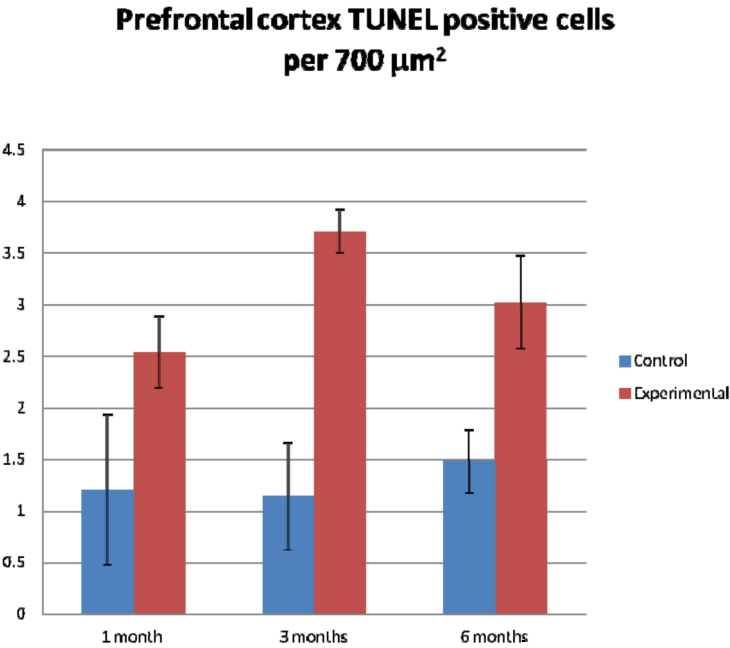


Fig. 2

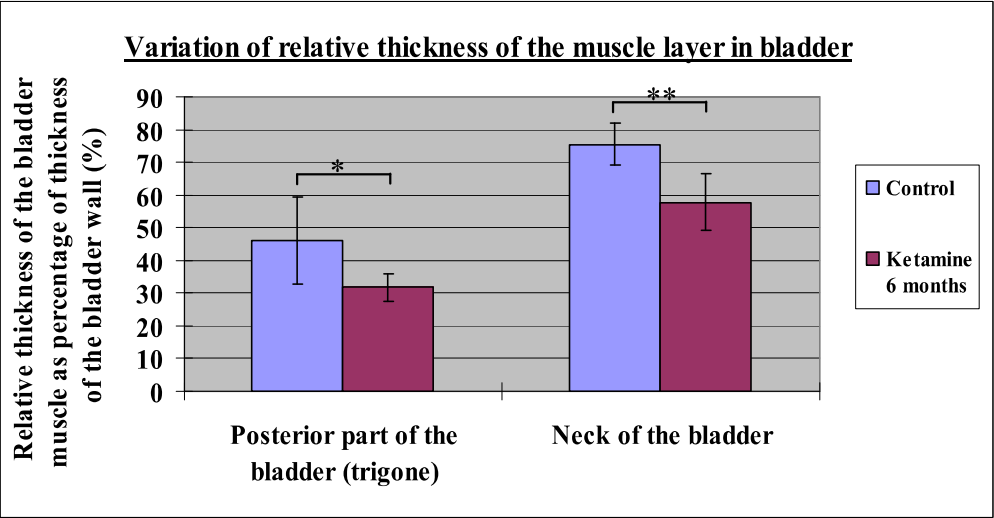
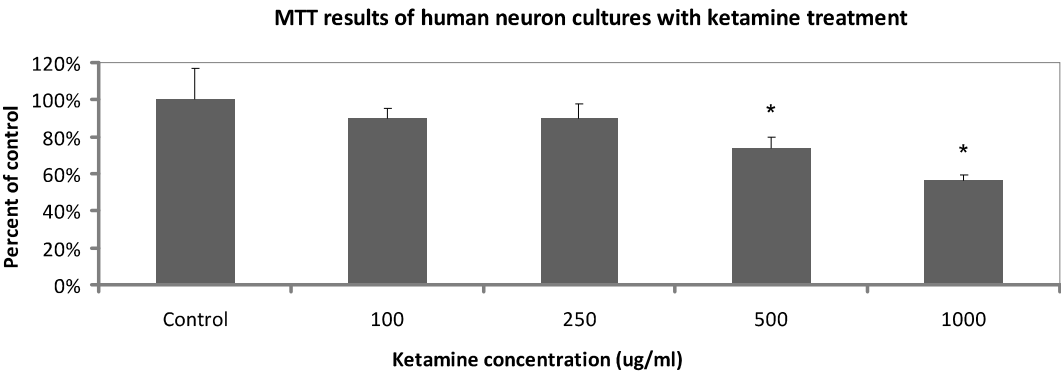


Fig. 3



This figure shows a section of the urinary bladder. Arrow points to severe fibrosis (red collagen fibers). Note that there is a comparatively smaller amount of muscles (yellow muscle fibers).

Fig. 4



Attachment 1



Mice are prone to kidney pathology after prolonged ketamine addiction

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Nerve fibers

ABSTRACT

ICR mice were injected with ketamine for 1, 3 and 6 months and the kidneys and urinary bladders were excised and processed for histology. Starting from 1 month, all addicted mice showed invasion of mononuclear white cells, either surrounding the glomerulus or the other tubules in the kidney. The aggregation of these cells extended all the way to the pelvis and ureter. As well, in the urinary bladder, the epithelium became thin and there was submucosal infiltration of mononuclear inflammatory cells. Silver staining revealed a loss of nerve fibers amongst the muscles of the urinary bladder of the treated. Immunohistochemistry on choline acetyltransferase which is a marker for cholinergic neurons also demonstrated a decrease of those cells.

We hypothesized that prolonged ketamine addiction resulted in the animals prone to urinary infection.

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1. Introduction

Ketamine addiction is an important abusive problem in the Far East. Ketamine is frequently employed as a recreational drug in this region, especially in rave party. Some of ketamine mechanisms on the nervous system have been identified and have been resolved to the glutamatergic N-methyl-D-aspartate (NMDA) receptor (White et al., 1982; Strayer and Nelson, 2008). Ketamine, as an NMDA non-competitive antagonist, can inhibit the reuptake of serotonin, dopamine, and norepinephrine, although the mechanism underlying this action is not entirely clear (Britt and Cance-Katz, 2005; Strayer and Nelson, 2008). Indeed, NMDA receptor blocker might cause neurotoxicity, especially in developmental period of synaptogenesis, NMDA antagonist could induce neurons to commit apoptosis (Ikonomidou et al., 1999; Gascon et al., 2007; Wang et al., 2008).

The effect of ketamine on other organs of the body is still far from clear. Just a year ago, two papers by Colebunders and Van Erps (2008) and Chu et al. (2008) have indicated that ketamine would perhaps affect the urinary system, in these cases the urinary bladder, resulting in an increase of urination frequency, nocturia, urgency, suprapubic discomfort during micturition and at times hematuria (Colebunders and Van Erps, 2008). Colebunders and Van Erps (2008) worked on only 1 case while Chu et al. (2008) had 59 cases but only 12 biopsies. The two studies relied on clinical observations and radiological findings and reported inflammatory

changes similar to interstitial cystitis (Chu et al., 2008). While the urinary signs and symptoms are prominent, histology and cytological involvements on both kidney and urinary bladder remain elusive (Chu et al., 2008). Here, in a study of ketamine addiction employing mice, we have observed histopathological changes in the kidneys and urinary bladders of these animals. The following is a brief report on the work which would represent possible changes in the model after ketamine addiction.

2. Materials and methods

2.1. Animals

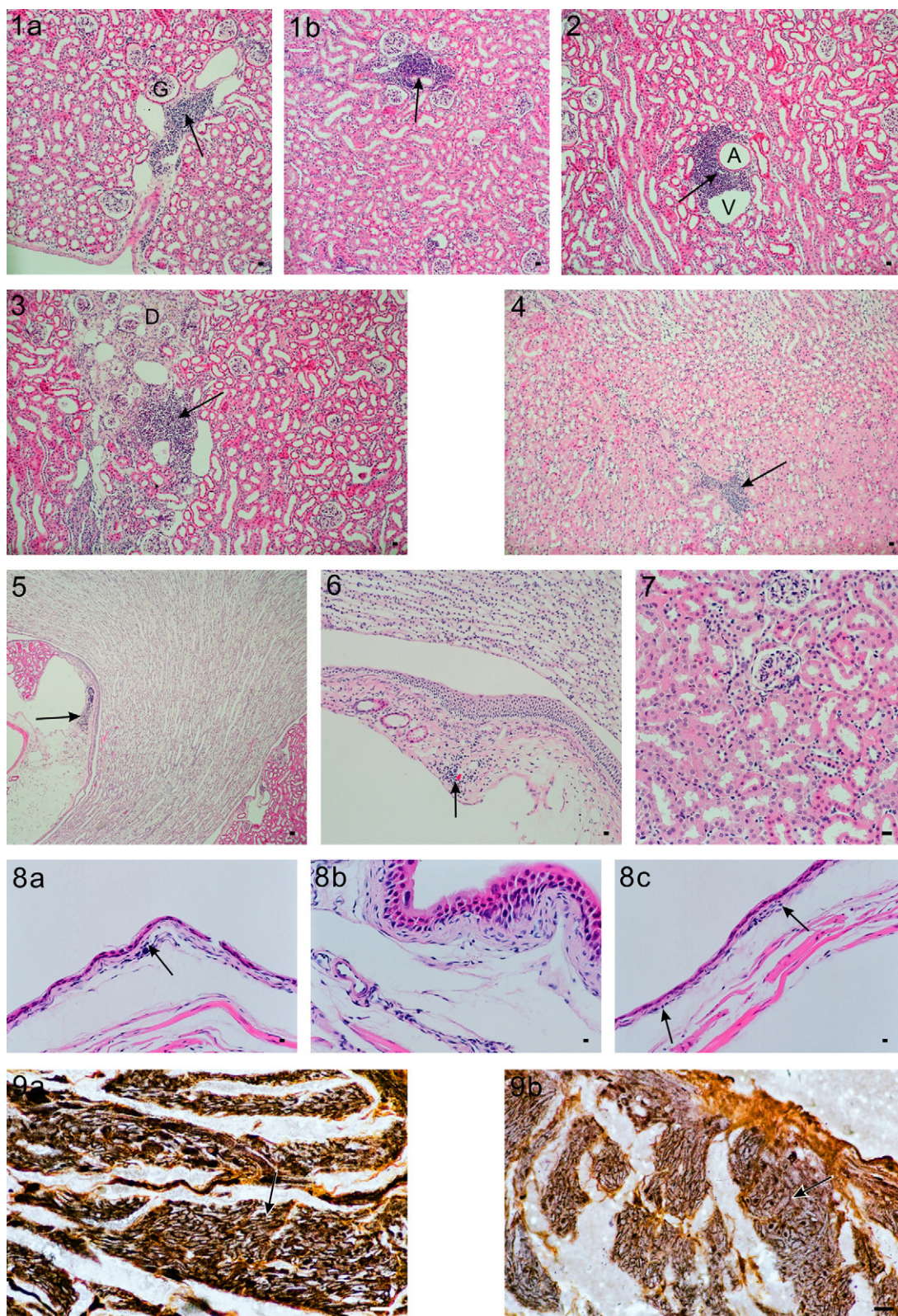
Mice (male ICR) of 2-month-old were obtained from the Animal House of Chinese University of Hong Kong (CUHK). The mice had free access to standard laboratory rodent chow (PicoLab Rodent Diet 20, PMI Nutrition Int., USA) and water. They were kept in rooms with 12 h light–dark cycle, with the temperature and humidity maintained at $22 \pm 1^\circ\text{C}$ and 45–55%, respectively. The animal experiments were approved by the Ethics Committee, CUHK.

2.2. Ketamine administration and tissue sampling

Ketamine, 30 mg/kg, was given as intraperitoneal injection daily, while controls mice were given injection of normal saline. Mice were weighed once in the beginning of every week for adjustment of the amount of ketamine administered. After 1, 3 or 6 months, mice were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) and perfused with saline solution through the left ventricle, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the left kidneys were removed, cut open midsagittally and further fixed overnight with the same fixative at room temperature. Afterwards, the specimens were processed through increasing concentration of ethanol, two changes of xylene, and three changes of paraffin. The tissue samples were finally embedded in paraffin blocks with a sagittal orientation so that serial sections of $5\ \mu\text{m}$ thick were obtained. Deparaffinized $5\ \mu\text{m}$ sections were stained with routine haematoxylin and eosin. Briefly, sections were placed in haematoxylin for 5 min, then washed in tap water and left for 5 min.

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Figs. 1–9. (1) Haematoxylin and eosin staining of sagittal section of kidney from a mouse treated with ketamine (30 mg/kg intraperitoneal injection daily) for 6 months. There were infiltrations of inflammatory mononuclear cells (arrows) (a) around and (b) between glomeruli in the kidney. “G” indicates a glomerulus. 100 \times . The scale bar represents 10 μ m. (2) Haematoxylin and eosin staining of sagittal section of kidney from a mouse treated with ketamine (30 mg/kg intraperitoneal injection daily) for 6 months. There was perivascular cuffing (arrow) by mononuclear inflammatory cells. “A” is artery and “V” is vein. 100 \times . The scale bar represents 10 μ m. (3) Haematoxylin and eosin staining of sagittal section of kidney from a mouse treated with ketamine (30 mg/kg intraperitoneal injection daily) for 6 months. There was an area of degeneration (D) and infiltration of mononuclear inflammatory cells (arrow) beside the area. 100 \times . The scale bar represents 10 μ m. (4) Haematoxylin and eosin staining of sagittal section of kidney from a mouse treated with ketamine (30 mg/kg intraperitoneal injection daily) for 3 months. There was infiltration of mononuclear inflammatory cells amongst tubules (arrow). 50 \times . The scale bar represents 20 μ m. (5) Haematoxylin and eosin staining of sagittal section of kidney from a mouse treated with ketamine (30 mg/kg intraperitoneal injection daily) for 6 months. There was

Sections were rinsed in 1% acid alcohol and washed in tap water. After that, sections were placed in eosin solution (1%) for 5 min, followed by washing in tap water, dehydrated and mounted with paramount. All stained sections were examined under light microscope.

In addition, the urinary bladder from both control and ketamine treated were removed and again cut midsagittally. They were processed as the kidneys and embedded in paraffin blocks, cut into 5 μm sections. The sections were stained with hematoxylin and eosin or Bielschowsky silver staining as performed by our laboratory previously (Wai et al., 2008).

Since preliminary observation on the silver staining specimens revealed difference in the density of nerve fibers in the muscular layer of the urinary bladder, a semi quantitative measurement was then conducted in the 6 months ketamine treated group and the control. Slides after silver impregnation were focused on the muscular layer of the urinary bladder and were taken as photographs with a Nikon photomicroscope under 200 \times magnification. The total area of the muscles was counted, as well as the total area of the positive silver nerve fibers using a computer program (CUHK morphometry, Prof. W.H. Kwong). The latter was expressed as a percentage of the total area of muscles (former) in each slide of the urinary bladder. Three consecutive midsagittal slides were observed from each control and ketamine specimens and in each control and ketamine treatment 6 months group, 4 animals were utilized. The total n is 12 from each group. The mean and standard deviations were computed and compared by Student's t -test.

As the same above approach, from three other consecutive slides of each specimen of each control and ketamine group, positive choline acetyltransferase (ChAT) neurons representing cholinergic cells were counted in the wall of the urinary bladder and expressed as a percentage of the total neurons present in the urinary bladder in that slide. The immunohistochemistry labeling of ChAT neurons was documented as in previous other studies of our group (Tiu et al., 2003).

3. Results

In all the kidneys of the mice addicted to ketamine for 1, 3 and 6 months, foci of infiltration of mononuclear white cells were observed. Some of these infiltrative patches were near to the glomeruli (Fig. 1a and b). Others were near to blood vessels (arteries or veins), forming perivascular cuffing (Fig. 2). Severe degenerating of parts of the kidney were evident in over 15% of all addicted cases (Fig. 3), while mononuclear infiltration was also demonstrated in the regions of the distal and collecting tubules of the medulla in all addicted cases (Fig. 4). The mononuclear infiltration could extend all the way into the papilla and the ureter (Figs. 5 and 6) of all addicted cases. The controls (1, 3 and 6 months) all had no significant infiltration of mononuclear white cells (Fig. 7).

The urinary bladder of the treated animals displayed some pathology when compared with control. The epithelium in the experimental mice was much thinner (1–2 layers thick) (Fig. 8a) than those of control (3–4 layers thick) (Fig. 8b). Infiltration of mononuclear cells (macrophages, lymphocytes, etc.) was frequently observed in the submucosa of the bladder wall (Fig. 8a and c). The other layers of the bladder were unremarkable. Silver staining, however, revealed abundant positive silver impregnated positive fibers in the muscle of control (Fig. 9a) while in the ketamine treated, the number of positive silver fibers were less in amount (Fig. 9b).

Morphometric evaluation revealed that in the control, the area occupied by the nerve fibers versus the total area of muscles in the urinary bladder was $34.26 \pm 6.95\%$ ($n = 12$, 3 slides from each of 4 animals in control and treated) while those of the 6 months ketamine treated group was $27.5 \pm 6.14\%$ ($n = 12$). Student's t -test revealed significant differences between the two groups ($p < 0.02$).

Our result on the percentage of ChAT positive neurons versus total neurons of the control group was $47.5 \pm 11.4\%$ while in the ketamine group was $23.33 \pm 8.96\%$ (mean \pm S.D.). There was a significant decrease ($p < 0.0008$) of cholinergic neurons in the ketamine treated, as ChAT is a marker for cholinergic neurons.

4. Discussion

Mononuclear infiltration is an indication of chronic inflammation of the urinary tract and is likely associated with interstitial inflammation (Erickson et al., 1994). Such changes in the mouse urinary system in these cases are probably not a direct effect of the ketamine on the urinary tract, but a secondary episode subsequent to pathophysiological changes induced by ketamine, e.g. changes in ion channel of the cells (Frenkel and Urban, 1992; Han et al., 2003; Kawano et al., 2005; Chen et al., 2009). Changes might also arise from alteration of muscular contractability in the urinary bladder. This can come as a change in neuromuscular activity as presented in this study or a possible decrease of nerve fibers. All these changes would subsequently lead to cellular degeneration, stasis of urine, dilatation of the urinary bladder and the subsequent migration of mononuclear white cells into the sites of lesion. The cells are usually monocytes, lymphocytes and macrophages. In the early phase, no chemical pathology might be detected from the urine or the blood (e.g. rise of creatinine) but prolonged alteration would finally change the biomarkers in the urine or blood of the animals (unpublished data of our laboratory).

Pathology of the urinary bladder indicates mononuclear infiltration, much similar to that of the clinical situation of interstitial cystitis. The thin walls of the bladders in the treated are probably as a consequence of the cystitis. The decrease of silver stainable fibers in the muscles of the urinary bladder is interesting. One would be tempted to suggest perhaps ketamine may be related to such decrease, i.e. degeneration of neuromuscular junctions and/or proprioceptive sensory fibers in chronic addiction. This is essentially a very important consequence if found correct but certainly needs much further investigation.

There was also a possible decrease in the cholinergic neurons in the urinary bladder of the ketamine treated animals. It is possible that damage induced via NMDA receptors play a role in the decrease of the cholinergic neurons or in the expression of acetylcholine (White et al., 1982; Strayer and Nelson, 2008).

The interstitial inflammatory disease in animal model after subject to ketamine addiction has never been described. In the bladder, it is similar to the human interstitial cystitis in that the epithelium is affected (Johansson and Fall, 1990) and there are submucosal changes with inflammatory cell aggregates (Lynes et al., 1990; Lépinard, 1998; Itano and Malek, 2001).

Our study of the ketamine addiction in the mouse substantiated the recent human cases that ketamine addiction exerts toxicity which will render the subject prone to urinary changes (Cobunders and Van Erps, 2008; Chu et al., 2008), which in turn affects not only the bladder but in addition the kidney as revealed

infiltration of mononuclear inflammatory cells (arrows) near the renal pyramid. 50 \times . The scale bar represents 20 μm . (6) Haematoxylin and eosin staining of sagittal section of kidney from a mouse treated with ketamine (30 mg/kg intraperitoneal injection daily) for 6 months. There was infiltration of mononuclear inflammatory cells (arrows) near the renal pelvis. 50 \times . The scale bar represents 20 μm . (7) Haematoxylin and eosin staining of sagittal section of kidney from a mouse that received no ketamine treatment. There was no significant mononuclear inflammatory cell infiltration. 200 \times . The scale bar represents 10 μm . (8) Silver staining of midsagittal sections of urinary bladder. (a) This section was from a mouse treated with ketamine (30 mg/kg intraperitoneal injection daily) for 1 month. There was infiltration of mononuclear cells (lymphocytes) (arrow) under the epithelium of urinary bladder. Note that the epithelium was thin. (b) This section was from a control mouse with no ketamine treatment and the epithelium of the urinary bladder was normal, which was thicker than that showed in (a). (c) This section was from a mouse treated with ketamine (30 mg/kg intraperitoneal injection daily) for 3 months. There was infiltration of mononuclear cells (lymphocytes) (arrows) under the epithelium of urinary bladder. The epithelium was thin as those of (a), and was thinner than the control in (b). 100 \times . The scale bar represents 10 μm . (9) Silver staining of midsagittal sections of the urinary bladder showing the nerve fibers in the muscles. (a) From a mouse with no ketamine treatment. The nerve fibers (arrow) showed profuse anastomosis. (b) From a mouse treated with ketamine (30 mg/kg intraperitoneal injection daily) for 1 month. The nerve fibers (arrow) showed loose arrangement. 400 \times . The scale bar represents 10 μm .

by the mice experiments in this work. This is an important information which could be used in the education to combat ketamine addictions.

Conflict of interest

None.

Acknowledgement

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Attachment 2

The Toxic Effect of Ketamine on SH-SY5Y Neuroblastoma Cell Line and Human Neuron

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KEY WORDS ketamine toxicity; neuron; apoptosis; Bax/Bcl-2 ratio

ABSTRACT Ketamine used as an injectable anesthetic in human and animal medicine is also a recreational drug used primarily by young adults often at all night dance parties in nightclubs. The percentage of ketamine users has grown very fast in the last 5 years worldwide. However, this leads to the serious question of the long-term adverse effects of ketamine on our nervous system, particularly the brain, because ketamine as an NMDA antagonist could cause neurons to commit apoptosis. Our study therefore aimed to find out the chronic effect of ketamine on neuron using prolonged incubation (48 h) of neuronal cells with ketamine in culture. Our results showed that differentiated neuronal cells were prone to the toxicity of ketamine but probably less susceptible than undifferentiated neuronal cells and fibroblasts. This suggested that the ketamine abuse would be harmful to many other organs as well as the brain. Our results also confirmed that the toxicity of ketamine is related to apoptosis via the Bax/Bcl-2 ratio pathway and caspase-3 in the differentiated neuronal cells. Therefore, long-term ketamine treated cell or animal models should be sought to study this multiorgan effects of ketamine. *Microsc. Res. Tech.* 73:195–201, 2010. © 2009 Wiley-Liss, Inc.

INTRODUCTION

Ketamine, a noncompetitive antagonist at the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor, is currently used in human and animal medicine as an injectable anesthetic especially in pediatric patients (Haas and Harper, 1992; Strayer and Nelson, 2008; Loxton et al., 2008; White et al., 1982). Ketamine is also a controlled substance, illegally used as a recreational drug. It is used primarily by young adults often at all night dance parties called “raves” that take place in nightclubs (Britt and Cance-Katz, 2005; Gable, 2004). In Hong Kong, The Central Registry of Drug Abuse database (CRDA) showed that the percentage of ketamine users has grown very fast in the last 5 years; from only few reported cases in 1999 to 70% of the age under 21-year-old drug abusers by 2002 (Joe Laidler, 2005). This trend is consistently with the findings in the United State of America and around the World (Lankenau et al., 2007). For use as a general anesthetic agent, ketamine is administered at a dose of 1–2 mg/kg intravenously or 5–10 mg/kg intramuscularly. For use in sedation or analgesia only, the suggested doses are 200–750 µg/kg intravenously or 2–4 mg/kg if given intramuscularly (Haas and Harper, 1992). 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 10–20% of subjects receiving ketamine (Strayer and Nelson, 2008). Furthermore, ketamine as one of the popular club drugs because of its anesthetic and hallucinate effects, the user might not be aware of injury induced or related physical assaults in addition to weird behavior that could be fatal (Gable, 2004).

The reason for prevalent use of ketamine is the short duration of action and lack of a physical dependence

(Haas and Harper, 1992; Lankenau et al., 2007). However, ketamine, as an NMDA antagonist, can inhibit the reuptake of serotonin, dopamine, and norepinephrine, although the mechanism underlying this action is not entirely clear (Britt and Cance-Katz 2005; Strayer and Nelson, 2008). Indeed, NMDA receptor blocker might cause neurotoxicity, and in developmental period of synaptogenesis, NMDA antagonist could cause neurons to commit apoptosis (Ikonomidou et al., 1999). A recent study shows that ketamine at 200 µM (50 µg/mL) significantly increased Bax, caspases 3, 6, 9, and cytochrome c in human HepG2 cells in culture leading to increases in DNA fragmentation and apoptosis (Lee et al., 2009). This study showed that ketamine induced apoptotic insults via the Bax-caspase pathway at a clinically relevant or an abused concentration that induced toxicity to hepatocytes (Lee et al., 2009). Other studies also showed that ketamine could increase significantly the neuronal apoptosis with increased Bax in forebrain culture from neonatal rat (Wang et al., 2005, 2008) and neonatal monkey (Wang et al., 2006). In addition, repeated administration of 20 mg/kg ketamine into postnatal day-7 rats caused significant increases in caspase-3 apoptotic neuronal cells in the frontal cortex as well as in other brain regions (Zou et al., 2009). These data also demonstrated that ketamine administration during development induced cell

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death were apoptotic in nature. Using primary neuron culture from day 0 rat, 10 $\mu\text{g/mL}$ of ketamine could induce cell death and dendritic arbor retraction (Vutskits et al., 2007). Most importantly, ketamine at concentrations as low as 0.01 $\mu\text{g/mL}$ could induce atrophy of neuronal dendritic architecture. These suggested that long-term administration of subanesthetic concentrations of ketamine, even if not affecting survival may impair neuronal network by interfering with fundamental contact in neurons and thus might lead to dysfunctions of the neural networks (Vutskits et al., 2007). Nevertheless, the concern of ketamine toxicity is the long-term effect of its use on the central nervous system (CNS), as evidences showed that ketamine could cause neuronal cell death in rats and monkeys infused with ketamine (Wang et al., 2008). In culture of rat forebrain neurons, incubation with ketamine of 10 μM (2.5 $\mu\text{g/mL}$) for prolong period of 24 h, which mimics the long-term in vivo exposure, indicated that ketamine is apoptotic to nervous cells (Gascon et al., 2007; Wang et al., 2008).

The human neuroblastoma cell line, SH-SY5Y, is a suitable and reliable model to test the neurotoxicity or neuroprotection of drugs (Nicolini et al., 1998, Akao et al., 2002). Together with the primary human neurons (ScienCell, Carlsbad, CA) in culture, we sought to investigate the long-term effect of different concentrations of ketamine, from subanesthetic, clinically relevant to abuse levels, on human neurons in culture. We also investigated the apoptotic effect of ketamine by examining the B-cell lymphoma 2-associated protein X (Bax) to B-cell leukemia/lymphoma-2 (Bcl-2) ratio, a crucial factor in determining the progress of cell apoptosis.

MATERIALS AND METHODS

Cell Cultures and Ketamine Treatment

The human neuroblastoma SH-SY5Y cell line (ATCC number CRL-2266) and NIH-3T3 fibroblast cells (ATCC number CRL-1658) (American Type Culture Collection (ATCC), Manassas, VA) were cultured in 96-well plate in Dulbecco's Modified Eagle/Nutrient Mixture F12 (HAM) 1:1 (DMEM/F12) medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (Invitrogen Corp., Carlsbad, CA). Differentiation of SH-SY5Y was performed with the addition of 10 μM of all-*trans*-retinoic acid into the culture medium for 5 days (Cuende et al., 2008, Encinas et al., 2000). Human neurons (Cat. No. 1521) were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured in a recommended neuronal medium from the supplier. After confluence of the cultures, ketamine or hydrogen peroxide (H_2O_2) of different concentrations was added to the culture medium, and incubation continued for 48 h.

MTT Assay

The MTT reduction assay was performed as previously published (Lü et al., 2008). Cultures were incubated for 48 h in different concentrations of ketamine and MTT reduction was started by addition of 10 μL of MTT stock solution (5 mg/mL in phosphate buffer saline (PBS)) (Sigma-Aldrich D4540, St. Louis, MO) in 90 μL of PBS per well after the medium was removed. After further 4 h incubation at 37°C, the reaction was terminated by addition of 100 μL of dimethyl sulfoxide

(DMSO). After a further incubation for 15 min, optical density (OD) at 550 nm was determined using an automatic microtiter plate reader (Epson LX-800, Molecular Devices). Results were means of replicates of six or eight wells of each ketamine or H_2O_2 concentration on the same plate. The OD result of zero concentration of ketamine or H_2O_2 concentration (OD_C) was taken as 100%, and percentages of MTT reductions at other concentrations were calculated as $[(\text{OD}_\text{S} - \text{OD}_0)/(\text{OD}_\text{C} - \text{OD}_0)] \times 100$, where OD_S is the OD result of different ketamine or H_2O_2 concentration, and OD_0 is the OD result of the eight MTT blank cell wells without MTT added.

Western Blot

For Western blots (Lü et al., 2008), confluent cells (a total of about 5×10^6 cells) after ketamine or H_2O_2 treatment were collected by trypsinization and centrifugation at 1,000g for 5 min. Collected cells were suspended in PBS precooled at 4°C and washed for two times. The cell pellets were homogenized with a homogenizer in 0.5 mL of lysis buffer, 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 0.1 mg/mL phenylmethylsulfonyl fluoride (PMSF), 1 mg/mL leupeptin, 1 mg/mL pepstatin A, and 5 mg/mL aprotinin. The homogenate was centrifuged at 14,000g for 30 min at 4°C, and the supernatant obtained was either immediately used or stored at -80°C until use. The protein concentration of the extract was determined by the Bio-Rad DC protein assay (500-0111, Bio-Rad Laboratories, Hercules, CA).

Equal amounts of protein (20 μg) samples were boiled in 2 \times loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 200 mM dithiothreitol, 0.2% bromophenol blue, and 20% glycerol) for 6 min before loading on 12% SDS-polyacrylamide gel. Electrophoresis was performed at 100 Volt (V) for 120 min followed by semidry transfer onto nitrocellulose membrane at 10 V for 1 h. The membrane was blocked for 1 h in blocking solution (5% nonfat dry milk, 0.05% Tween-20 in PBS) and then incubated overnight at 4°C with either one of the following antisera at 1:2,000 dilution: antiactin (sc-1616), anti-Bax (sc-526), and anti-Bcl-2 (sc-783). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The following day, after three sequential 5-min washes with 0.05% Tween-20 in PBS (PBST), the membrane was incubated with the appropriate IRDYE[®] FC conjugated secondary antibody 1:2,000 for 1 h (IRDYE[®] 700DX antigoat FC 605-430-003 for Bax and Bcl-2, IRDYE[®] 800CW antirabbit FC 611-131-003 for actin) (LI-COR Biosciences, Lincoln, NE). After washing three times for 10 min with PBST, the bound antibodies were then visualized and recorded using the ODYSSEY Infrared Imaging System (LI-COR Biosciences). Band density values of individual proteins were normalized to that of the actin. Results were means of three replicates of experiments.

Caspase-3 Activity

The caspase-3 activity was measured using the Fluorimetric Caspase 3 Assay Kit (CASP3F, Sigma-Aldrich Corp., St. Louis, MO) according to manufacturer's instructions. Briefly, the cells were washed with phosphate-buffered saline and harvested with trypsiniza-

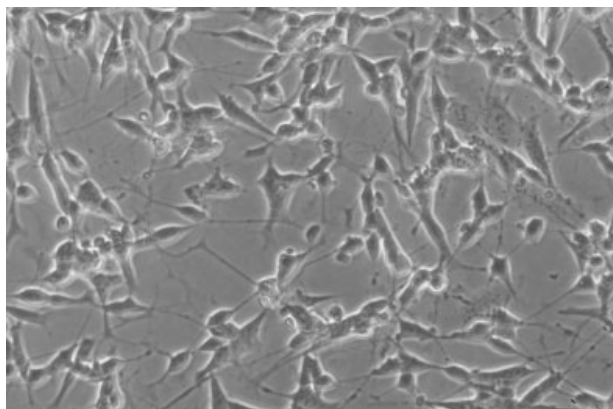


Fig. 1. The human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's Modified Eagle/Nutrient Mixture F12 (HAM) 1:1 (DMEM/F12) medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Differentiation was performed with the incubation of 10 µM of all-*trans*-retinoic acid for 5 days. The cells showed more neuronal appearance with neuritic extension.

tion using 0.25% of trypsin solution in 0.1% EDTA (59429C, Sigma-Aldrich Corp.). Cell pellets collected were lysed using the 1× lysis buffer as provided in the kit. Supernatant after centrifugation at 14,000g for 10 min at 4°C was used for caspase-3 activity assay. Standards, positive controls, samples and the inhibited samples were set up according to the kit's menu. The fluorescent 7-amino-4-methylcoumarin (AMC) generated from the hydrolysis of acetyl Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) by caspase-3 was measured with excitation and emission wavelengths at 355 nm and 460 nm, respectively, using the Wallace 1420 Victor² Multilable Counter (Perkin-Elmer, Waltham, MA). The concentration of the AMC released was calculated from the AMC standards, and the caspase-3 activity was expressed as nmol AMC/min/mg protein. The protein concentration of the samples was determined using the Bio-Rad DC protein assay (500-0111, Bio-Rad Laboratories).

Statistical Method

All data were expressed as mean ± standard deviation (SD). Results of the ketamine treated cultures were compared with the controls (without ketamine treatment) using student's *t*-test. The difference between groups was considered as statistically significant when *P*-value was less than 0.05.

RESULTS

MTT Cell Death Test

Differentiation of SH-SY5Y cell cultures was induced by retinoic acid; the cells differentiated into a more neuronal appearance with neuritic extensions (Fig. 1). In undifferentiated SH-SY5Y, treatment with ketamine at 250 µg/mL or higher caused significant ($P < 0.05$) cell death as compared to the control without ketamine treatment (Fig. 2A). MTT reduction was $54.3\% \pm 17.4\%$ for 250 µg/mL in undifferentiated SH-SY5Y, which was significantly ($P = 0.0154$) different from control. For differentiated SH-SY5Y, treatment with

ketamine of 1,000 µg/mL (or higher) caused significant ($P = 0.000005$) cell death (MTT reduction $47.3\% \pm 12.8\%$) as compared to control (Fig. 2B). In fibroblasts (NIH-3T3) culture, treatment with ketamine of 100 µg/mL or above could cause significant ($P < 0.05$) cell death as compared to the control (Fig. 2C). MTT reduction was $72.3\% \pm 6.4\%$ for 100 µg/mL in NIH-3T3, which was significantly ($P = 0.0057$) different from control. In other words, for fibroblasts significant cell death could be caused by lower ketamine concentrations than undifferentiated and differentiated SH-SY5Y. For human neuron cultures, treatment with ketamine of 500 µg/mL caused significant ($P = 0.00201$) cell death (MTT reduction $73.7\% \pm 5.8\%$) as compared to the control (Fig. 2D). The use of H₂O₂ of 500 mM was a positive control for apoptosis and indeed treatment with H₂O₂ of 500 mM caused significant ($P < 0.05$) cell death in undifferentiated SH-SY5Y ($52.8\% \pm 8.6\%$), differentiated SH-SY5Y ($11.2\% \pm 0.7\%$), human neuron ($63.9\% \pm 8.7\%$), and NIH-3T3 ($45.0\% \pm 8.0\%$) cultures as compared to their respective controls (Fig. 2).

Bax/Bcl-2 Ratio and Caspase-3 Activity Results

The Bax to Bcl-2 (Bax/Bcl-2) ratio Western blot results are shown in Figure 3A. Bax/Bcl-2 ratios were significantly increased in undifferentiated SH-SY5Y (2.49 ± 0.147), differentiated SH-SY5Y (3.18 ± 1.96), and NIH-3T3 (5.78 ± 1.50) cultures treated with 2,500 µg/mL of ketamine as compared to their respective control cultures without ketamine treatment (0.26 ± 0.20 , 0.38 ± 0.35 , 1.01 ± 0.42 for undifferentiated SH-SY5Y, differentiated SH-SY5Y, and fibroblast NIH-3T3 respectively). Active caspase-3 activities were measured in undifferentiated and differentiated SH-SY5Y cultures treated with ketamine of 2,500 µg/mL comparing to control cultures with no ketamine treatment. Caspase-3 activity was increased significantly ($P = 0.000113$) in treated differentiated SH-SY5Y culture (0.194 ± 0.018) as compared with control culture (0.010 ± 0.006) (Fig. 3B). However, caspase-3 activities showed no significant difference between undifferentiated SH-SY5Y cultures treated with or without ketamine.

DISCUSSION

Ketamine becomes a popular club drug due to its rapid onset and short duration of effects (Haas and Harper, 1992; Gable, 2004). However, this opens the serious question of the long-term adverse effects of ketamine on our nervous system, the brain in particular. Our study therefore aimed to find out the chronic effect of ketamine on neuron using prolonged incubation (48 h) of neuronal cells with ketamine in culture (Vutskits et al., 2007). Our results showed that treatment with ketamine at 250 µg/mL (1 mM) induced significant cell death in undifferentiated SH-SY5Y cultures (Fig. 2A), a human neuroblastoma cell line. Undifferentiated SH-SY5Y cells are not neurons but cancerous neuronal cells; its origin was from neuroblasts that they might possess both properties as cancer and neuronal cells (Edsjö et al., 2007; Reynolds et al., 1986). SH-SY5Y cells are undifferentiated in culture, and different substances such as retinoic acid could induce the differentiation into more neuronal like cells (Claggett-Dame et al., 2006; Edsjö et al., 2007; Uemura

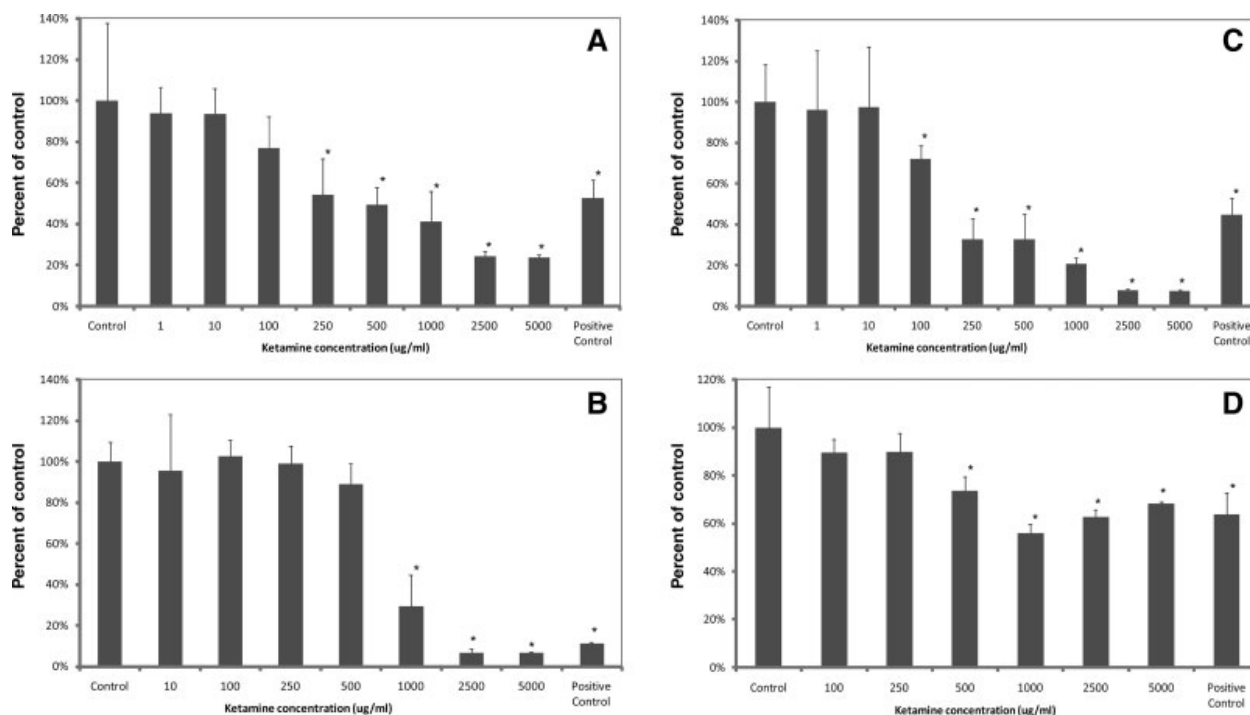


Fig. 2. Cell death by MTT reduction in different types of cell cultures treated with various ketamine concentrations. The positive control was treated with 500 mM hydrogen peroxide. (A) Undifferentiated SH-SY5Y; (B) differentiated SH-SY5Y induced by retinoic acid; (C) fibroblast (NIH-3T3); and (D) human neurons. * $P < 0.05$ as compared with the control with no ketamine treatment.

et al., 2003). Differentiated SH-SY5Y cells still might not totally resemble neurons, but they are more close to neurons than the undifferentiated SH-SY5Y cells. Our results showed that in differentiated SH-SY5Y culture, ketamine of 1,000 µg/mL (4 mM) caused significant cell death (Fig. 2B), and this concentration was higher than that of undifferentiated SH-SY5Y. Using primary human neurons in culture, treatment with ketamine at 500 µg/mL (2 mM) induced significant cell death (Fig. 2D). Therefore, it seemed that neuron and more neuronal like cells could be more resistant to the toxic effect of ketamine than cancerous cells. Our results showed also that for fibroblasts NIH-3T3 culture, a lower concentration of ketamine of 100 µg/mL (0.4 mM) could cause significant cell death (Fig. 2C). This concentration of ketamine (100 µg/mL or 400 µM) was similar to the concentration of 50 µg/mL (200 µM) which showed significant apoptotic effect to HepG2, a human hepatoma cell line (Lee et al., 2009). Furthermore, if comparing HepG2 with the aforementioned study (Lee et al., 2009) and undifferentiated SH-SY5Y from our study, neuroblastoma was more resistant to ketamine toxicity and requiring higher concentration (250 µg/mL) than hepatoma (50 µg/mL), to have significant cell death although the incubation time in our study was longer (48 h as compared to 24 h in Lee et al.'s study (2009)). Taken together, differentiated neuronal cells of human cell line are prone to the toxicity of ketamine but probably less susceptible than undifferentiated neuronal cells, fibroblasts, and hepatocytes.

The toxicity of ketamine is believed to be related to the apoptotic pathway (Zou et al., 2009; Wang et al., 2008; Lee et al., 2009). MTT is only a cell viability test; the causes of cell death can be different (Lü et al., 2008; Shearman et al., 1994), therefore, to relate the cell death with apoptosis, we needed to analyze the changes of apoptotic markers. According to the aforementioned results, we treated the fibroblasts, undifferentiated and differentiated SH-SY5Y cultures with high ketamine concentration of 2,500 µg/mL to make sure cell death was induced in all cell types, and then the treated cultures were assayed for the Bax/Bcl-2 ratio, a crucial apoptotic marker. This high ketamine concentration of 2,500 µg/mL (40 mM) was used only for induction of cell death in this study; this is not clinically relevant. Our results showed that the Bax/Bcl-2 ratios were increased significantly in cultures treated with ketamine as compared to their controls with no ketamine treatment (Fig. 3A). In the apoptotic pathways, Bax is well-known as proapoptotic by interacting with Bcl-2, which is regarded as antiapoptotic (Oltvai et al., 1993). Mitochondria play a pivotal role in apoptosis in both the intrinsic and extrinsic pathways (Sastre et al., 2000; Sastre and Rao, 2002). Oligomers of Bax could insert into the outer mitochondrial membrane triggering cytochrome c release for promoting apoptosis. On the other hand, Bcl-2 forms complexes with Bax in such a way that the release of cytochrome c is inhibited and thus preventing apoptosis. In this way, the ratio of Bax/Bcl-2 ratio is a crucial factor in determining the progress of cell apoptosis (Raghupathi, 2004; Woo

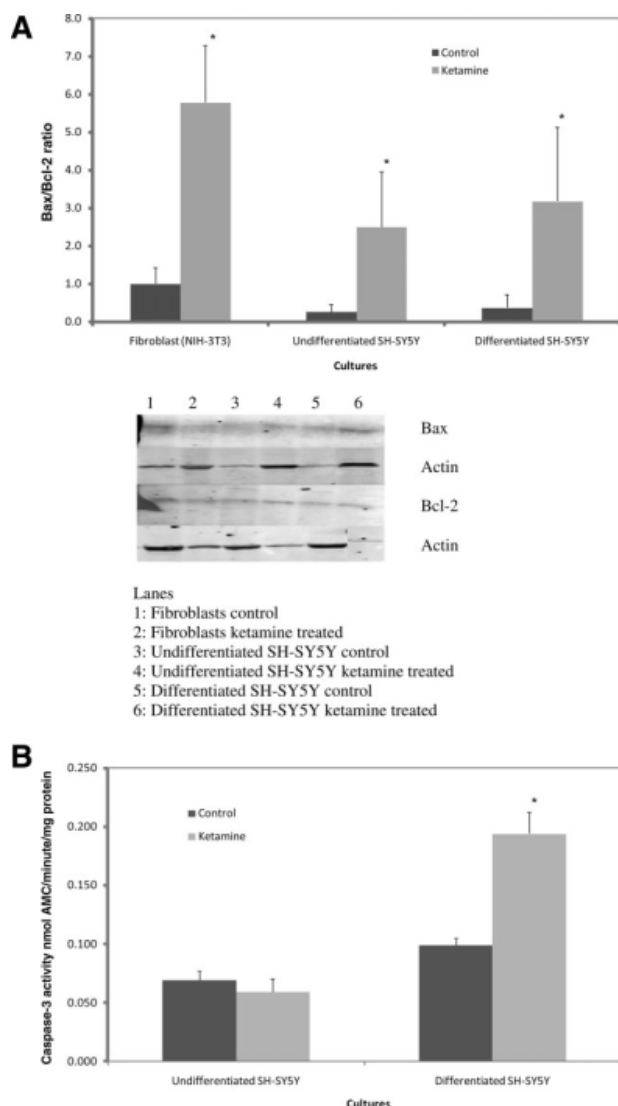


Fig. 3. Results of apoptotic markers in undifferentiated and differentiated SH-SY5Y and fibroblasts NIH-3T3 cultures treated with ketamine (2500 $\mu\text{g/mL}$) as compared to controls without ketamine treatment. (A) Bax/Bcl-2 ratio with a panel showing representative Western blot results (Note 1); 1: fibroblasts control, 2: fibroblasts ketamine treated, 3: undifferentiated SH-SY5Y control, 4: undifferentiated SH-SY5Y ketamine treated, 5: differentiated SH-SY5Y control, 6: differentiated SH-SY5Y ketamine treated. (B) Caspase-3 activity. * $P < 0.05$ versus control. Note: Because of the limited number of cells in culture, the quantities of samples for Western blot analysis were small; the final Bax and Bcl-2 bands were not too strong to visual eyes. The protein concentrations of these samples were also variable and the amounts of proteins applied for electrophoresis might not be the same (20 μg). Therefore, even the actin bands did not show consistent intensities for all samples. Nevertheless, the ODYSSEY Infrared Imaging System could record and measure the intensities of all the bands.

et al., 2000). The significant increases of Bax/Bcl-2 ratio supported that the toxicity of ketamine in this study is related to apoptosis.

Downstream to Bax and Bcl-2 activation, caspase-3 activity was increased significantly in differentiated SH-SY5Y (Fig. 3B) with ketamine-induced apoptosis,

while caspase-3 activity did not increase in undifferentiated SH-SY5Y. This seemed to be suggesting that different effectors may be involved in the apoptosis of differentiated and undifferentiated forms of SH-SY5Y; caspase-3 for the differentiated and other activators/ effectors for the undifferentiated forms. Other effectors might include caspase-6 and -7 as well as the activator caspase-9 (Milosevic et al., 2003; Riedl and Shi, 2005). The other possibility is that the differentiated SH-SY5Y but not the undifferentiated cells possess the NMDA receptor to which ketamine is a noncompetitive antagonistic blocker leading to cell death and apoptosis (Ikonomidou et al., 1999; Young et al., 2005). However, it has been shown that both the undifferentiated and differentiated (induced by retinoic acid) SH-SY5Y cells showed the expression of NMDA receptor (NR) subunit NR1 (Kulikov et al., 2007). Although retinoic acid differentiation increased the NR1 expression, this supported that differentiation of SH-SY5Y cells are more neuronal like than the undifferentiated cells. In the same study, in fact, retinoic acid differentiation caused also expression of NRs in adipocytes. Investigation is needed to elucidate further details of the pathways involved in particular in the undifferentiated SH-SY5Y.

Glia and neuron are the two most abundant cell types in the brain; it is also important to understand how ketamine might affect the glial cells. From recent studies, ketamine could influence glial cells including microglia and astrocytes in response to inflammatory insults (Chang et al., 2009; Shibakawa et al., 2005). This might in turn affect the survival of neuronal cells. In other words, ketamine might have an indirect effect on the well being of neurons. Further studies are required to find out whether or not ketamine is toxic to glial cells, as to neurons in this study; if so, ketamine would have a more global detrimental effect on the whole brain.

Studies of toxicity of ketamine used mostly neonatal animals or cultures derived from neonatal/fetal tissues, and most studies examined the short term or acute effect of ketamine (Ikonomidou et al., 1999; Scallet et al., 2004; Farber and Olney, 2003; Takadera et al., 2006; Vutskits et al., 2007; Ke et al., 2008; Fredriksson et al., 2007; Zou et al., 2009; Young et al., 2005; Gascon et al., 2007; Wang et al., 2006, 2008). This is due to the fact that ketamine is frequently used as anesthetic especially in pediatric patients (Haas and Harper, 1992; Strayer and Nelson, 2008), and the concern of the neurotoxic effects of related short-term administration of ketamine. However, despite the fact that ketamine is being increasingly used as a club drug by youngsters and adults (Gable 2004, Strayer and Nelson, 2008, Loxton et al., 2008), long-term effect of ketamine on the nervous system should be investigated in adult animal or human tissues. Ketamine is considered as safe at the anesthetic or subanesthetic levels (Haas and Harper, 1992); high levels and their toxic effect as resulted from abuse are certainly undesirable. For example, a typical recreational dose through intranasal inhalation, in powdered form, is about 60 mg (Gable, 2004). Given also that ketamine is distributed to highly perfused organs, including brain, the ketamine could achieve to levels several folds of in plasma (Haas and Harper, 1992). Ketamine levels of mM,

which from our results as above causing cell deaths, could be attained in particular in the brain. Therefore, the toxic effects of these high levels of ketamine in chronic and abuse use should be studied and documented. Our study was designed to aim at finding out such relevant results. We used ketamine from very low to very high concentrations in the treatments of cells derived from adult tissues, and also with a long incubation time of 48 h to mimic the chronic effect (Vutskits et al., 2007) and we were able to show that ketamine was toxic to fibroblasts, neuroblastoma, and primary neurons. Importantly, fibroblasts were more susceptible to ketamine toxicity than neuronal cells suggesting the ketamine abuse would be harmful to many other organs as well as the brain. Indeed, Lee et al. (2009) showed that ketamine was toxic to hepatocytes at also low concentrations. In addition, from a recent study, ketamine abusers could have destruction of their lower urinary tract including syndrome of cystitis and contracted bladder, and secondary damage might be irreversible (Chu et al., 2008). Therefore, long-term ketamine treated cell or animal models should be sought to study this multiorgan effects of ketamine. Our and other results showed that apoptosis was involved in ketamine's toxic effect, and any future therapeutic approach might consider targeting this pathway (Takadera et al., 2006).

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Attachment 3



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 ketamine 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.

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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-

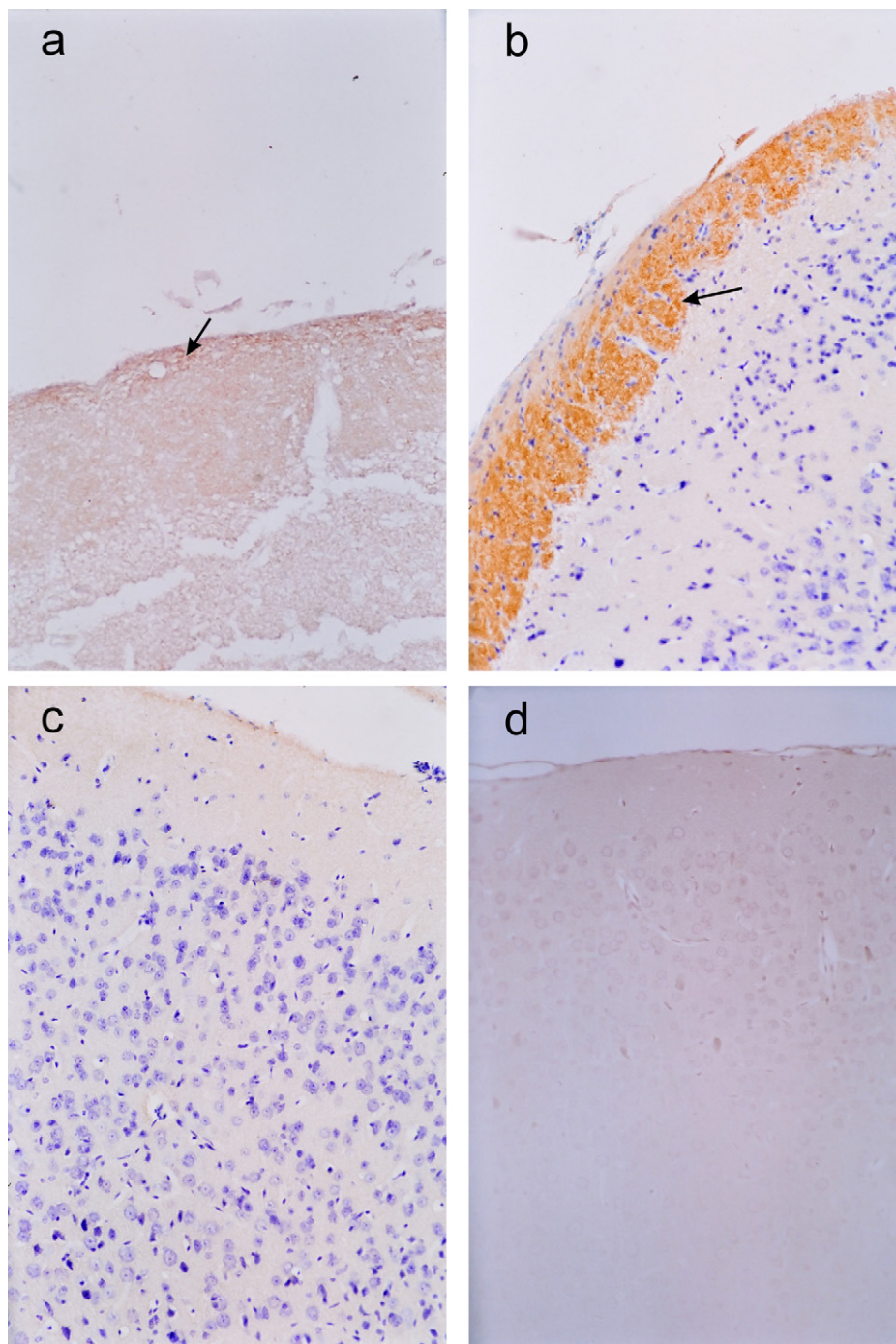


Fig. 1. Hyperphosphorylated tau immunostaining of sections from the prefrontal and entorhinal cortices of mice with administration of ketamine and control mice receiving no ketamine. (a) A section of the prefrontal cortex from a mouse treated with ketamine for 3 months showed there were some positively stained sites (arrow) for hyperphosphorylated tau in layer I of the prefrontal cortex. $\times 400$. (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. $\times 400$. (c) There were no stained positive sites for hyperphosphorylated tau in this section of prefrontal cortex from a control mouse. $\times 400$. (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. $\times 400$.

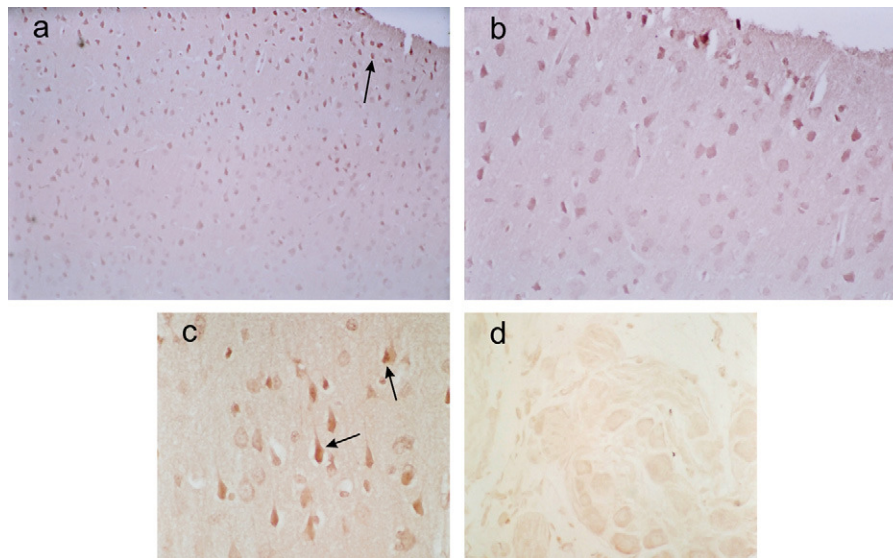


Fig. 2. Hyperphosphorylated tau immunostaining of sections from the prefrontal and entorhinal cortices of monkeys with administration of ketamine for 6 months and control monkeys receiving no ketamine. (a) This section shows hyperphosphorylated tau positively stained cells were in the outer layers of the prefrontal cortex. $\times 200$. (b) Same as (a). $\times 400$. (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. $\times 400$. (d) A section of the cortex from a control monkey showed no cell stained positive for hyperphosphorylated tau. $\times 400$.

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; BioSourceTM, 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 $1500 \mu\text{m}^2$ 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 \pm 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 $14.6 \pm 6.4\%$ (mean \pm SD) of TUNEL positive cells were hyperphosphorylated tau positive. Thus, not all the cells

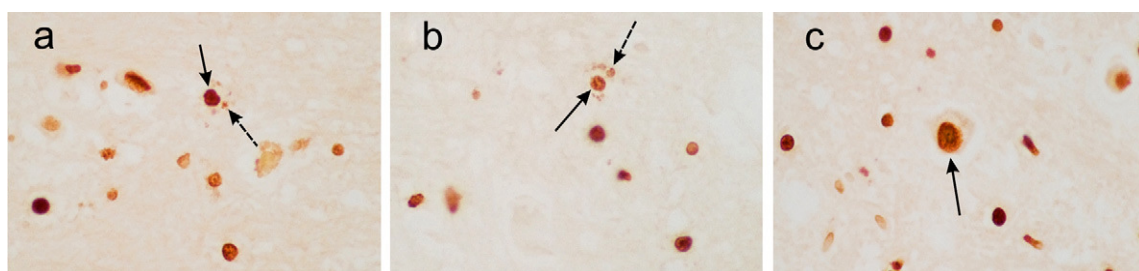


Fig. 3. Double staining of hyperphosphorylated tau and TUNEL was performed with sections from the prefrontal cortex of monkeys after administration of ketamine for 6 months (a and b) and control monkeys receiving no ketamine (c). These sections show cells positive for the double staining of hyperphosphorylated 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. $\times 200$. (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. $\times 200$.

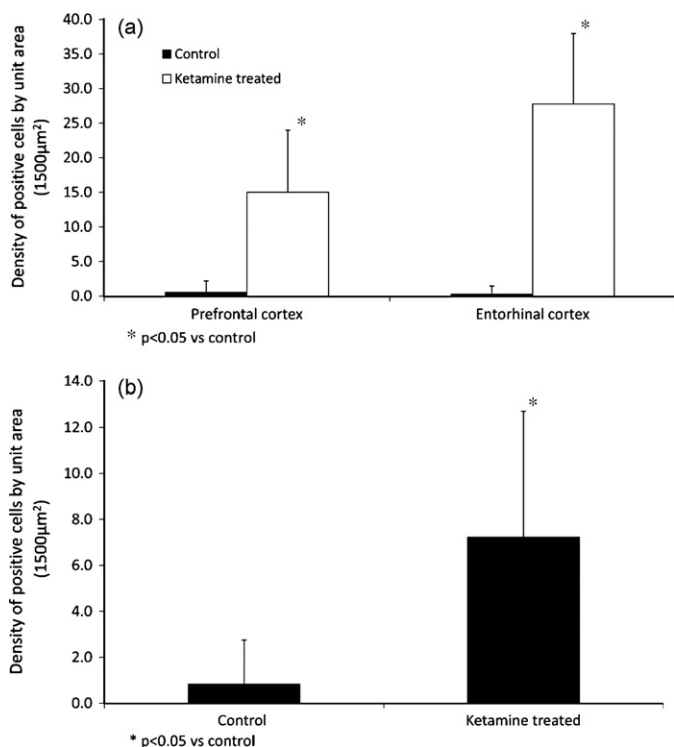


Fig. 4. Densities of the hyperphosphorylated tau positive cells were evaluated using morphometric method with sections from the prefrontal and entorhinal cortices of monkeys after administration of ketamine for 6 months and control monkeys receiving no ketamine. (a) Histogram shows the densities of the 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 tan-

gles (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 immunohistochemistry, 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 preneurofibrillary 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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Attachment 4

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Permanent deficits in brain functions caused by long-term ketamine treatment in mice

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Permanent deficits in brain functions caused by long-term ketamine treatment in mice

L Sun^{1,2}, WP Lam², YW Wong², LH Lam², HC Tang², MS Wai², YT Mak², F Pan¹ and DT Yew²

Abstract

Ketamine, an injectable anesthetic, is also a popular recreational drug used by young adults worldwide. Ketamine is a non-competitive antagonist of *N*-methyl-D-aspartate receptor, which plays important roles in synaptic plasticity and neuronal learning. Most previous studies have examined the immediate and short-term effects of ketamine, which include learning and cognitive deficits plus impairment of working memory, whereas little is known about the long-term effects of repeated ketamine injections of common or usual recreational doses. Therefore, we aimed to evaluate the deficits in brain functions with behavioral tests, including wire hang, hot plate and water maze tests, plus examine prefrontal cortex apoptotic markers, including Bax, Bcl-2 and caspase-3, in mice treated with 6 months of daily ketamine administration. In our study, following 6 months of ketamine injection, mice showed significant deterioration in neuromuscular strength and nociception 4 hours post-dose, but learning and working memory were not affected nor was there significant apoptosis in the prefrontal cortex. Our research revealed the important clinical finding that long-term ketamine abuse with usual recreational doses can detrimentally affect neuromuscular strength and nociception as part of measurable, stable and persistent deficits in brain function.

Keywords

noncompetitive NMDA antagonist, prefrontal cortex, behavioral test, apoptosis, brain deficits

Introduction

Ketamine was first synthesized in 1962 as a short-acting anesthetic in human and veterinary medicine.^{1,2} It produces a unique anesthetic state called dissociative anesthesia. When ketamine is used in smaller doses, it can produce a psychedelic experience of incredible intensity.^{2,3} Because of these side reactions, it has become increasingly popular in recent years as a recreational drug in night-clubs.^{4–6} Recent surveys suggested that 10% of drug users in the United Kingdom use ketamine on a regular basis in night-clubs.⁷ The percentage of ketamine users has rapidly increased 4-fold between 1997 and 2001 among night-club patrons, particularly among young people. Currently, Ketamine is a controlled (Schedule III) substance in the United States (<http://www.anesthesia.com.mx/articulo/keta.html>) and other countries (<http://laws.justice.gc.ca/eng/C-38.8/20100120/index.html?rp2=HOME&rp3=SI&rp1=ketamine&rp4=all&rp9=cs&rp10=L&rp13=50>).

Ketamine is a non-competitive antagonist of *N*-methyl-D-aspartate (NMDA) receptor, which is considered to be one of the vitally important receptors in mechanisms of synaptic plasticity and neuronal learning, such as long-term potentiation and long-term depression.⁸ Age-related decrease in NMDA receptor function is also related to memory decline in the elderly.⁹ Ketamine-mediated hypofunction of NMDA-receptor results in a combination of enhancement of thalamo-limbic systems and inhibition of thalamo-neocortical systems.^{1,10} NMDA receptor

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antagonist with repetitive ketamine administration has been shown to reduce the function of the prefrontal dopaminergic system in rats, which plays a critical role in sustaining working memory tasks and executive functions.¹¹ Most previous studies have examined the immediate and short-term effects of ketamine in healthy individuals, resulting in impairment of working memory, learning and cognition,¹² whereas little is known about the long-term effects of repeated ketamine administration on the brain. Recently, some studies performed with rodents have shown that prolonged exposure to ketamine increased cell death in brain areas including the prefrontal cortex of the central nervous system (CNS).^{11,13} These findings suggested that chronic exposure to ketamine in the CNS might interfere with learning and memory through a neurotoxic effect related to activation of apoptotic pathways. Prefrontal cortex is implicated in a variety of cognitive and executive processes, including working memory, decision-making, inhibitory response control, attentional set shifting and temporal integration of voluntary behavior constituting the so-called 'pleasure centers.'¹⁴⁻¹⁹ All of these abilities depend on proper prefrontal cortex neuronal network connections, which are highly sensitive to their neurochemical environment. Disruption to this neurochemical environment would obviously damage the functions of prefrontal cortex, but the effect of long-term ketamine (as an NMDA receptor antagonist) on prefrontal cortex is still largely unknown.²⁰

In view of the importance of prefrontal cortex, our study explored the effects of long-term ketamine administration on functional changes in mice prefrontal cortex, including sensorimotor and cognitive performance using behavioral tests, as well as the changes in expressions of proteins regulating the extrinsic (caspase-3) and the mitochondrial (Bcl-2 and Bax) apoptotic pathways. We hypothesized that long-term ketamine use could permanently damage the sensorimotor processes of prefrontal cortex, therefore we investigated whether the damage was related to apoptosis or not in our study of the long-term effects of repeated ketamine injection of usual recreational dose on the mouse brain.

Materials and methods

Experimental animals, groups and treatments

Four-week old male ICR (Institute of Cancer Research) mice bred in the Chinese University of Hong Kong (CUHK) animal facility were used

because rodents are extensively used in studies of ketamine effects^{11,21} and the appropriate facilities were available for the behavioral tests. Mice were housed 10 per cage with water and food pellets available *ad libitum* in a room kept at $22 \pm 1^\circ\text{C}$ and exposed to 12:12 hour (h) light/dark cycles. This study was approved by the Institutional Animal Care Committee of CUHK. For evaluation of the behavioral and neurochemical effects induced by chronic ketamine administration, 90 mice were randomly divided into 3 groups receiving treatment doses of 1-, 3- or 6-month of daily intra-peritoneal injections of ketamine at 30 mg/kg (Alfasan ketamine 10% injectables, Holland) or placebo of equal amounts of saline. We used a sub-anesthetic dose of 30 mg/kg of ketamine, which is regarded as the recreational dose for rodents with a LD_{50} of 600 mg/kg⁴ and is consistent with dosages reported in the literature.^{21,22}

In each group of 30 mice, 20 were injected with ketamine and 10 with saline as controls. The weights of mice were recorded every week for adjustment of ketamine injection and monitoring of the well-being of the animals. At the end of 1, 3 or 6 months, all mice were used for the behavioral tests and then sacrificed for subsequent analysis of brain samples. In each of the 1-, 3- or 6-month groups, 3 control and 6 ketamine-treated mice were used for terminal dUTP nick end labeling (TUNEL) staining, and 7 control and 14 ketamine-treated mice were used for prefrontal cortex samples for Western blot assay.

Behavioral studies

We performed the following three behavioral tests 4 h after the administration of ketamine or saline on the final day of each treatment or control group, with the water maze test requiring training and testing on consecutive 3 days immediately before the final day. Because of the short half-life, ketamine and its metabolites are cleared by urinary excretion 4 h after the dose.^{1,2} The aim was to study effects due to possible stable and persistent damages to the brain caused by ketamine rather than its immediate effect after ketamine injection.

Wire hang test. Neuromuscular strength was evaluated by placing the animals on a wire cage lid.^{23,24} The lid was then turned upside down approximately 50 centimeter (cm) above the surface of a soft bedding material and regularly waved (4 strokes per second (sec)) in the air so that the mouse had to grip the wire to

avoid falling off. The latency time in sec to fall from the lid onto the bedding was recorded, with 60 sec as the cut-off time.

Hot plate test. Sensitivity to a painful stimulus was evaluated by the hot plate test, one of the most commonly used tests for determining the analgesic effects of experimental drugs in rodents.²⁵ The mouse was placed on the flat aluminum surface of the hot plate maintained at $50 \pm 1^\circ\text{C}$. Ambulation of the mouse on the plate was limited by a 16 cm high non-opaque Plexiglas frame of $11 \times 9 \text{ cm}^2$ area. The frame enclosed the surface so that the mouse could not jump out. The numbers of times the mouse licked its fore and hind paws, fluttered, shook or jumped up within 25 sec were recorded. We have observed that these are the four types of movements the mice would do in response to placement on a hot plate. After the trial, the animal was removed from the hot plate immediately to prevent tissue damage.

Water maze test. Swim tasks are used to measure spatial navigation learning and memory in mice.²⁶⁻²⁸ A black circular tank of 150 cm diameter was filled to a depth of 25 cm of water and maintained at $23 \pm 1^\circ\text{C}$. The water was changed for each day's experiment. The animals were to learn the best swimming strategy to find the visible white platform exposed about 1 cm above the surface of the water in the center of the circular tank. The position of the platform remained the same throughout the whole experiment. Several prominent fluorescent cues were placed around the tank at four fixed points, with one in each quadrant, including a T-shape (the starting point where the mouse was put into the tank), a Y-shape, a moon and a triangle. During the experiment, these cues were intended to facilitate mouse spatial orientation in the dark environment when the tank was closed with a long curtain hung over it from the ceiling above. Each trial began with the mouse being placed at the starting point along the edge of the pool facing the wall to avoid seeing the platform. The escape latency time for the mouse to locate and climb onto the platform was recorded. If a mouse did not find the platform after 90 sec, it would be gently guided to the platform and allowed to stay on the platform for 10 sec to recognize the location. The mouse would have three such consecutive trials each day with 30 sec of rest time between trials. Escape latency time to reach the visible platform or 90 sec if failed was recorded. All groups of mice were trained for

3 days with three trials per day, starting 4 h after ketamine or saline injection, and were then tested on the day after the training, which was the final day of treatment/control in each group. We used this water maze test to test the working memory and visual acuity.^{29,30}

TUNEL assay

The DNA fragmentation indicative of apoptosis was examined using TUNEL^{31,32} that could detect early-stage apoptosis and examine the topographic distribution of apoptotic cells. Mice were sacrificed for TUNEL as we previously reported.³³ Mice were anesthetized with 0.5 mL of 7.0% chloral hydrate (1 mg/kg), and then exsanguinations were performed with 50 mL of saline through the cardiac apex of the left ventricle till the liver turned pale pink. Perfusion was continued with 50 mL of 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS; pH 7.4) through the same position. The PFA solution could keep the tissue's shape from changing before embedding; then, various tissue samples were removed and immersed into 4% PFA for 24 h for further fixation. The tissue samples were then dehydrated and embedded in paraffin wax. Blocks were cut into 5 μm sections with a microtome.

TUNEL was performed using ApopTag[®] Peroxidase In Situ Apoptosis Detection Kit (Millipore Corporation, Billerica, Massachusetts, USA) according to the manufacturer's instruction including both positive and negative controls as we previously reported.³³ Sections were first deparaffinized, rehydrated and treated with proteinase K (20 $\mu\text{g}/\text{mL}$). After quenching with 3.0% hydrogen peroxide, the sections were treated with biotin-deoxyuridine triphosphate in the working solution of deoxynucleotidyl transferase (TdT) for 1 h at 37°C . The reaction was stopped with the stop/wash buffer. To detect the binding of dioxigenin-11-dUTP, sections were treated with the anti-digoxigenin conjugate for 30 min. Finally, after visualization with dimethylaminoazobenzene, apoptotic cells including the cellular DNA fragmentation were photographed with a digital camera (Zeiss Axiocam MRc5). In each assay, negative controls were included using the same incubation procedure but omitting the TdT, while positive controls were prepared by incubating the pretreated sections with DNase I (Sigma, DN 25, 1.0 $\mu\text{g}/\text{mL}$) that hydrolyzed DNA preferentially at sites adjacent to pyrimidine nucleotides. We counted TUNEL-positive cells from 10 sections of each mouse prefrontal cortex sample. Sections were selected from

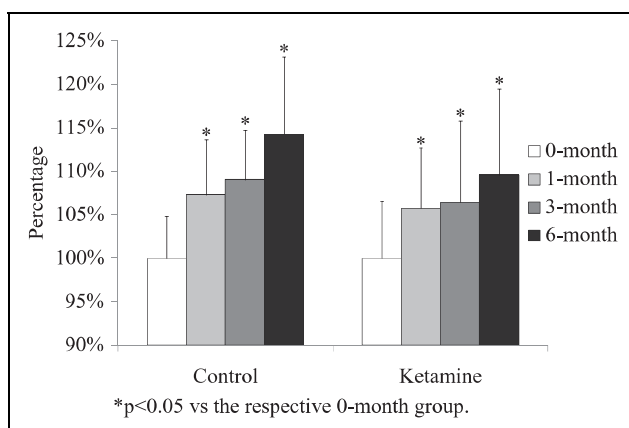


Figure 1. Body weights of different mouse groups. The body weights of mice were recorded weekly. The final body weights were expressed as the percentage of the body weights at 0-month (taken as 100%) when the experiment started. All ketamine-treated groups showed less gains in body weights than the respective control groups; however, the differences were not statistically significant. Results were group means \pm standard deviations.

comparable layers of prefrontal cortex. A total area of at least 15 mm² was counted for each sample. Results were expressed in number of TUNEL positive cells per mm².

Western blot assay

Western blot assay was modified as we previously published.³⁴ For Western blot assay, mice were sacrificed as we previously reported.³⁵ Briefly, mice were killed by cervical dislocation. The prefrontal cortex was immediately removed and snap-frozen in liquid nitrogen and stored at -80°C until processed for Western blot assay. Brain tissue samples were homogenized in 0.5 mL of lysis buffer, 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 0.1 mg/mL phenylmethylsulphonyl fluoride, 1 mg/mL leupeptin, 1 mg/mL pepstatin A and 5 mg/mL aprotinin. The homogenate was centrifuged at 14,000 *g* for 30 min at 4°C and the supernatant obtained was either immediately used or stored at -80°C until use. The protein concentration of the extract was determined by the Bio-Rad DC protein assay (500-0111, Bio-Rad Laboratories, Hercules, California).

Equal amounts of protein (100 μg) samples were boiled in $2 \times$ Laemmli loading buffer (final concentrations: 0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue)³⁶ for 6 min before loading on 12%

SDS-polyacrylamide gel. After electrophoresis and semi-dry transfer, the nitrocellulose membrane was blocked (5% non-fat dry milk, 0.05% Tween-20 in PBS) and then incubated overnight at 4°C with either of the following anti-sera (dilution): actin (1:20,000) (MAB1501, Millipore Corp.), Bax (1:1,000; sc-526, Santa Cruz Biotechnology Inc., Santa Cruz, California), or Bcl-2 (1:1,000; sc-783, Santa Cruz Biotechnology Inc.), caspase-3 (1:1,000) (9662, Cell Signaling Technology Inc., Danvers, Massachusetts). The following day, after three sequential 5 min washes with 0.05% Tween-20 in PBS (PBST), the membrane was incubated with the appropriate IRDYE[®] FC conjugated secondary antibody 1:10000 for 1 h (IRDYE[®] 700DX anti-rabbit FC 605-430-003 for Bax, Bcl-2 and caspase-3; IRDYE[®] 800CW anti-mouse FC 611-131-003 for actin; LI-COR Biosciences, Lincoln, Nebraska 68504-0425). After washing three times for 10 min with PBST, the bound antibodies were then visualized and recorded using the ODYSSEY Infrared Imaging System (LI-COR Biosciences). Band density value of individual proteins was normalized to that of the actin of the same sample. Bax to Bcl-2 ratio (Bax/Bcl-2) was calculated from the Bax and Bcl-2 results of the same sample.

Statistical method

All data were expressed as mean \pm standard deviation (SD) or median (10th to 90th percentile) for the groups. One-month, 3-month and 6-month ketamine groups were compared to their respective control groups unless specified. The Wilcoxon or Student-*t* (two-tailed) were used when appropriate for non-normally or normally distributed datasets, respectively. Specifically, Student-*t* test was used for comparison of body weight and Western blot results between groups, while Wilcoxon test was used for comparisons of behavioral test and TUNEL results. The difference between groups was considered statistically significant when *p*-value was less than 0.05.

Results

Body weight of mice

Setting body weights at 0-month as the baseline of 100%, the percentage increases in body weight for both the ketamine and control groups at 1, 3 and 6 months were significant ($p < 0.05$; Figure 1). Although the percentage increases were less among all ketamine groups than the percentage increases of

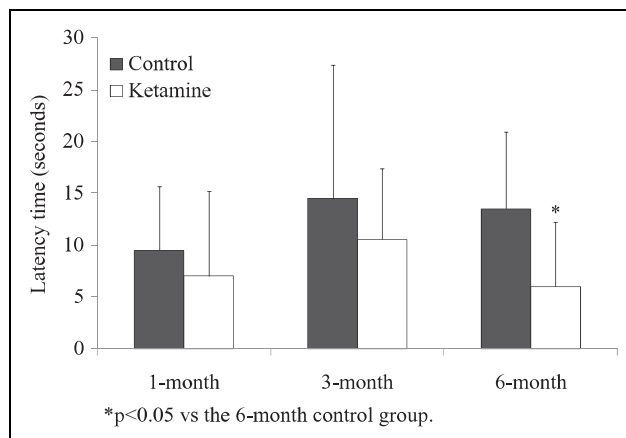


Figure 2. Wire hang test results of different mouse groups. Wire hang test was performed 4 hours after the last ketamine or saline injection to the mouse. The 6-month ketamine group showed significantly shorter latency time ($p < 0.05$) to fall from the wire than the 6-month control group. Results were group medians + 90th percentiles.

the respective control groups, for example, the percentage increase was $109.6\% \pm 9.9\%$ (mean \pm SD) in the 6-month ketamine group versus the $114.3\% \pm 8.9\%$ in the 6-month control group, the differences in percentage increases between ketamine groups and their respective control groups were not statistically significant.

Behavioral studies

All behavioral tests were performed 4 h after the last dose of ketamine, therefore, the results reflected not the immediate effects but the stable and persistent effects of long-term ketamine.

Wire hang test. The latency times (in sec) of falling from the wire for 1-, 3- and 6-months ketamine groups were consistently less than the latency times of their respective control groups (Figure 2). After 6 months' ketamine administration, the latency time of the 6-month ketamine group (6.0, 2.9–12.2 [median, 10th–90th percentile]) was significantly less ($p < 0.05$) than the 6-month control group (13.5, 4.8–20.9).

Hot plate test

For the 6-month ketamine group, the total number of movements of the mice (14.0, 9.9–18.2 [median, 10th–90th percentile]) on the hotplate was significantly less ($p < 0.05$) than the number of the control group (19.5, 13.8–25.2; Figure 3). This showed that

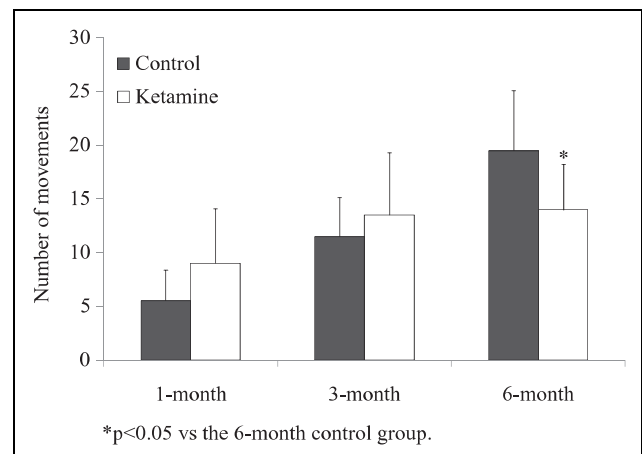


Figure 3. Hot plate test results of different mouse groups. Hot plate test was performed 4 hours after the last ketamine or saline injection to the mouse. The 6-month ketamine group showed significantly less in the number of movements ($p < 0.05$) on the hot plate ($50 \pm 1^\circ\text{C}$) than the 6-month control group. Results were group medians + 90th percentiles.

after 6 months of ketamine injection, the sensory perception of heat was damaged. However, there were no significant differences or consistent trends of change in ketamine-treated mice for 1 and 3 months as compared to their respective controls.

Water maze test. The escape latency time (in sec) for the mice to locate and climb onto the platform showed no statistically significant differences between the 1-, 3- and 6-month ketamine groups as compared to their respective control groups (Figure 4), although the ketamine-treated mice consistently used more time to climb onto the platform in all groups.

TUNEL and Western blot assay

There were no statistically significant differences in the TUNEL-positive cell counts in the prefrontal cortex between the ketamine groups of 1-, 3- and 6-months and their respective control groups (Figure 5). Although Western blot results showed consistently that all 1-, 3- and 6-month ketamine groups had higher Bax, lower Bcl-2, higher Bax/Bcl-2 and higher caspase-3 (to actin ratio) levels in prefrontal cortex than their respective control groups (Table 1), these differences were not statistically significant. Representative images of Western blot results are shown in Figure 6. The antibody for caspase-3 we used detects both the full length (35 kDa) and the cleaved (17 kDa) forms. The cleaved caspase-3, i.e. the active form, was not detected, indicating that

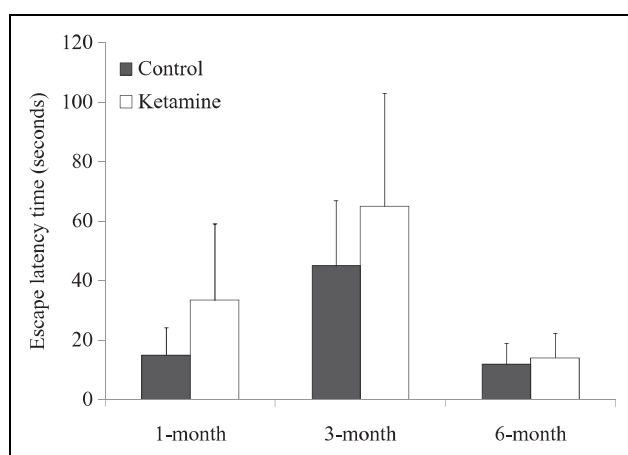


Figure 4. Water maze test results of different mouse groups. After training for 3 days, water maze test was performed 4 hours after the last ketamine or saline injection to the mouse. The escape latency time of all ketamine-treated groups were not significantly different from their respective control groups. Results were group medians + 90th percentiles.

apoptosis did not occur in the prefrontal cortex samples we took.

Discussion

Although there were no significant differences between the gains in body weights in ketamine groups as compared to the control groups, the consistently lower gain in body weight with ketamine treatment (Figure 1) may possibly indicate the presence of somatic effect associated with ketamine. The CNS plays important roles in the control of appetite and maintenance of body weight.^{37,38} With long-term ketamine administration, disruption of brain function is not unexpected, with possible effects on the hormonal and neuronal signals for appetite and body weight control in young animals. Indeed, in another series of experiments (unpublished data) in our laboratory after 6 months ketamine administration to adolescent monkeys, functional magnetic resonance (fMRI) imaging revealed that the ventral tegmental area (VTA) was damaged; VTA is involved in the reward circuitry for food intake.³⁷ This might occur in mice with long-term ketamine administration, affecting appetite and resulting in reduced body weight gain over time as compared to control groups. Further detailed studies are necessary to elucidate this possible somatic effect of ketamine using different dosages and examining the mechanism of action in more depth.

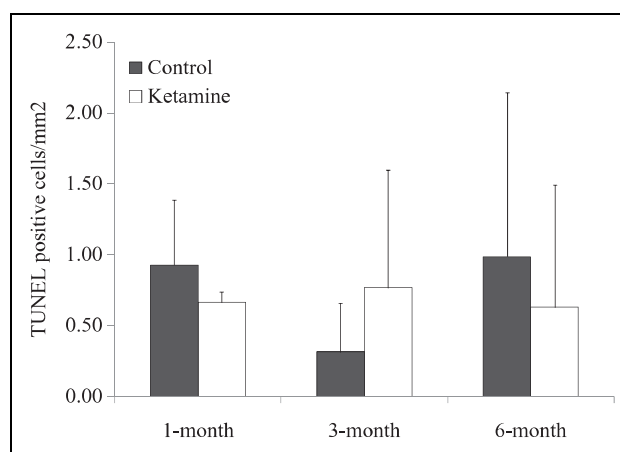


Figure 5. Terminal dUTP nick end labeling (TUNEL) results of prefrontal cortex samples from different mouse groups. TUNEL-positive cell counts in the prefrontal cortex samples were determined. The cell counts were not significantly different between all ketamine-treated groups and their respective control groups. Results were group medians + 90th percentiles.

Although the somatic effect of ketamine was not truly significant in our study, the 6 months of treatment with ketamine showed statistically significant and permanent impairments in neuromuscular strength and nociception by the wire hang test (Figure 2) and hot plate test (Figure 3), respectively. It is possible that significant muscle weakness echoed and manifested the effects of less body weight gain in mice treated with ketamine for 6 months; less gain in body weight of 6-month ketamine treatment group compared to controls might indicate less muscle mass and strength leading to significantly poor performance in the wire hang and hot plate tests. In addition, it is possible that the motor nerve conduction velocity was reduced or damaged³⁹ by ketamine after 6 months' administration, potentially leading to impaired physical abilities. Further studies are necessary to elucidate the relationship and rationale between body weight, behavioral tests and physical performance impairments following long-term ketamine use.

Learning and working memory were not significantly affected, as in the water maze test results (Figure 4) for all 1-, 3- and 6-month groups, although the ketamine treatment groups consistently showed longer but not significant escape latency times than the respective control groups. Results of both the TUNEL and apoptotic markers (including Bax, Bcl-2 and caspase-3) showed no differences between the ketamine and control groups in the prefrontal cortex. Apparently, the

Table 1. Western blot results of prefrontal cortex samples from different mouse groups. Bax, Bcl-2 and caspase-3 (to actin ratio) were performed and Bax/Bcl-2 was calculated from the Bax and Bcl-2 results of the same sample

| Protein | 1 Month | | 3 Months | | 6 Months | |
|-----------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| | Control | Ketamine | Control | Ketamine | Control | Ketamine |
| Bax | 0.0027 \pm 0.0010 | 0.0042 \pm 0.0026 | 0.0035 \pm 0.0027 | 0.0040 \pm 0.0026 | 0.0026 \pm 0.0011 | 0.0031 \pm 0.0018 |
| Bcl-2 | 0.040 \pm 0.025 | 0.024 \pm 0.018 | 0.056 \pm 0.031 | 0.032 \pm 0.026 | 0.043 \pm 0.029 | 0.030 \pm 0.025 |
| Caspase-3 | 0.053 \pm 0.026 | 0.075 \pm 0.036 | 0.040 \pm 0.021 | 0.046 \pm 0.018 | 0.031 \pm 0.012 | 0.045 \pm 0.022 |

^a Please note the consistent changes of higher Bax, lower Bcl-2, higher Bax/Bcl-2 and higher caspase-3 in ketamine-treated groups as compared with their respective control groups, suggesting a possible tendency of more apoptosis associated with ketamine. However, the differences were not statistically significant. Results were group means \pm standard deviations (SD).

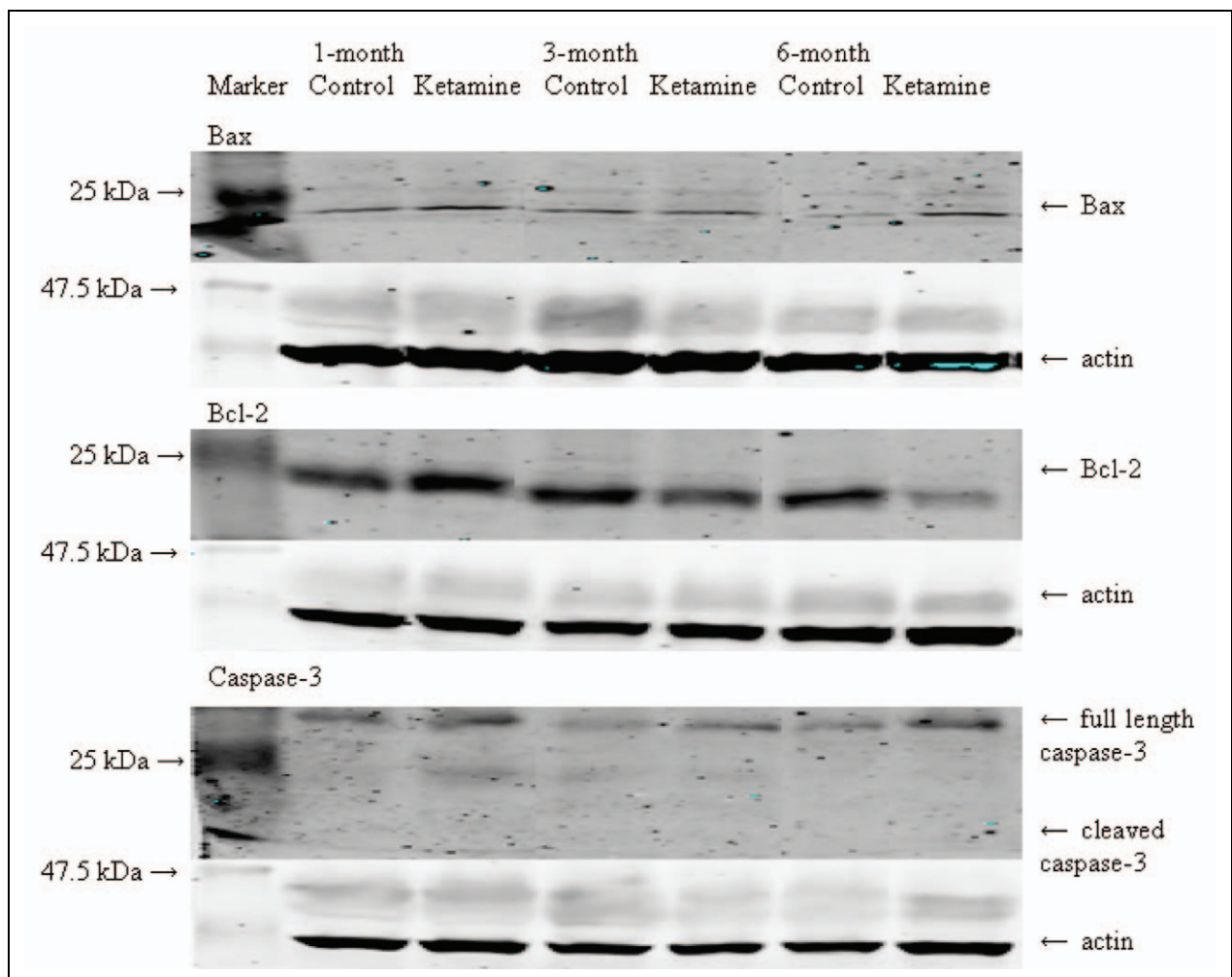


Figure 6. Representative images of Western blot results for Bax, Bcl-2 and caspase-3 of prefrontal cortex samples from control and ketamine mice. Please note the cleaved caspase-3, i.e. the active form (17 kDa), was not detected. Only the full length caspase-3 (35 kDa) was visualized.

function of prefrontal cortex with respect to learning and working memory did not appear permanently damaged, bearing in mind that we tested the mice 4 h after ketamine injection. However, other studies have shown significantly more apoptosis occurring in different brain

regions, including the prefrontal cortex, in ketamine-administrated animals as compared to controls.^{11,13,21,22} Water maze is generally considered to be a test of spatial learning and working memory,⁴⁰ but this might not always be the case because the performance of the mice

could depend on other factors such as the use of different search strategies,⁴¹ fatigue⁴² and hypothermia.⁴³ Indeed, it has been shown that impairments in working memory induced by ketamine were not attributable to dysfunction of motivational, motor, short-term or spatial memory processes.²¹ Probably, further validation of the water maze test should be elucidated for more precise examination of the learning and working memory function of mice.²⁹

We deliberately performed the behavioral tests 4 h after ketamine injection because we were looking for stable and persistent damages in the brain caused by long-term ketamine rather than its immediate effects on the brain function of that particular daily injection. Ketamine is known to be short-acting and short-lived,^{1,2} with no obvious outcomes even in cases of overdose.⁴ Most studies have examined the effects immediately after its injection.^{21,22,44,45} In this study, we used a sub-anesthetic dose of 30 mg/kg, which is regarded as the recreational dose for rodents.⁴ In a recent unpublished pilot study, we experimented with different doses of ketamine at 30, 60 and 90 mg/kg. At 60 and 90 mg/kg of ketamine, there were mice fatalities after 1 month of ketamine injection. Also, ketamine was given only once daily to the mice, while in young human ketamine abusers, use of the drug would likely not be limited to only one time per day. Therefore, the significant and permanent damaging effects of ketamine on neuromuscular strength, nociception and possibly on body weight could be even more serious in human youngsters. This is a new finding and an important message illustrating that even at a reasonable recreational dose (one dose per day), the long-term use of ketamine is harmful to the brain and its functions, with definite permanent damage. This is a convincing message that could be used in anti-ketamine campaigns.

Using TUNEL and Western blot testing, we examined the prefrontal cortex for the presence of apoptotic lesions possibly caused by ketamine. There were no significant differences for all TUNEL and Western blot results between the ketamine and control groups; however, it may be worthwhile to note the consistent trends of higher Bax, lower Bcl-2, higher Bax/Bcl-2 and higher caspase-3 (to actin ratio; Table 1) showing the tendency of more apoptosis possibly associated with ketamine treatment. However, this does not exclude the possibility that the functions of prefrontal cortex may be affected without significant cellular damage. Indeed, it has been reported that in the rat, ketamine inhibited a subclass of fast spiking

interneurons in prefrontal cortex that regulated the motivation of various behaviors.²² Hypofunction of the prefrontal cortex as induced by drugs could cause dysfunction leading to altered decision making by neglecting negative future consequences for immediate reward.⁴⁶ Another study showed that even without detectable cell death, ketamine produced aberrant diffuse network noise in multiple cortical and subcortical structures, including the prefrontal cortex, which might cause dysfunction of brain operations, including impairments in cognition and sensorimotor integration.⁴⁷ We have also made similar findings: a previous study by our group³³ reported significant increase of hyperphosphorylated tau in Layer I of the prefrontal cortex of mice treated with ketamine for 6 months. Layer I contains mainly communicating fibers; the presence of hyperphosphorylated tau in fibers would block the transmission through the fibers between cortical regions causing disruption of prefrontal cortex functions. Neuronal cell death may only occur secondarily with the eventual blockage of fiber transmission. Our previous results³³ also showed species differences: in monkey treated with ketamine (1 mg/kg) for 6 months, significant increases of hyperphosphorylated tau were found in neurons of the deeper layers of the prefrontal cortex. Such presence of hyperphosphorylated tau might signify immediate degeneration or death of the neurons themselves rather than the possible secondary cell damage or death as in mouse.

In our other series of experiments with long-term ketamine in mice (unpublished results), we found there was significant apoptosis in the cerebellum, suggesting there might be regional differences in response between different CNS regions in the mouse. In the present study, our results showed ketamine did not cause significant cellular damage or apoptosis in the prefrontal cortex, therefore further studies should examine brain function by *in vivo* or microdialysis methods,^{22,48} comparing other regions of the brain outside the prefrontal cortex such as cerebellum. For instance, one direction of study on mechanisms of motor weakness due to ketamine could examine the known increase in glutamate release in nucleus accumbens in rat that leads to disruption of motor behavior and latent inhibition.⁴⁴

In conclusion, our research showed for the first time an important finding of ketamine use that 6 months of the usual recreational dose created stable and persistent damage in brain function. Despite no significant apoptosis detected in prefrontal cortex,

young mice showed significant deterioration in neuromuscular strength and nociception following chronic use of ketamine. Further research is needed to determine the extent and mechanisms of potentially serious brain damage caused by this recreational drug.

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Attachment 5

索K唔會high，
只會令你down得快！

記憶力衰退
精神錯亂
幻聽
胡言亂語
妄想
抑鬱
動作不協調
腎積水
胃衰竭
血尿
小便赤痛
用成人紙尿片

大腦功能缺損
幻覺
情緒失控
疑神疑鬼
焦慮
驚恐
胃痛
手震
膽管脹大
膀胱縮小
頻尿
尿液倒流

索K致命

氯胺酮（K仔）是這五年間吸食量最多的危害精神毒品，屬於問題嚴重，香港政府已於二〇〇八年開始，直接定性將K仔類毒品為「極毒」，顯示K仔正逐步殘害青少年的身心，逐步殘害香港社會。

醫院管理局新界東聯網總監馮康醫生，聯同一群誠意為義的社工及醫護人員，以他們接觸過K仔青少年的各式個案，研究和經驗，寫成《香港濫K》此書，正面顯示問題的嚴重性，但願香港早日遠離K害。



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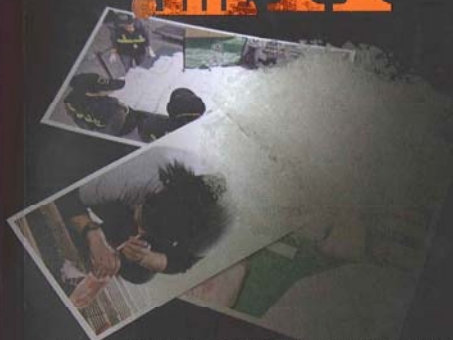
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編著

馮康醫生（醫院管理局新界東聯網總監）

贊助 北區醫院慈善信託基金



Attachment 6

Methods: Nine single-nucleotide polymorphisms (SNPs) of the NET gene ([rs7194148, rs28386840, rs2242446] in promoter region, [rs1532701, rs40434, rs13333066] in intron 1, [rs187714] in intron 3, [rs5569] in exon 9, and [rs42460] in exon 14) were analyzed in total 965 Han Chinese subjects. The Chinese version Tridimensional Personality Questionnaire was introduced to assess personality traits in HD patients and examined the association between personality traits and these SNPs of NET gene.

Results: No statistically significant differences in genotype frequencies of NET polymorphisms between HD patients and controls, although, individuals with A allele of rs1532701 and T allele of rs13333066 have significant protective effect in the development of HD after multiple logistic regression analysis. Moreover, the AATA haplotype frequency in block (rs1532701-rs40434-rs13333066-rs187714) has a significant association between HD patients and controls. However, the nine polymorphisms of NET gene did not influence novelty seeking and harm avoidance scores in HD patients.

Conclusion: The AATA haplotype (rs1532701-rs40434-rs13333066-rs187714) of NET gene possibly plays a protective factor in the development of HD, but NET gene is not associated with the specific personality trait in HD patients.

Policy of full disclosure: None.

P-01.046 Chronic ketamine abuse causes dysfunctions of different brain areas relevant to neurodevelopmental psychiatric disorders: Evidence from fMRI in a primate model

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Objective: Ketamine is a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, and illegal use of it as a recreational drug among adolescence and young adult is rapidly growing. Many studies have showed that prefrontal dopaminergic system is particularly vulnerable to the toxic effects on cognitive functions. This suggests that chronic ketamine abuse in young people may cause a severe disruption of different brain areas relevant to psychiatric disorders.

Methods: We established a chronic ketamine abuse model using the adolescent cynomolgus monkeys administrated with ketamine once a day (1 mg/kg, i.v.) for 6 months. Blood oxygenation level dependent (BOLD) contrast images were generated through stimulating the function of somatosensory area using functional magnetic resonance imaging (fMRI). Parallel and successive behavioral were observed.

Results: Chronic ketamine abuse in younger monkeys caused obvious deficits in the ventral tegmental area (VTA)/substantia nigra (SN), parietal cortex, and cingulate cortex. Besides, some increased activities were observed in striatum (lentiform nucleus, LN), fusiform gyrus (FG) and entorhinal cortex (Ent) on the right side of the brain in the chronic ketamine administrated monkeys. Behaviour results showed significant differences of the movement in both control and ketamine groups with general and consistent decreased trend with time periods of ketamine administration.

Conclusion: Dysfunction of a projection from Ent to LN could play a role in ketamine abuse or induce epilepsy. We also found that deficit of cortical visual area in ketamine abuse model might cause a "positive schizophrenic syndrome". Moreover, hypofunction of mesocortical dopamine pathway may induce a negative symptom in psychosis, or attention deficit disorder (ADD).

Policy of full disclosure: None.

P-02. Animal Models

P-02.001 Alzheimer's disease drug galantamine, but not donepezil, improves social isolation rearing-induced deficits in prepulse inhibition via muscarinic acetylcholine receptors

Y. Ago¹, K. Koda¹, K. Yano¹, K. Takuma¹, T. Matsuda¹. ¹Osaka University, Suita, Japan

Objective: Galantamine and donepezil are used in the treatment of Alzheimer's disease. Galantamine is a rather weak acetylcholine (ACh) esterase inhibitor, but it has additional allosteric potentiating effects at nicotinic ACh receptors (nAChRs). Clinical studies show that galantamine improves negative and cognitive symptoms in schizophrenia, while donepezil does not. In this circumstance, we have found that both galantamine and donepezil improved deficits in prepulse inhibition (PPI), sensory information-processing deficits, in apomorphine-treated mice but only galantamine improved PPI deficits in socially isolation-reared mice (Psychopharmacology, 2008). This paper investigates the mechanism of the beneficial effect of galantamine in a model of social isolation rearing-induced PPI deficits.

Methods: Three-week-old male ddY mice were housed either in groups or isolated in cages of the same size for more than 6 weeks. PPI, microdialysis and Ca²⁺ imaging experiments were done as previously reported (Br. J. Pharmacol., 2005; 2009).

Results: Galantamine-induced improvements of PPI deficits were blocked by the muscarinic ACh receptor (mAChR) antagonists scopolamine and telenzepine (preferential for M1 subtype), but not by the nAChR antagonists. Like galantamine, the mAChR agonists oxotremorine and N-desmethyloclozapine (selective for M1 subtype) improved isolation rearing-induced PPI deficits. Although M1-mAChR expression in the prefrontal cortex and hippocampus was not altered by isolation rearing, N-desmethyloclozapine-induced increases in prefrontal dopamine levels was reduced in isolation-reared mice, indicating the reduction of M1-mAChR function. With regard to the mechanisms of galantamine-induced activation of mAChRs, we found that galantamine, like donepezil, increased extracellular ACh levels in the prefrontal cortex. However, donepezil inhibited carbachol-induced Ca²⁺ signal, which was blocked by telenzepine, in SH-SY5Y cells, whereas galantamine did not. This suggests that donepezil has antagonistic activity for M1-mAChRs.

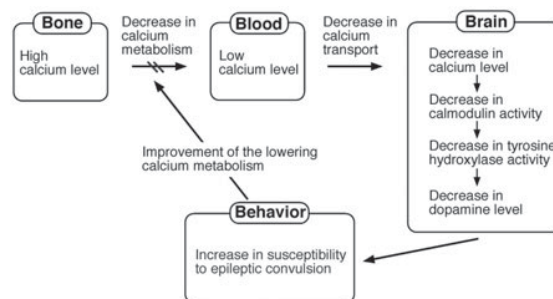
Conclusion: Galantamine improves isolation rearing-induced PPI deficits via mAChRs, and the different effects of galantamine and donepezil on M1-mAChRs may explain the difference in the clinical effects of these drugs.

Policy of full disclosure: None.

P-02.002 Disorders of dopaminergic functions in epileptic mice

K. Akiyama¹, D. Sutoo¹. ¹University of Tsukuba, Japan

Objective: EL mouse is an inbred strain, derived from ddY mice, with convulsive tendencies. Violent tonic-clonic convulsions occur in adult EL mice as a result of postural stimulation. Neurochemical disorders in EL mice were investigated as a possible model for seizure mechanism.



Methods: Calcium levels were measured biochemically. Dopamine and its related substances were analyzed quantitative

Attachment 7



EAST ASIAN ARCHIVES OF PSYCHIATRY 东亚精神科学志

The Second Joint International Conference of
the Hong Kong College of Psychiatrists and
the Royal College of Psychiatrists (UK)

Brain, Behaviour and Mind 2010

**Advancing Psychiatric Care in the East:
Moving On – From Science to Service**

11-13 December 2010

Official Journal of the
Hong Kong
College of Psychiatrists
香港精神科医学院期刊



结果：共41名患者成功于每个时间点完成所有评估。临床和认知参数从T1到T3都有所改善。认知功能的改善模式，与临床参数和内省力的改善模式有所不同。在T1，内省力和任何参数间没有相互关联。在T2，内省力分数与WCST的持续性错误、阴性症状和一般症状有相关性。使用线性回归倒退模型，WCST的持续性错误和PANSS一般症状的分数是重要的模型，它们能解释38.2%的变量。在T3，只有PANSS阴性和一般分数与内省力相关。PANSS阴性和一般分数的模型解释了54.3%内省力变量。由T1至T3，自省力的改善与一般PANSS和阳性分数相关，但在Bonferroni校正后并不显著。

讨论：结果显示，临床参数和认知功能对不同阶段的精神疾病的自省力有不同模式的贡献。这可解释文献结果不一的原因。这可能也表明，在疾病的不同阶段，其自省力不足的成因或有所不同。虽然WCST的持续性错误在T2与自省力显著相关，但随着时间，它的改善却与自省力改善没有相关性。以较多样本进行的首发精神分裂症纵向研究，在未来将是检视自省力障碍成因的重要途径。

Gender Differences in First-episode Psychosis: A Three-year Follow-up Study in Hong Kong

S2.1.4

WC CHANG

Department of Psychiatry, Tai Po Hospital, Hong Kong, China

Background: Gender differences have been observed in psychosis with respect to clinical manifestations and illness outcome. Majority of studies focused mainly on chronic inpatient samples with schizophrenia diagnosis. Thus, we aimed at investigating gender differences in a sample of young people with first-episode psychosis.

Methods: A total of 700 (male, $n = 360$; female, $n = 340$) subjects aged 15 to 25 years consecutively enrolled in a territory-wide first-episode psychosis treatment programme in Hong Kong from 1 July 2001 to 31 August 2003 were studied. Baseline and follow-up characteristics were collected via systematic review of records. Clinical Global Impressions scale (CGI-S) and Social and Occupational Functioning Assessment Scale (SOFAS) were applied for symptoms and functional assessments, respectively.

Results: At entry, females had significantly shorter median duration of untreated psychosis (DUP) [$p < 0.001$], achieved higher educational level ($p < 0.01$), were more likely to have past suicidal attempts, exhibited less negative symptoms (CGI-S; $p < 0.05$), and more affective symptoms (CGI-S; $p < 0.01$) than males. At 3-year follow-up, males had more severe negative symptoms (CGI-S; $p < 0.001$), less affective symptoms (CGI-S; $p < 0.01$), more violent behaviour and forensic records ($p < 0.01$), and higher rate of comorbid substance abuse ($p < 0.01$). Females had superior psychosocial functioning (SOFAS, $p < 0.001$) and were more likely to be employed ($p < 0.01$) when compared with males with first-episode psychosis.

Conclusions: Notable gender differences in clinical profiles, illness trajectory and functional outcome were demonstrated in Chinese young people suffering from first-episode psychosis. Differential needs between men and women and gender-specific therapeutic strategies should be considered in early intervention service.

首发思觉失调的性别差异：香港三年随访

研究

张颖宗

香港大埔医院精神科

背景：在思觉失调的临床表现和疾病预后上，应注意性别差异的因素。大部分研究都集中患有慢性精神分裂症的住院病人。因此，我们探讨首次确诊的思觉失调青少年患者样本上的性别差异。

方法：2001年7月1日至2003年8月31期间，我们对参与全港「治疗首发思觉失调计划」的700名（360名男性和340名女性）介乎15至25岁的持续样本进行研究。通过有系统的纪录审查，收集参与者最初和其后跟进的特点。我们分别使用临床总体印象量表（简称CGI-S）和社会及职业功能量表（简称SOFAS）对症状和功能作出评估。

结果：研究开始时，相对于男性，女性的精神病未治疗时间（简称DUP）中位数较短（ $p < 0.001$ ）、教育水平较高（ $p < 0.01$ ）、过去曾有自杀企图的机会较大，以及明显表现较少的阴性/负面症状（CGI-S; $p < 0.05$ ）和较多情感表达（CGI-S; $p < 0.01$ ）。在3年的随访后，男性出现较多负面症状（CGI-S; $p < 0.001$ ）、较少情感表达（CGI-S; $p < 0.01$ ）、较多暴力行为和法庭记录（ $p < 0.01$ ），而滥用药物的共存病患亦呈现较高比率（ $p < 0.01$ ）。与男性患者相比，女性有较好心理社会功能（SOFAS, $p < 0.001$ ），工作上也有较大机会获雇用（ $p < 0.01$ ）。

结论：性别差异在临床概况上有显著差别，中国首发思觉失调青年人说明其疾病轨迹和功能性结果。我们在早期介入服务中，应顾及较适用于不同性别的治疗策略和需要。

Toxicity of Ketamine on Animal Models — A Review of Studies in our Laboratory

S2.2.1

DTW YEOW

School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China

Ketamine toxicity had been studied on cultures, mouse and monkey models. Culture studies employing neuroblastoma cultures revealed direct toxicity on the differentiated (retinoic acid-induced differentiation) and undifferentiated neuroblastoma cells, as well as in the primary human neurons. Increased cell death was observed. In the prefrontal cortices of chronic ketamine-treated (1 mg/kg per day) mice and monkeys, groups of neurons demonstrated presence of hyperphosphorylated tau in their cell bodies. There was also an increase in neurons with activated caspase 3, indicating the way towards apoptosis. Activated fMRI areas in the ketamine-treated animals included entorhinal cortex, fusiform gyrus, corpus striatum, and the mesencephalon. Apart from the central nervous system (CNS) responses, kidney and liver damages were observed in both chronic- and acute-treated (2 weeks, high dose) mice. The situation had worsened after combined ketamine treatment and alcohol ingestion. Microarray on the brains of chronic models of mice pointed out that expression of transmitters / enzymes of transmitters were affected after as little as 3 months of ketamine treatment. These results illustrate a 'real' concern of ketamine toxicity, especially subsequent to chronic treatment.

氯胺酮在动物模型上之毒性研究

姚大卫

香港中文大学生物医学学院

关于氯胺酮的毒性，曾经有人利用细胞培植、老鼠和猴子模型进行研究。培植研究采用神经母细胞瘤，分化成神经细胞瘤细胞和人体主要的神经细胞的培养，区别直接毒性分化的差异（维甲酸诱导分化）。我们观察到细胞死亡有所增加。在老鼠和猴子的前额叶皮质慢性注入氯胺酮（每日1 mg/kg）下，其胞体有一组神经元出现过度磷酸化的T形物。同时，含有活性化的caspase 3神经元有所增加，这说明细胞凋亡的状况。氯胺酮中的活性化功能磁共振成像（简称fMRI）区域治疗了动物的内嗅皮质、梭状回、纹状体和中脑。除了中枢神经系统有反应，在对老鼠的慢性和急性治疗上，都观察到对其肾脏和肝脏的损害（2周，高剂量）。若氯胺酮与酒精同时摄取，情况会进一步恶化。由老鼠的大脑基因芯片的慢性模型中可知，短短3个月的氯胺酮处理，会影响神经递质及其酶之表达。这些结果说明了，尤其经长期影响下，氯胺酮的「真正」毒性。

Partnership with Community Partners in Specialist Substance Abuse Treatment

S2.2.2

WH CHEUNG

Substance Abuse Assessment Unit, Kwai Chung Hospital,
Hong Kong, China

Over the past decade, there was a trend of increasing prevalence of psychoactive substance abuse, especially among the youth. The myth of the relatively harmless nature of the psychoactive substance and the hidden nature of the habit were believed to be some of the major hurdles in tackling the problems.

To face the challenge from the new drug scene and the different characteristic of the substance abusers, the traditional clinic-based specialist substance abuse treatment services has to be evolved into a more efficient and cost-effective model of service delivery.

Different models of partnership between a Substance Abuse Assessment Unit in a local mental hospital and its community partners and their implications in future service development will be discussed.

在滥用药物的专科治疗上与社区伙伴的合作关系

张伟谦

香港葵涌医院滥用药物评估中心

过去10年，滥用危害精神毒品日渐流行，这情况于青少年群中更为明显。误信滥用精神科药物无害和隐蔽性的滥药习惯，是解决问题的主要障碍。

传统以诊所为基础的滥用药物专科治疗服务，必须发展一个具效率和成本效益的服务模式，以面对新毒品的出现和滥药者不同特点的挑战。

本文就本地精神病院的滥用药物评估中心与其社区伙伴不同模式的合作关系，以及对未来服务发展之影响进行讨论。

Neuropsychological Functioning of Adolescent Substance Abusers and its Implication on Treatment

S2.2.3

SSY CHANG

Kwai Chung Hospital, Hong Kong, China

Adolescent abuse of psychotropic drug has been a

growing problem in Hong Kong since the past decade. The physical dependence and adverse effects of substance abuse have widely been reported. The awareness of the neuropsychological impairments resulted from substance abuse has also increased in recent years. The phenomenon of ketamine misuse and abuse in Hong Kong among the teenagers is alarming.

In the present presentation, profiles of neuropsychological functioning of adolescents abusing ketamine and poly-substances are compared with profiles of teenagers who do not abuse drugs. Implication for neuropsychological impairment on individuals' functioning will be discussed. In addition, application of the neuropsychological assessment as a treatment component in the substance abuse problems is highlighted.

青少年滥用药物者的神经心理功能和其治疗的影响

郑淑仪

香港葵涌医院

青少年滥用危害精神毒品是香港过去10年来日益严重的问题。滥用药物带来的躯体依赖和不良影响已被广泛报道。近年来，药物滥用所造成神经心理障碍的意识也有所提高。香港青少年对氯胺酮误用和滥用的现象是惊人的。

这简报比较滥用氯胺酮和多种物质的青少年，与不使用药物青少年的神经心理功能数据，也讨论氯胺酮和多种物质对个人神经心理功能障碍的启示。简报也会强调神经心理学，作为评估药物滥用问题治疗上的应用。

Cognitive Dysfunction in Primarily Ketamine Users

S2.2.4

WK TANG¹, HJ LIANG¹, AKL TANG²

¹ Department of Psychiatry, The Chinese University of
Hong Kong, Hong Kong, China

² Department of Psychiatry, Prince of Wales Hospital,
Hong Kong, China

Objectives: To evaluate the cognitive dysfunction in young primarily ketamine users in Hong Kong.

Methods: Cross-sectional design was used. A total of 40 subjects aged 16 to 30 years were recruited from 3 counselling centres for psychotropic substance abuse in Hong Kong. Ketamine group (n = 17) and health control group (n = 23) were matched in age, education and gender. Each subject completed a battery of cognitive function tests covering the domains of general intelligence, working memory, verbal memory, executive function, language and visual memory (including Digit Symbol-Coding, Arithmetic, Information, Stroop Test, Modified Verbal Fluency Test, Wisconsin Card Sorting Test, Digital Span Forward and Backward, WMS-III-Logical Memory, WMS-III-Word List, Rey Osterrieth Complex Figure, and Modified Boston Naming). Scores of cognitive function tests were standardised using z score.

Results: Compared with the control group, ketamine users had significantly poorer performance in the general intelligence (p = 0.004), verbal memory (p = 0.007) and visual memory (p = 0.008), even after adjusted for age, sex, and education. There was also a trend that ketamine users had poorer performance in the executive function (p = 0.528), working memory (p = 0.099), and language (p = 0.308) domains.

Attachment 8



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Ketamine Abuse and Toxicity

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Dr. Anthony E. JAMES

Miss Stephanie L. Y. Yeung

Miss Angel W. P. Lam

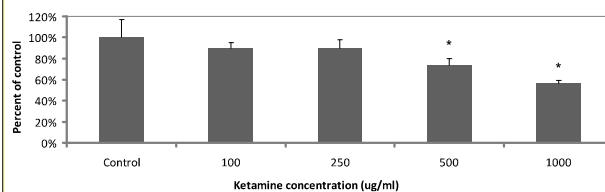
Miss Nicole L. H. Lam

Miss Karena Y. W. Wong

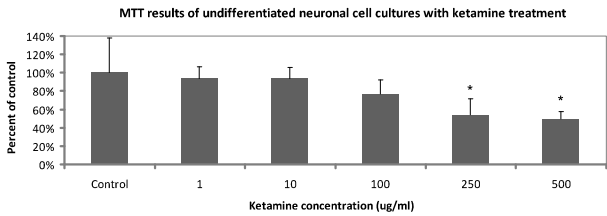
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Miss Karen L. K. Yeung

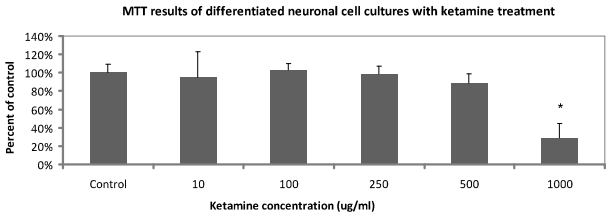
MTT results of human neuron cultures with ketamine treatment



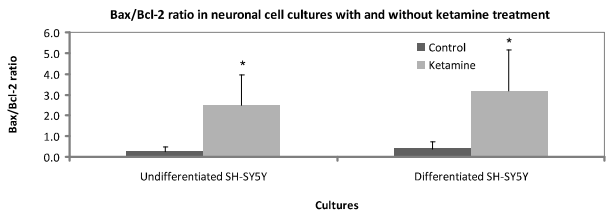
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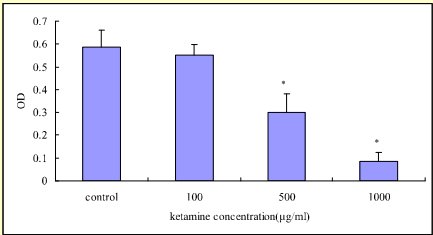


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Cell Viability Measured by MTT Assay of PC-12 Cells after Treatment with Different Concentrations of Ketamine



*p<0.001

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Dosages

Human Children: < 15mg/kg

Rodents (high metabolism): 10X

Primate: equivalent to human dosage

- 1) University of California (San Francisco)
Comparative medicine: 200mg/kg
- 2) Irifune et al. (2009)
< 150mg/kg
> 60mg/kg affects righting reflex

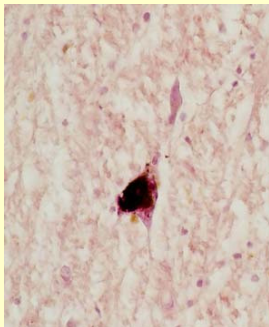


Control



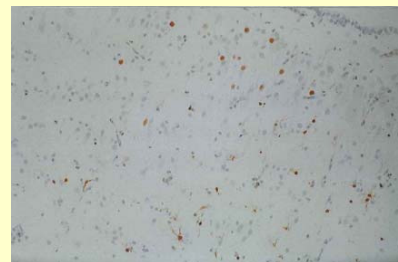
Ketamine treatment (40 mg)

Prefrontal



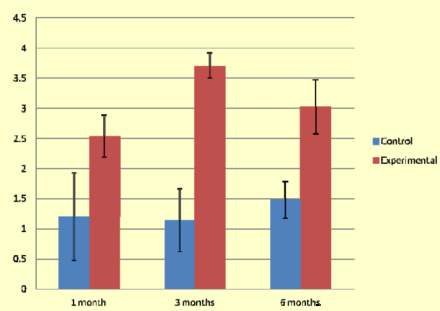
Activated Caspase 3 in Monkey

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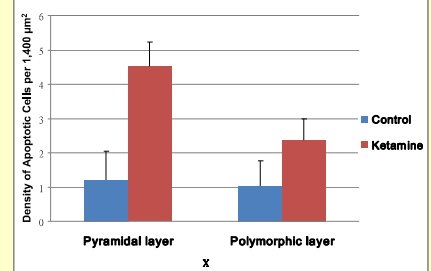
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Prefrontal cortex TUNEL positive cells per 700 μm^2



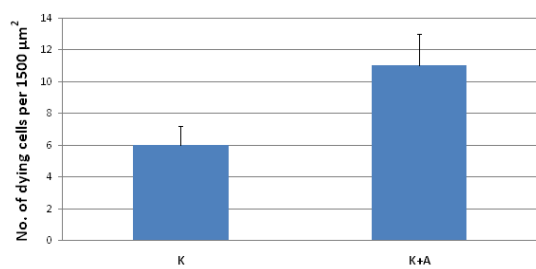
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Hippocampus



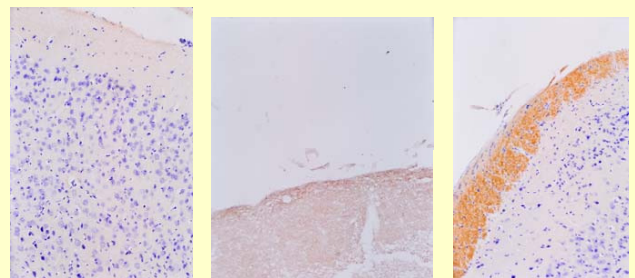
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Prefrontal



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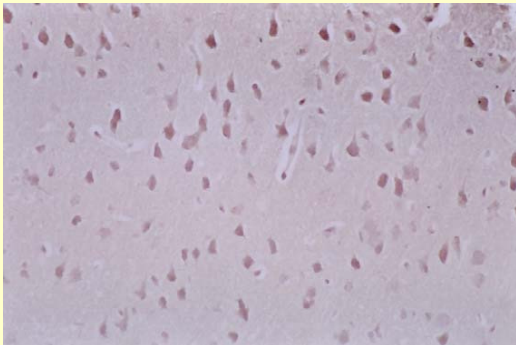
Hyperphosphorylated tau



Mice

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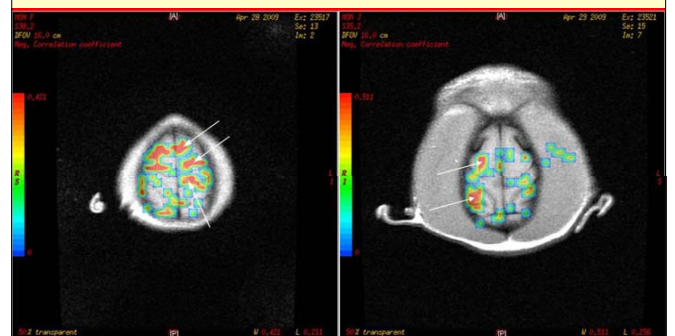
Hyperphosphorylated tau



Monkey

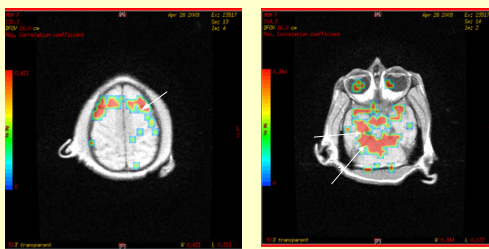
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fMRI - Monkey



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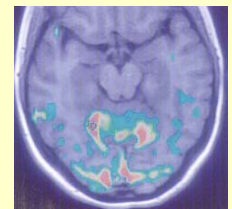
fMRI - Monkey



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Ketamine abused

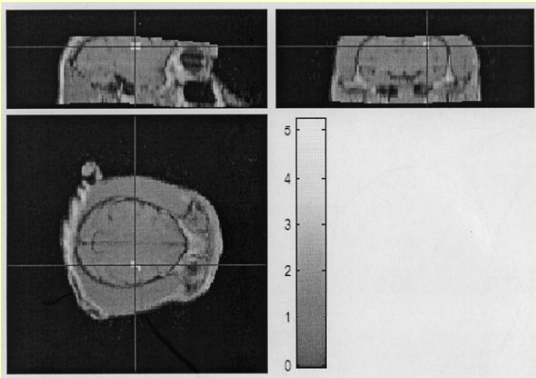


Control

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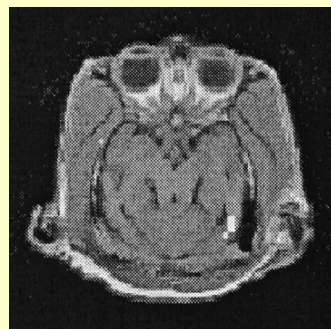
Ketamine

Most affected areas after long-term addiction:



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Ketamine



Entorhinal cortex - spatial memory
Fusiform cortex - love and hate?

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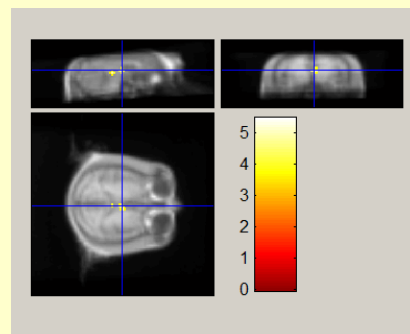
Ketamine



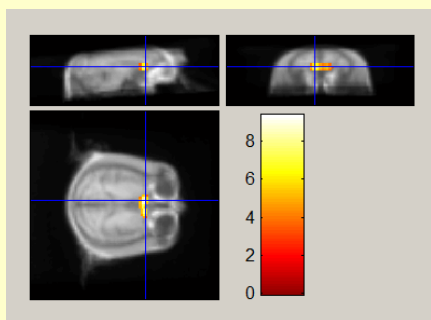
Corpus Striatum - tuning of movement

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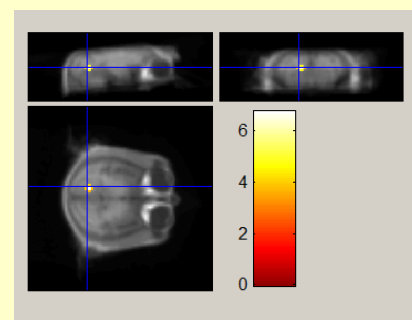
3-Month > 6-Month



6-Month > Control



Control > 6-Month



RNA Expression

Numbers of Different Expressed Genes
in the Brains of Prolonged Ketamine Treated Mice

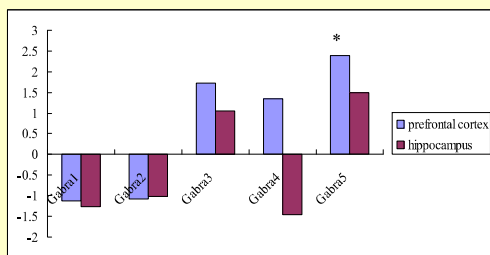
| Fold Change | Total No. of Genes | No. of Up-regulated | No. of Down-regulated |
|-------------|--------------------|---------------------|-----------------------|
| >2.0 | 12 | 2 | 10 |
| >1.9 | 21 | 3 | 18 |
| >1.8 | 27 | 5 | 22 |
| >1.7 | 37 | 12 | 25 |
| >1.6 | 45 | 18 | 27 |
| >1.5 | 64 | 25 | 39 |
| >1.4 | 103 | 41 | 62 |
| >1.3 | 246 | 110 | 136 |

RNA Expression

GO Analysis

| GO ACCESSION | GO Term | p-value | Corrected p-value |
|--------------|---|-------------|-------------------|
| GO:0042136 | Neurotransmitter biosynthetic process | 0,000175264 | 0,29003784 |
| GO:0007214 | Gamma-aminobutyric acid signaling pathway | 0,000382054 | 0,29003784 |
| GO:0042401 | Biogenic amine biosynthetic process | 0,000563124 | 0,29003784 |
| GO:0004890 | GABA-A receptor activity | 0,000666473 | 0,29003784 |
| GO:0042398 | Amino acid derivative biosynthetic process | 0,000721329 | 0,29003784 |
| GO:0016917 | GABA receptor activity | 0,000837374 | 0,29003784 |
| GO:0042133 | Neurotransmitter metabolic process | 0,001027183 | 0,29003784 |
| GO:0003677 | DNA binding | 0,001408004 | 0,29003784 |
| GO:0008292 | Acetylcholine biosynthetic process | 0,001535487 | 0,29003784 |
| GO:0004753 | Saccharopine dehydrogenase activity | 0,001535487 | 0,29003784 |
| GO:0047131 | Saccharopine dehydrogenase (NAD+, L-glutamate-forming) activity | 0,001535487 | 0,29003784 |
| GO:0005307 | Choline:sodium symporter activity | 0,001535487 | 0,29003784 |

**Gene Expression Changes in
6 α GABA Receptors as Revealed by Real-time PCR**

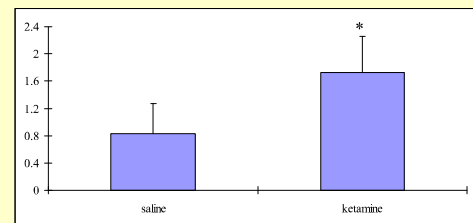
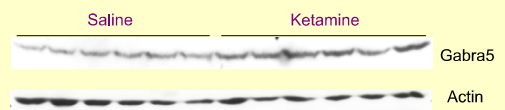


* $p < 0.05$

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1-Month Ketamine + Control Western Blot (n=6)

Prefrontal Cortex



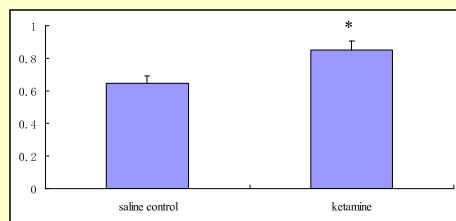
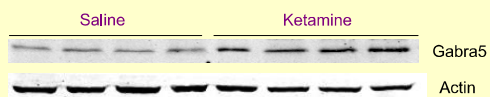
* $p = 0.01$

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Western Blot for α GABA Receptor 5

3 Months Saline Control and Ketamine Groups (n=4 for each group)

Prefrontal Cortex

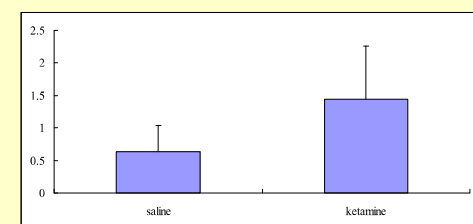
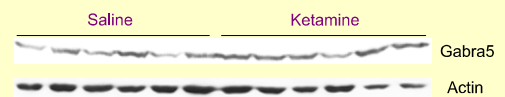


* $p = 0.0016$

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1-Month Ketamine + Control Western Blot (n=6)

Hippocampus

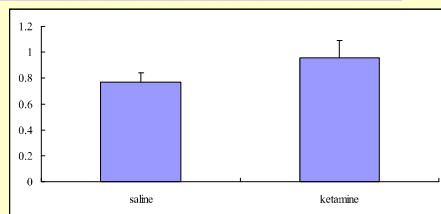
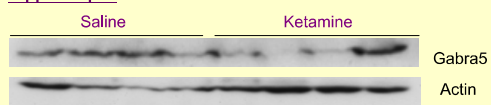


$p = 0.055$

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Western Blot for α GABA Receptor 5 3 Months Saline Control and Ketamine Groups (n=4 for each group)

Hippocampus

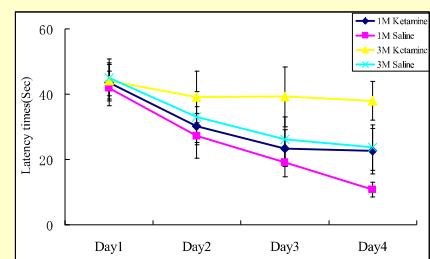


$p = 0.05$

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Different Performance in the Morris Water Maze between Ketamine-treated and Saline groups

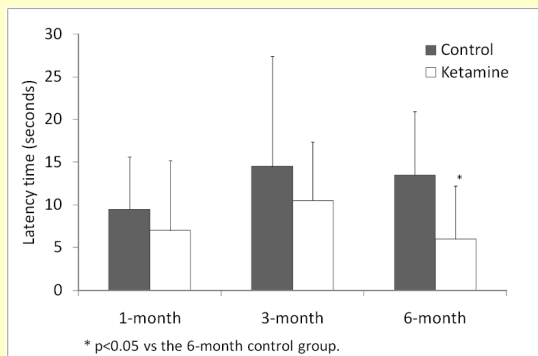
Spatial Acquisition



Significant difference in both the 1-month and 3-month groups ($p < 0.05$ by Repeated Measures ANOVA)

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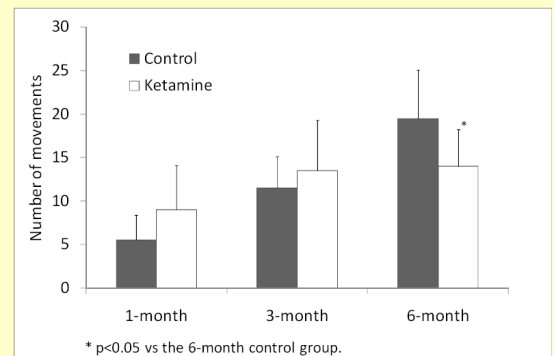
Wire Hang Test Results of Different Mouse Groups



* $p < 0.05$ vs the 6-month control group.

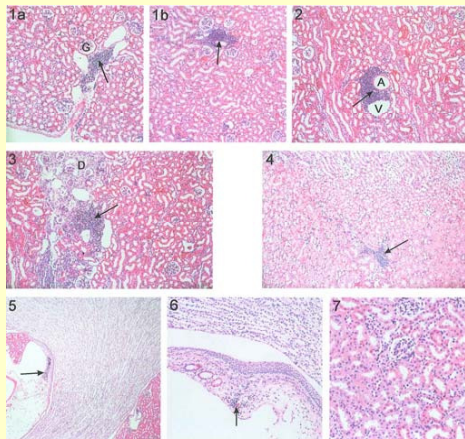
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Hot-plate Test Results of Different Mouse Groups



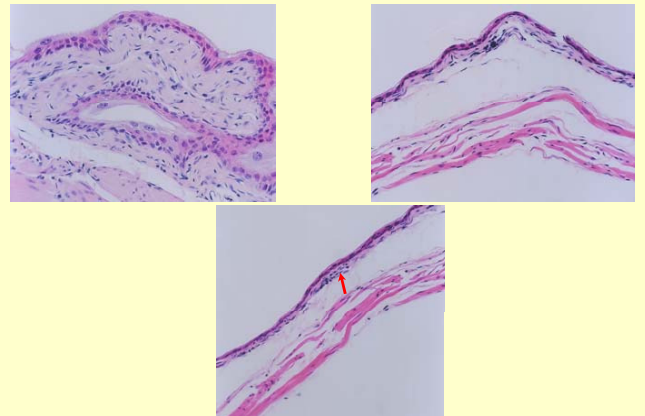
* $p < 0.05$ vs the 6-month control group.

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Epidermal Cells of the Gall Bladder of Ketamine-treated Animal

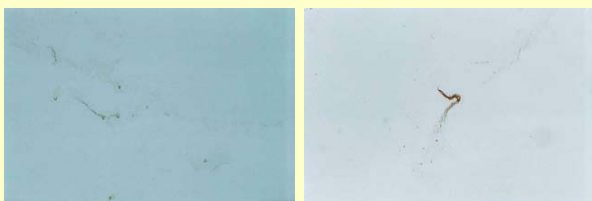


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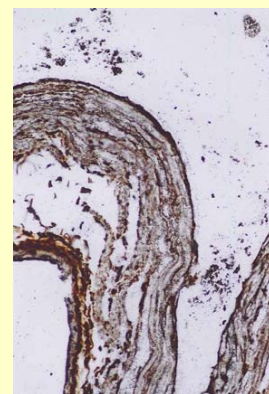
1) Axonal Degeneration – No Degeneration of Myelin

2) ↓ Transmitters (Immunocytochemistry for ChAT)

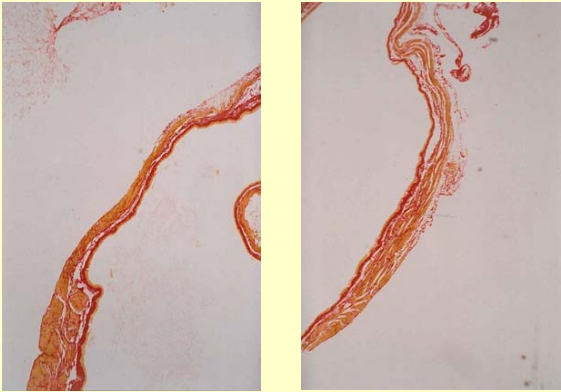
Optical Density per Unit Area: 21.85 ± 3.29 (Control)
 10.45 ± 0.77 (Ketamine)



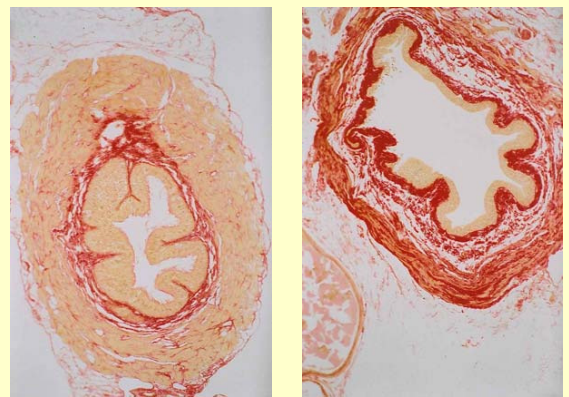
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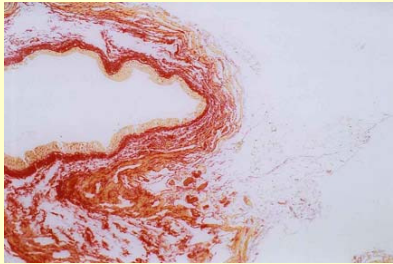
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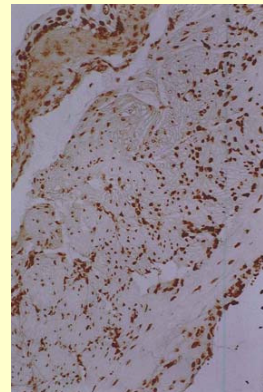
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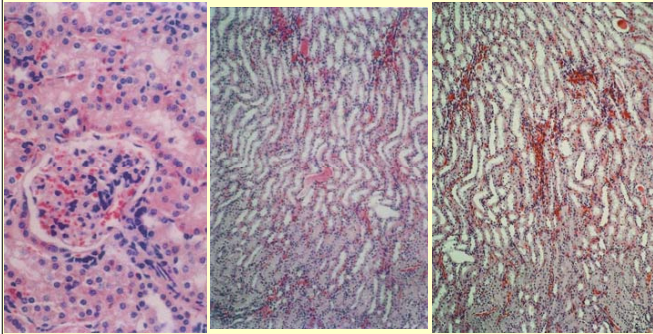
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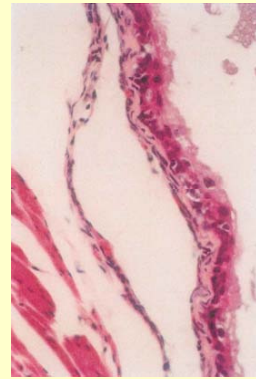
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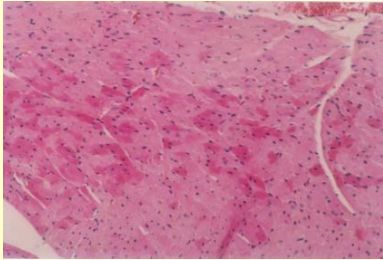
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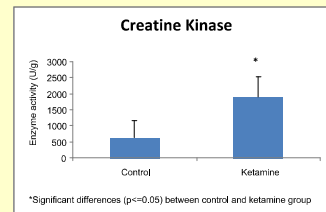
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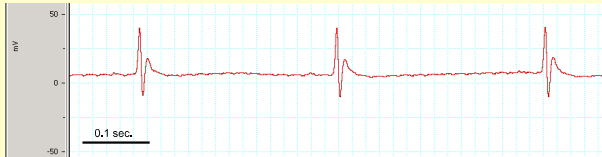
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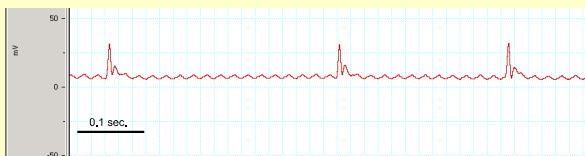
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ECG

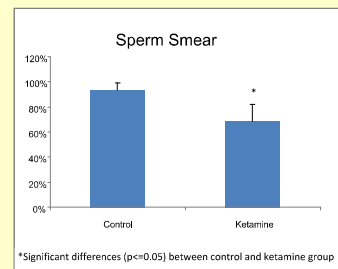
ICR: control



ICR: ketamine (30 mg/kg) + 12 % ethanol



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Acknowledgements

- Beat Drugs Fund Association, Narcotics Division, Security Bureau, HKSAR
[Projects: BDF080048 & BDF100052]
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- 中國科技部
- 雲南省科技部
- 雲南省公安廳法醫研究所

~Thank You~