รายงานวิจัยฉบับสมบูรณ์

การศึกษาความสัมพันธ์ของยีน glutamate transporter กับการติดยาเสพติดเมทแอมเฟตามีน

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มีนาคม 2553

สัญญาเลขที่ RMU5080011

รายงานวิจัยฉบับสมบูรณ์

โครงการ

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ดิฉัน ผู้ช่วยศาสตราจารย์ ดร.สุทิสา ถาน้อย หัวหน้าโครงการวิจัย “การศึกษาความสัมพันธ์ของยีน glutamate transporter กับการติดยาเสพติดเมทแอมเฟตามีน” ขอแสดงความขอบคุณต่อทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลางในสถาบันอุดมศึกษา สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) และสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่มอบโอกาส และทุนสนับสนุนโครงการวิจัยนี้จนสามารถดำเนินการได้สำเร็จ และเป็นแนวทางที่จะค้นคว้า วิจัยในระดับสูงต่อไป

ขอขอบคุณ Professor Gavin Reynolds จาก Department of Psychiatry, Queen’s University Belfast UK ที่ให้คำปรึกษา และข้อเสนอแนะที่เป็นประโยชน์ต่อการดำเนินโครงการวิจัย และการตีพิมพ์เผยแพร่ผลงานวิจัยในระดับสากล รวมทั้งขอขอบคุณ ศาสตราจารย์ ดร.ประเสริฐ โศภณ ภาควิชากายวิภาคศาสตร์ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ซึ่งเป็นผู้ริเริ่ม จุดประกายการทำวิจัยอย่างต่อเนื่องภายหลังจากเรียนจบปริญญาเอกแล้ว และทำให้ดิฉันได้รับประสบการณ์ในการทำงานวิจัยเพิ่มมากขึ้น นอกจากนั้นดิฉันขอขอบคุณ รองศาสตราจารย์ ดร.เสมอ ถาน้อย คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร ที่ ให้คำปรึกษาด้านเทคนิควิธีวิจัยเกี่ยวกับระบบสืบพันธุ์ ที่ได้ศึกษาเพิ่มเติมในสัตว์ทดลอง เพื่อการใช้ตัวอย่างทดลองอย่างมีคุณค่า และขอขอบคุณ นายแพทย์ปริทัศน์ วาทิกทินกร ที่ช่วยในการดูแล และเก็บข้อมูลพื้นฐานจากผู้ป่วย

ขอขอบคุณทัณฑสถานบำบัดพิเศษกลาง และบุคลากรที่ให้ความอนุเคราะห์ และอำนวยความสะดวกในการเก็บตัวอย่าง นอกจากนั้นยังขอขอบคุณ คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร ที่เอื้อเฟื้อสถานที่ และเครื่องมือวิจัยสำหรับการดำเนินโครงการวิจัยให้สำเร็จด้วยดี

ท้ายที่สุดที่สำคัญ ต้องขอขอบคุณเป็นอย่างมากสำหรับผู้ป่วย และอาสาสมัคร ที่ให้ความอนุเคราะห์ตัวอย่างและข้อมูลสำหรับการวิจัยครั้งนี้ ความเสียสละของท่านทั้งหลายนี้จะเป็นประโยชน์อย่างมากในการวิจัยทางการแพทย์ต่อไปในอนาคต

ผู้ช่วยศาสตราจารย์ ดร.สุทิสา ถาน้อย
หัวหน้าโครงการวิจัย

บทคัดย่อ

รหัสโครงการ : RMU5080011

ชื่อโครงการ : การศึกษาความสัมพันธ์ของยีน glutamate transporter กับการติดยาเสพติดเมทแอมเฟตามีน

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เมทแอมเฟตามีน เป็นสารกระตุ้นจิตและประสาททำให้เกิดการเปลี่ยนแปลงต่อนสารสื่อประสาท และเกิดความผิดปกติทางด้านกระบวนการคิด และการแสดงออกทางพฤติกรรม มีการศึกษาถึงผลกระทบของการได้รับสารเสพติดเมทแอมเฟตามีนทั้งในระบบประสาท และระบบสืบพันธุ์ ดังนั้นในงานวิจัยนี้จึงมีวัตถุประสงค์ในการศึกษาถึงผลของการได้รับเมทแอมเฟตามีนต่อการทำงานของระบบสารสื่อประสาทกลูตาเมท โดยศึกษาที่ตัวรับสารสื่อประสาทกลูตาเมทชนิด NMDA receptor และตัวเก็บกลับสารสื่อประสาทกลูตาเมทชนิด glutamate transporter EAAT3 รวมทั้งศึกษาการเปลี่ยนแปลงของรูปร่าง ปริมาณ และการตายของเซลล์อสุจิ ภายหลังจากการได้รับสารเสพติดเมทแอมเฟตามีนในสัตว์ทดลอง นอกจากนั้นแล้วยังศึกษาการเปลี่ยนแปลงลำดับเบสของยีนตัวเก็บกลับสารสื่อประสาทกลูตาเมทชนิด glutamate transporter SLC1A2 gene ในผู้ติดสารเสพติดเมทแอมเฟตามีน

จากการศึกษาพบว่ามีการเปลี่ยนแปลงของการแสดงออกของตัวรับกลูตาเมท NMDA receptor และตัวเก็บกลับกลูตาเมท glutamate transporter EAAT3 ในสมองของสัตว์ทดลองที่ได้รับสารเสพติดเมทแอมเฟตามีน นอกจากนั้นแล้วในการศึกษาในระบบสืบพันธ์ พบว่าร้อยละของจำนวนของเซลล์อสุจิที่มีรูปร่างปกติ และปริมาณเซลล์อสุจิทั้งหมดนั้นลดลงอย่างมีนัยสำคัญ ในสัตว์ทดลองที่ได้รับสารเสพติดเมทแอมเฟตามีน นอกจากนั้นยังพบว่ามีการเพิ่มปริมาณการตายแบบ apoptosis ของเซลล์อสุจิในสัตว์ทดลองที่ได้รับสารเสพติดเมทแอมเฟตามีนเช่นกัน ในการศึกษาในผู้ป่วยที่ติดสารเสพติดเมทแอมเฟตามีน พบว่ามีความสัมพันธ์การเปลี่ยนแปลงลำดับเบส (single nucleotide polymorphisms; SNP) ของยีนตัวเก็บกลับกลูตาเมท glutamate transporter SLC1A2 gene ในผู้ติดสารเสพติดเมทแอมเฟตามีน นอกจากนั้นความสัมพันธ์ของการเปลี่ยนแปลงลำดับเบสใน glutamate transporter ยีนนี้ยังพบในผู้ติดสารเสพติดเมทแอมเฟตามีนที่มีภาวะทางจิตร่วมด้วย (methamphetamine dependence with psychosis)

จากผลการศึกษาพบว่าการเปลี่ยนแปลงการแสดงออกของตัวรับกลูตาเมท NMDA receptor และตัวเก็บกลับกลูตาเมท glutamate transporter EAAT3 นั้นเป็นการตอบสนองต่อการผิดปกติของระบบสารสื่อประสาทกลูตาเมทในสมอง นอกจากนั้นแล้วยังพบความสัมพันธ์ของยีน glutamate transporter กับภาวะการติดสารเสพติดเมทแอมเฟตามีน ซึ่งเป็นการยืนยันถึงยีน glutamate transporter นั้นมีบทบาทสำคัญที่เกี่ยวข้องกับกลไกการติดสารเสพติดเมทแอมเฟตามีน นอกจากนั้นแล้วผลของการศึกษาแสดงให้เห็นว่าสารเสพติดเมทแอมเฟตามีนนั้นไม่ได้เหนี่ยวนำให้เกิดความเป็นพิษต่อระบบประสาทเท่านั้น ยังแสดงผลถึงความเป็นพิษต่อกระบวนการสร้างเซลล์สืบพันธุ์อีกด้วย

คำสำคัญ: เมทแอมเฟตามีน; สารสื่อประสาท; กลูตาเมท; ระบบประสาท; ระบบสืบพันธุ์

Abstract

Project Code : RMU5080011

Project Title : The association study of glutamate transporter gene and methamphetamine dependence

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Project Period : 3 years 4 months (1 December 2006 – 31 March 2010)

There are areas of interest on the effects of methamphetamine in the changes in nervous tissues and reproductive organs. Therefore, the present study was designed to investigate the effects of methamphetamine (METH) on the alteration of glutamate/NMDA receptor subunit1 (NMDAR1) and neuronal glutamate transporter (EAAT3) densities in frontal cortex, hippocampus, and striatum of rat brains in order to understand the mechanism of glutamatergic neurotransmission. Moreover, the present study has also focused on the EAAT2 gene (SLC1A2; solute carrier family 1 member 2) in association studies of METH dependence. In addition, the present study has determined the effect of METH on the change of gametogenesis as well as to study apoptosis in the seminiferous tubules in the testis of male rats in order to better understanding the mechanisms of METH on reproductive system.

In the animal studies, rats were injected intraperitoneally with a single dose of 8 mg/kg METH, 4 mg/kg/day METH for 14 days and saline in acute, subacute and control groups, respectively. NMDAR1 and EAAT3 immunoreactivities were determined by western blotting. An alteration of NMDAR1 and EAAT3 immunoreactivities was found in the brain areas of METH-treated animals. The concentration of spermatozoa was determined using Neuabaur's counting chamber. Apoptotic activities within the seminiferous tubules were studied using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay. The results showed a reduction of percentage of normal sperm morphology and total sperm count in METH-treated animals. Apoptotic activities were also significantly increased in the seminiferous tubules of male rats after exposure to METH. In the human studies, the genotypic study of seven single nucleotide polymorphisms (SNPs) in *SLC1A2* gene were carried out by PCR and followed by either direct sequencing or restriction fragment length polymorphism (RFLP) methods. There was a significant difference in the genotypic and allele frequencies of SNPs in *SLC1A2* gene. Moreover, we found a significant difference between METH dependence patients with psychosis and control subjects in the genotypic and allelic frequencies of SNPs in *SLC1A2* gene.

The results in the present study suggest that an alteration of NMDA receptor and glutamate transporter expression may be a response to possible deficits of glutamatergic neurotransmission induced by METH. Moreover, the variation of glutamate transporter gene *SLC1A2* may play a genetic risk factor for susceptibility to METH dependence. The results of METH-induced toxicity in reproductive system suggest that METH not only works as drug of abuse in the central nervous system, but also in gametogenesis of males.

Keywords: Methamphetamine; NMDA receptor; Glutamate transporter; SLC1A2 gene; spermatogenesis

หน้าสรุปโครงการ (Executive Summary)

1.ชื่อโครงการ

(ภาษาไทย) : การศึกษาความสัมพันธ์ของยีน glutamate transporter กับการติดยาเสพติดเมทแอมเฟตามีน

(ภาษาอังกฤษ) : The association study of glutamate transporter gene and methamphetamine dependence

2. รหัสโครงการ RMU5080011

3. ชื่อหัวหน้าโครงการ หน่วยงานที่สังกัด ที่อยู่ หมายเลขโทรศัพท์ โทรสาร และ e-mail

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4. สาขาวิชาที่ทำการวิจัย Neurobiology and Reproductive biology

7. ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

In recent years, a dramatic escalation in the addictive drugs in Thailand has been observed. It is well established that addictive drugs, amphetamine, methamphetamine (METH), ecstasy etc., can produce psychoactive effects such as pleasant perceptual changes. Many factors such as social, psychological and biological factors are the cause of the substance dependence. It has become increasingly evident that glutamate is also involved in addiction, and glutamatergic mechanisms may be responsible for plastic changes in the brain that lead to addictive behaviour. METH may also produce alterations of striatal and cortical glutamate/NMDA receptors (Eisch et al., 1996). Several studies support the role of glutamate in the development of METH toxicity (Mark et al., 2007; Shoblock et al., 2003; Raudensky and Yamamoto 2007; Xue et al., 1996; Cadet et al., 2003; Abekawa et al., 1996). Those

observations suggest that glutamate/NMDA receptors may play a critical role in the effects of drug addiction. Moreover, an increase in vesicular glutamate transporter 1 (VGLUT1), a subtype of vesicular glutamate transporter responsible for regulating glutamate release, has been observed in striatal and cortical regions after METH exposure (Mark et al., 2007). Taken together, these changes in the indicators of glutamatergic system suggest that the other components of glutamatergic neurotransmission may be abnormal in the development of METH toxicity including excitatory amino acid transporters (EAATs).

Genetic factors are also involved in the susceptibility to substance dependence. Gene regulating glutamate neurotransmission have become attractive candidates for genetic association studies of substance abuse. These include the excitatory amino acid transporter (EAATs). Recent studies have shown that glutamate transporters play a critical role in the development of morphine tolerance, abnormal pain sensitivity and withdrawal syndrome (Nakagawa et al. 2001; Mao et al. 2002) suggesting that glutamate transporters may contribute to the neural mechanisms of substance abuse, but how the activity of glutamate transporters in brain is regulated during substance abuse remains to be investigated.

It has been established that the development and expression of behavioural sensitisation is coincident with functional changes in limbic-motor circuitry (Robinson and Berridge, 1993; Pierce and Kalivas, 1997). Therefore, it has been hypothesized that mediate the response to addictive drugs, sexual behaviour, and feeding behaviour may be functionally related in the same neural mechanisms (Robinson and Berridege, 1993; Pierce and Kalivas, 1997). Therefore, there may be an interaction between changes of neural mechanism and reproductive system after taken drug of abuse.

8. วัตถุประสงค์

The core aim of the present study was to understand the neuronal mechanism underlying glutamatergic neurotransmission and genetic risk of METH dependence and an interaction between changes of neural mechanism and reproductive system after taken METH.

The specific objectives are:

1. To determine the effects of METH administration on the alteration of glutamate/NMDA receptor and neuronal glutamate transporter EAAT3 densities in frontal cortex, hippocampus, and striatum of rat brains
2. To determine the association of polymorphisms in the EAAT2 gene (SLC1A2; solute carrier family 1 member 2) with METH dependence.

3. To determine the effect of METH on the change of gametogenesis as well as to study apoptosis in the seminiferous tubules in the testis of male.

เนื้อหางานวิจัย

Introduction

In recent years, a dramatic escalation in the addictive drugs in Thailand has been observed. Addictive drugs are characterized by their ability to induce tolerance, somatic dependence and psychological dependence that manifests itself as compulsive drug-seeking behavior, drug-taking and loss of control over actually voluntary acts. The majority of newly drug-dependent subjects are teenagers and the mostly used drugs are methamphetamine (METH). It is well established that addictive drugs, amphetamine, METH, ecstasy etc., can produce psychoactive effects such as pleasant perceptual changes. Many factors such as social, psychological and biological factors are the cause of the substance dependence.

It has become increasingly evident that glutamate is also involved in addiction, and glutamatergic mechanisms may be responsible for plastic changes in the brain that lead to addictive behaviour. It has also been reported that amphetamine, dopamine agonist, can produce long-term behavioural changes including sensitisation, tolerance, and dependence (Klawans et al., 1978; Segal et al., 1980; Robinson and Becker, 1986). Moreover, the administration of METH, a derivative of amphetamine, can damage neurotoxicity to dopaminergic and serotonergic nerve terminals in several brain areas (Morgan and Gibb, 1980; Ricaurte et al., 1980; O'Callaghan 1991; O'Dell et al., 1991). In addition, METH-induced dopamine depletion is blocked by glutamate N-methyl-D-aspartate (NMDA) receptor antagonist (Sonsalla et al., 1989; Shoblock et al., 2003). Furthermore, excitatory amino acids such as glutamate also play a crucial role in modulating dopamine release in the prefrontal cortex (Hata et al., 1990; Jedema and Moghaddam 1994), in the striatum (Krebs et al., 1991; Wang 1991; Sacaan et al., 1992), and in the nucleus accumbens (Imperato et al., 1990; Youngren et al., 1993; Taber and Fibiger, 1995).

METH may also produce alterations of striatal and cortical glutamate/NMDA receptors (Eisch et al., 1996). The glutamate/NMDA receptors seem to be critically involved in synaptic formation and plasticity both during early development of the CNS (Cline et al. 1987; Udin and Scherer, 1990) and in the adult animals. The glutamate/NMDA receptors may be important in aspects of long-term potentiation (e.g. Coan et al., 1987; Davies et al., 1989), which may in turn underlie some forms of learning. The activation of the glutamate/NMDA receptors seems to account for a significant proportion of the neuronal damage and degeneration, which results from hypoxia, ischemia and hypoglycemia in the central nervous system (CNS) (Choi 1988). Several studies support the role of glutamate in the development of METH toxicity. METH enhances glutamate release observed in several brain regions, such as striatum (Mark et al., 2007), cerebral cortex (Shoblock et al., 2003), hippocampus (Raudensky and Yamamoto

2007), ventral tegmental area and nucleus accumbens (Xue et al., 1996). An elevation of extracellular glutamate can activate glutamate receptors which mediate excitotoxicity leading to apoptosis and/or neuronal cell death (Cadet et al., 2003). With regards to the glutamate receptors, glutamate/N-methyl-D-aspartate (NMDA) receptors which play a principle role in excitatory neurotransmission, brain development and synaptic plasticity have been reported to be implicated in METH neurotoxicity (Abekawa et al., 1996). Furthermore, a reduction of glutamate/NMDA receptors has been reported in substantia nigra, nucleus accumbens and medial prefrontal cortex during amphetamine withdrawal (Lu et al., 1999). Those observations suggest that glutamate/NMDA receptors may play a critical role in the effects of drug addiction.

Moreover, an increase in vesicular glutamate transporter 1 (VGLUT1), a subtype of vesicular glutamate transporter responsible for regulating glutamate release, has been observed in striatal and cortical regions after METH exposure (Mark et al., 2007). Taken together, these changes in the indicators of glutamatergic system suggest that the other components of glutamatergic neurotransmission may be abnormal in the development of METH toxicity including excitatory amino acid transporters (EAATs). EAATs are a group of molecules required for normal glutamatergic neurotransmission which have specific patterns of cellular localization: EAAT1 and EAAT2 have been localized to astroglia, whereas EAAT3 and EAAT4 have been localized to neurons (Shigeri et al., 2004). EAAT3 is localized to both post-and presynaptic neurons and is responsible for up to 40% of glutamate transport in the rat brain (Rothstein et al., 1994).

Genetic factors are also involved in the susceptibility to substance dependence and the manifestation of substance-induced psychiatric disorders from the results of family, twin and adoption studies (Yates et al. 1996; van den Bree et al. 1998) even though the underlying pathogenesis of substance dependence is still unknown. Tsuang et al. (1996) have reported that genetic factors accounted for 34% of the variance in an individual's risk of developing a drug use disorder and 44% for developing a specific stimulant abuse disorder. Therefore, genetic contributions to the etiology of substance dependence are topics of major interest.

Gene regulating glutamate neurotransmission have become attractive candidates for genetic association studies of substance abuse. These include the excitatory amino acid transporter (EAATs). EAATs potentially affect glutamatergic neurotransmission by maintaining extracellular glutamate concentrations within physiological levels. EAATs play a role in reuptake the synaptically released glutamate. Several findings show that glutamate transporter may be involved in memory formation and induction of long-term potentiation (Levenson et al. 2002), suggesting that regulation of the glutamate transporter system may be a general

component of plasticity at glutamate mediated synapses. A deficient uptake has been implicated in the pathogenesis of several diseases such as ischemic brain damage (Kuwahara et al. 1992) and neurodegenerative diseases (Rothstein et al. 1996). There are five subtypes of glutamate transporters (EAAT1-EAAT5). Their cellular localizations are different: EAAT1 and EAAT2 are located in astroglia, whereas EAAT3, EAAT4 and EAAT5 are located in neuron (Danbolt 2001). Although GLT1, glutamate transporter type I (EAAT2), and GLAST, glutamate/aspartate transporter (EAAT1), have been shown to be expressed in glial cells normally, and EAAC1 (EAAT3) in neurons, a neuron-expressed variant form of GLT1 has been observed (Chen et al. 2002). Recent studies have shown that glutamate transporters play a critical role in the development of morphine tolerance, abnormal pain sensitivity and withdrawal syndrome (Nakagawa et al. 2001; Mao et al. 2002) suggesting that glutamate transporters may contribute to the neural mechanisms of substance abuse, but how the activity of glutamate transporters in brain is regulated during substance abuse remains to be investigated.

It has been established that the development and expression of behavioural sensitisation are coincident with functional changes in limbic-motor circuitry (Robinson and Berridge, 1993; Pierce and Kalivas, 1997). Therefore, it has been hypothesized that mediate the response to addictive drugs, sexual behaviour, and feeding behaviour may be functionally related in the same neural mechanisms (Robinson and Berridege, 1993; Pierce and Kalivas, 1997). In addition, it has been reported that METH has the effects in reproductive organs. Yamamoto et al. (2002) reported that METH induces apoptosis in seminiferous tubules in male mice testis. Apoptotic cells were detected in the seminiferous tubules of male mice 24 hours after a single treatment with 5, 10, and 15 mg/kg METH. Moreover, it has been reported that gonadal steroid hormones play an important role in modulating METH neurotoxicity (Dluzen et al., 2002; Dluzen and McDermott 2002). Treatment of gonadectomized females with a physiological regimen of estrogen significantly diminished the amount of striatal dopamine (DA) depletion to METH compared with non-estrogen treated mice suggesting that estrogen serves as a neuroprotectant (Dluzen and McDermott 2002). In contrast, testosterone tends to increase METH-evoked dopamine responses (Dluzen and McDermott 2002). Therefore, there may be an interaction between changes of neural mechanism and reproductive system after taken drug abuse.

From review literatures, there are areas of interest on the effects of METH in the changes in nervous tissues and reproductive organs. The present study was designed to investigate the effects of METH on the alteration of glutamate/NMDA receptor and neuronal glutamate transporter EAAT3 densities in frontal cortex, hippocampus, and striatum of rat brains in order

to understand the mechanism of glutamatergic neurotransmission. Moreover, the present study has also focused on the EAAT2 gene (*SLC1A2*; solute carrier family 1 member 2) in association studies of METH dependence since EAAT2 has been accounted for 90% of glutamate reuptake in the brain (Rothstein et al. 1995). *SLC1A2* has been mapped to 11p13-p12 (Li and Francke 1995) and consists of 11 exons spanning over 165 kb. The present study has tested associations of METH dependence with 7 SNPs distributed in *SLC1A2* (Deng et al. 2004).

In addition, the changes in reproductive organs were also studied. The present study has determined the effect of METH on gametogenesis as well as to study apoptosis in the seminiferous tubules in the testis of male rats in order to better understanding the mechanisms of METH on reproductive system.

Materials and Methods

Human Studies

1. Subjects

The study group included 200 subjects. The group consisted of 100 subjects diagnosed as METH dependence and 100 control subjects. The METH-dependent patients are derived from the Central Correctional Institutional for Drug Addicts, Bangkok, Thailand. Consensus on the diagnosis, according to the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV; American Psychiatric Association, 1994) criteria for METH dependence was achieved by a board-certified psychiatrist, and a form of interview was administered to all patients. The diagnosis of METH dependence was based on clinical assessment according to the criteria of DSM-IV (APA 1994). The control subjects were 100 psychiatrically normal subjects not addicted to tobacco, smoking, alcohol and coffee drinking, and drug usage. The Human Ethics Committee of Naresuan University has approved the study protocol. A written informed consent was obtained from all the participants.

2. Blood samples

Blood samples were collected on filter paper known as FTA® Cards (Whatman Inc.) according the manufacturer's instruction and as described by Li et al (2004). The whole blood (about 125 µl per 1 inch) from a finger-stick participant was dropped onto FTA® Cards. The blood samples were dried at room temperature. Two 3-mm-diameter disks were punched from the dried blood spot of each filter paper. The disks were then transferred to separate 0.2-ml PCR tubes, washed in the PCR tube three times with 200 µl of FTA purification reagent (Whatman, Inc) and twice with Tris-EDTA buffer, and then air dried in the same tube. The filter paper disks were subsequently subjected to PCR amplification.

3. Genotype determination

Single nucleotide polymorphism (SNP) selection in the glutamate transporter *SLCIA2* region was adopted from the work of Deng et al (2004). SNP information has been collected from dbSNP database http://www.ncbi.nml.nih.gov/SNP/. Optimized PCR conditions of 7 SNPs were performed.

The regions of interest were amplified by PCR. The PCR reaction mixture was prepared in a total volume 25 µl with genomic DNA on the sample disks. 10 pmol of each primer, 2.5 mM of MgCl2, 0.2 mM of each dNTP and 0.2 U of Taq DNA polymerase. The thermocycling program for PCR was involved about 30-40 cycles of 94 $^{\circ}$C 30s, $\geq$60 $^{\circ}$C 30s, $\geq$72 $^{\circ}$C 30s.

Table 1 Detail information of PCR primers for genotyping of SNPs

SNP	Primer sequence (5'-3')	Product size (bp)	Genotyping method
rs1923295	Forward CAAGAAAAAGAGGGGAAAACA Reverse TGGTGCAGACAGGATTGAGA	164	Sequencing analysis
rs4755404	Forward GGCAAAGAGGGAAAGTCCTC Reverse AATGGAGGCATGAAAACAGG	220	Real-time PCR-RFLP
rs4756224	Forward GTCAGCCCAGATGACAAGGT Reverse CCCACCCAATTTTGTGAGTC	223	Sequencing analysis
rs1923298	Forward CAAATCCACAGCAAACTGGA Reverse AACAAGCCAAATGTGAAGCA	187	Sequencing analysis
rs1885343	Forward TTTCCCTTATCCCAATGCTG Reverse CTGCCACAAGGGAAAAATTC	173	Real-time PCR-RFLP
rs752949	Forward GGCCAAATCTCAGCTCTATGC Reverse CAGCTGGCCTACCTAAGACG	224	PCR-RFLP
rs1042113	Forward CTGGGGATTGATAAGCGTGT Reverse TAGTGTGGAGGGGAAACAGG	209	PCR-RFLP

3.1 Restriction Fragment Length Polymorphism (RFLP) analysis

The genotyping was performed using restriction fragment length polymorphism (RFLP) analysis. PCR product was incubated with the restriction enzymes according to the manufacturer's instruction. Resulting products were detected by ethidium bromide staining after electrophoresis on an agarose gel.

3.2 Sequencing analysis

Three SNPs such as rs1923295, rs4756224, and rs1923298 were genotyped by cycle sequencing with ABI PRISM® 3700 DNA analyzer.

3.3 Statistical analysis

The Statistical Package for the Social Sciences (SPSS) computer program was performed for the statistical analysis of the clinical and genetic data. Chi-square (x2) or Fisher exact tests were applied for comparisons of allele and genotype frequencies between control subjects and meamphetamine-dependent patients. Analysis of the Hardy–Weinberg equilibrium (HWE), Pair-wise linkage disequilibrium (LD) and haplotype were carried out using SNPstats software (http://bioinfo.iconcologia.net/SNPstats.). All statistic significances were considered at *p*-value < 0.05 and a haplotype with a frequency lower than 3% was excluded.

Animal Studies

1. Animals and methamphetamine administration

Male Sprague-Dawley rats (250–280 g) were obtained from the National Animal Center, Mahidol University, Thailand. The animals were housed 2-3 per cage and maintained at 24±1°C under a 12 h light/dark cycle with free access to water and food. All animal procedures were carried out in compliance with Mahidol University Code of Practice and the National Institutes of Health (USA) Guidelines for treatment of laboratory animals. The protocol for this study was approved by the Animal Research Committee of Naresuan University, Thailand.

Drug treatment consisted of D-methamphetamine HCl (Alltech, IL) with permission of Ministry of Public Health. Animals in acute METH group were received vehicle for 13 days and followed with one injection of METH (8 mg/kg, i.p.) on the day 14. Animals were treated with METH for 14 days (4 mg/kg/day, i.p.) in sub-acute group. For the control group, animals were treated with vehicle for 14 days. All rats were decapitated at 24 h after the last dose. The striatum, frontal cortex and hippocampal formation were isolated, and then tissues were stored at -70 °C until used.

2. Immunodetection for glutamate N-Methyl-D-aspartate (NMDA) receptor subunit 1

NMDA receptor subunit 1 immunoreactivity (NMDAR1-IR) was determined following the method previously reported (Nudmamud-Thanoi and Reynolds 2004) Briefly, tissue was homogenized in 5 mM Tris-HCl containing 20 mM NaCl, pH 8.0 and the homogenate was then centrifuged. The pellet was homogenized again in lysis buffer. Protein concentration in the tissue lysate was estimated by the bicinchoninic acid assay (BCA) (Pierce, IL). Tissue lysate was then boiled for 5 min with an equal volume of 2x sample buffer. The supernatants containing 50 µg original protein, chosen to give results within the linear range for NMDAR1 estimations, were analyzed by polyacrylamide gel electrophoresis. The proteins were then electrotransferred on to polyvinylidene fluoride (PVDF) membranes (Amershams, IL). Membranes were incubated with protein blocking solution and subsequently incubated with NMDAR1 antibody (Sigma-Aldrich, MO). The membranes were then incubated with biotinylated secondary antibody (Vector, CA) and avidin biotinylated horseradish peroxidase complexes (ABC kit) (Vector , CA). The protein immunoreactivity was visualized using 3,3',5,5'-tetramethylbenzidine (TMB) (Promega, USA). Immunoblotting for β-actin (Santa Cruz

, CA) was used as an internal standard to confirm equal protein loading and sample transferring. Expression of NMDAR1 protein was normalized against that of β-actin.

3. Immunodetection for excitatory amino acid transporter EAAT3

Tissues were prepared as described previously (Kerdsan et al., 2009). Briefly, tissue was homogenized in 5 mM Tris-HCl containing 20 mM NaCl, pH 8.0 and the homogenate was then centrifuged. The pellet was homogenized again in lysis buffer. Protein concentration in the tissue lyses was estimated by bicinchoninic acid assay (BCA) (Pierce, Rockford, IL). Tissue lysate was then boiled at 100° C for 5 min with an equal volume of 2x sample buffer containing 250 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 20 mM dithiothreitol, 0.01% bromophenol blue. The supernatant containing 80 µg original protein, chosen to give results within the linear range for EAAT3 estimations, were separated by polyacrylamide gel electrophoresis. The proteins were then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Amersham, Arlington Heights, IL). Membranes were incubated with protein blocking solution containing 5% bovine serum albumin in phosphate buffered saline (PBS) for overnight and subsequently incubated for 3 h at room temperature with polyclonal EAAT3 antibody (Santa Cruz Biotechnology, Inc., USA). The membranes were then incubated for 2 h at room temperature with biotinylated secondary antibody and followed by incubated with avidin biotinylated horseradish peroxidase complexes (Vectastain ABC kit, Vector Laboratory, In, Burlingame, CA). The protein immunoreactivity was visualized using 3,3',5,5'-tetramethylbenzidine (TMB) (Promega, USA). Immunoblotting for β-actin (Santa Cruz , CA) was used as an internal standard to confirm equal protein loading and sample transferring. Expression of EAAT3 protein was normalized against that of β-actin.

3. Immunoreactive data analysis

The immunostained membranes were scanned into a computer and integrated optical density (IOD) was measured by Scion Image program based on NIH image (v. Alpha 4.0.3.2; www.scioncorp.com; 2000-2001). The value is the sum of the optical densities of all pixels in the region. All immunoreactive bands were measured with the same dimension to obtain their integrated optical density (Nudmamud-Thanoi and Reynolds 2004; Raudensky and Yamamoto 2007) Data were expressed as percentage of control group and all data were represented as mean ± S.E.M. Differences between groups were analyzed using ANOVA followed by Dunnett post hoc test.

4. Sperm collection

Each testis and excurrent duct was separated via midline incision. The epididymis was then dissected free of the testis and placed in pre-warm phosphate buffer saline (PBS). Blood

contamination was removed by washing the tissue in PBS. The tissue was minced with scissors to release spermatozoa.

5. Sperm morphology and concentration

The cauda sperm suspensions were diluted 1:10 with 10% neutral buffered formalin in PBS. The cauda sperm suspensions were diluted in 2% aqueous solution of eosin and sperm heads were stained, and then the spermatozoa were evaluated for individual sperm morphology. Two hundred spermatozoa per animal were evaluated by bright field microscopy (40X objective). An individual spermatozoa was classified as normal when a sperm was seen normally shaped head with normal flagellum (Figure 1A). Classifications of individual spermatozoa were abnormal when a sperm was seen as a bent neck sperm (Figure1B), a bent tail sperm (Figure 1C), a coiled tail sperm (Figure 1D), a tailless sperm (Figure 1E), and a headless sperm (Figure 1F). The concentration of spermatozoa was determined using Neuabaur's counting chamber. An aliquot of sperm suspension was charged into the counting chamber and sperms were counted. The sperm count was expressed in millions/ml.

6. Apoptotic activity study

Small pieces of testes from six animals in each group were fixed in 10% formalin in PBS. Tissues were dehydrated in graded ethanol, embedded in paraffin, and sectioned (4 μm thickness) on serial coronal plane. Apoptotic activities within the seminiferous tubules were studied using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay (Dead End™ Colorimetric TUNEL System for quantitative study and Dead End™ Fluorometric TUNEL System for qualitative study; Promega, Madison). The protocols were followed by manufacture instructions. Two slides from each animal were used for quantitative study. In cross section, 240 seminiferous tubules from each group were counted for the numbers of apoptotic cell under light microscope (40X). DNA fragmentation detected as DNA ladder in nucleus of spermatogenic cells was used as an evidence of apoptotic cell in seminiferous tubules. Apoptotic index, modified from the study of Fazlioglu et al. (2008), was defined as an average apoptotic cell per tubule (Apoptotic index = total apoptotic cells/240).

7. Statistical analysis

The data were expressed as mean ± SD and compared for statistical significance using analysis of variance (ANOVA) followed by Bonferroni's post hoc test. P value <0.05 was considered as a significant difference.

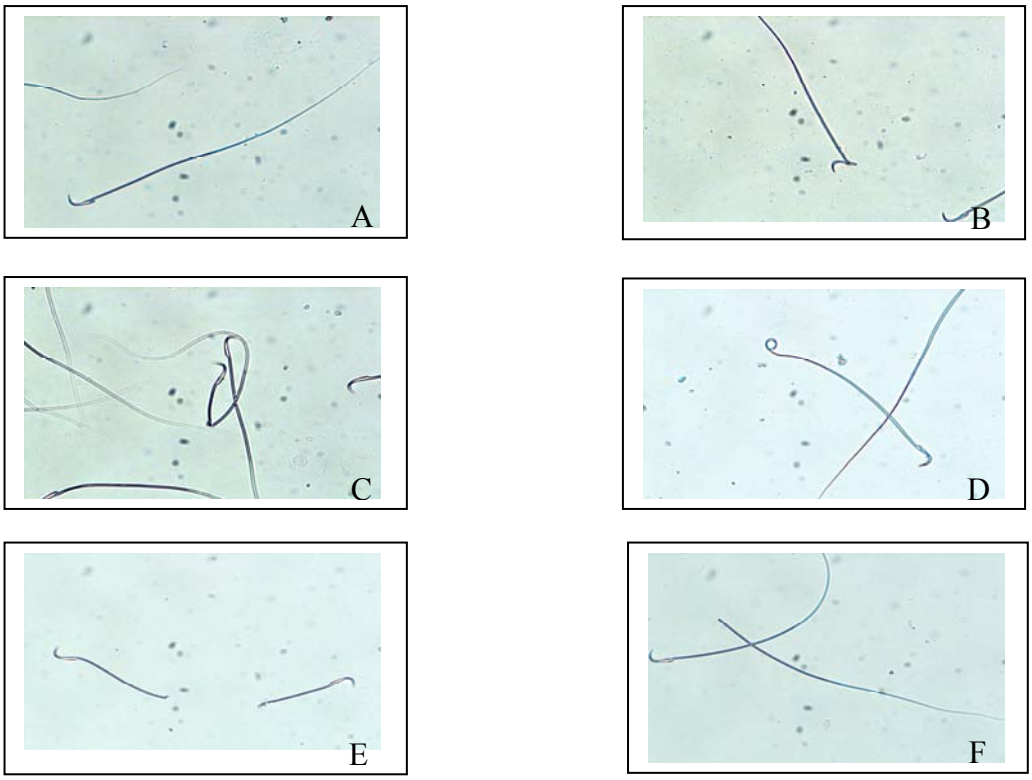

Figure 1. Sperm morphology; normal sperm (A), bent neck sperm (B), bent tail sperm (C), coiled tail sperm (D), tailless sperm (E) and headless sperm (F).

Results

Human Studies

Glutamate transporter EAAT2 gene (*SLC1A2*) polymorphisms in METH dependence

The demographic data of patients with METH dependence and control subjects are shown in Table 2. There was no significant difference between the METH dependent patients and controls in gender and age. Genotype and allelic distribution of *SLC1A2* polymorphisms are shown in Table 3, 4, 5, and 6. The distribution of the genotypes for each polymorphisms in the METH dependence and control groups were all in Hardy-Weinberg equilibrium.

For the rs1923295, rs4756224, and rs1923298 SNPs, neither the genotypes nor alleles were significantly associated with METH dependence (Table 3). There was a significant difference in the genotypic and allele frequencies of rs752949 and rs1042113 in SLC1A2 gene (Table 4). Moreover, a significant increase in the presence of A allele of rs752949 was found in METH dependent patients (96%) when compared with control subjects (81.3%) (p=0.002) (Figure 2). For the rs1042113, a significant increase in the G allele occurrence of rs1042113 was observed in METH dependence patients (39%) compared with controls (21.3%) (p=0.014) (Figure 2).

The present study has further investigated the SLC1A2 gene polymorphisms in METH dependent patients with psychosis. We found a significant difference between METH dependent patients with psychosis and control subjects in the genotypic and allelic frequencies of rs752949 and rs1042113 (data are shown in Table 4). In addition, allelic occurrence analysis in rs752949 showed a significant difference in the presence of A allele between METH dependence with psychosis and controls (*P*=0.014). Furthermore, there was a significant difference in the G allele occurrence of rs1042113 between METH dependence with psychosis and control subjects (p=0.001) (Figure 3).

For the rs4755404, there was no significant difference in either genotype or allele distribution between METH dependence and control (p=0.198; p=0.611 respectively). For the allelic occurrence analysis, although there was no significant difference in the presence of G allele of rs4755404 between METH dependence and control, a significant difference of G allele occurrence was found in METH dependence with tolerance (p=0.026), with no awareness (p=0.058), with decrease of social activity (p=0.032), and with physiological dependence (p=0.041) (Table 5).

For the rs1885343, a significant difference in genotype frequency between METH dependent patients and controls (p=0.024) was observed but not in allele frequency (p=0159).

Moreover, a significant difference in genotype distribution was also found in METH dependent patients with psychosis (p=0.007), with mental disorder (p=0.008), with tolerance (p=0.006), with withdrawal (p=0.016), with decrease of social activity (p=0.023), and with physiological dependence (p=0.004). Interestingly, a significant difference in A allele occurrence was also found in METH dependent patients (p=0.010) and in all symptoms classified (as shown in Table 6).

Table 2. Demographic data of METH dependence and control subjects

	Patients	**Controls**
Sex	male	male
Age (n: Patients = 100, Controls = 75)		
mean±SD	29.45±4.55	29.68±5.57
range	21-45 y	20-42 y
Age onset of METH dependence (n = 100)		
mean±SD	19.52±6.53	-
range	12-41 y	-
Duration of METH dependence (n = 100)		
mean±SD	9.35±5.26	-
range	1-23 y	-

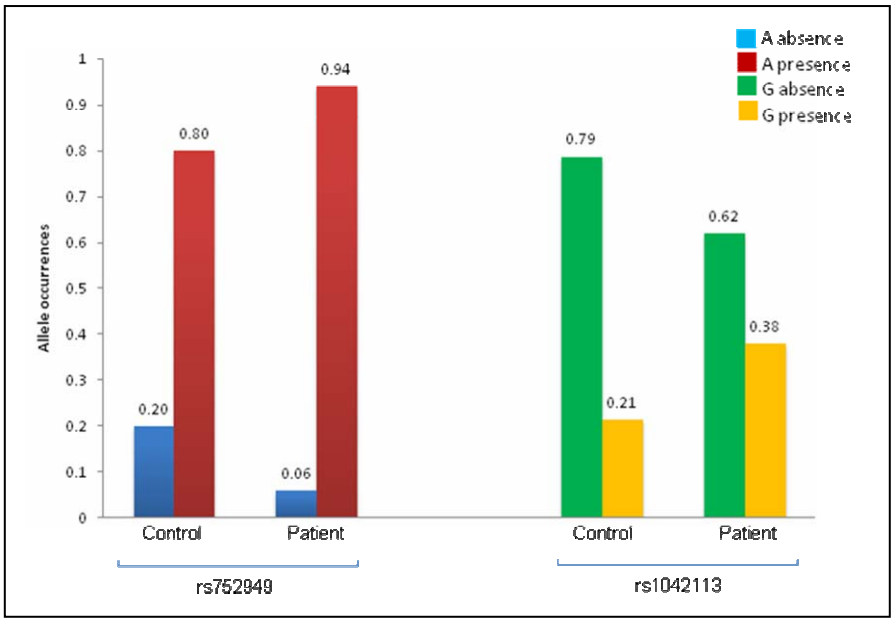

Figure 2 Occurrence of A allele in rs752949 and G allele in rs1042113 of *SLC1A2*

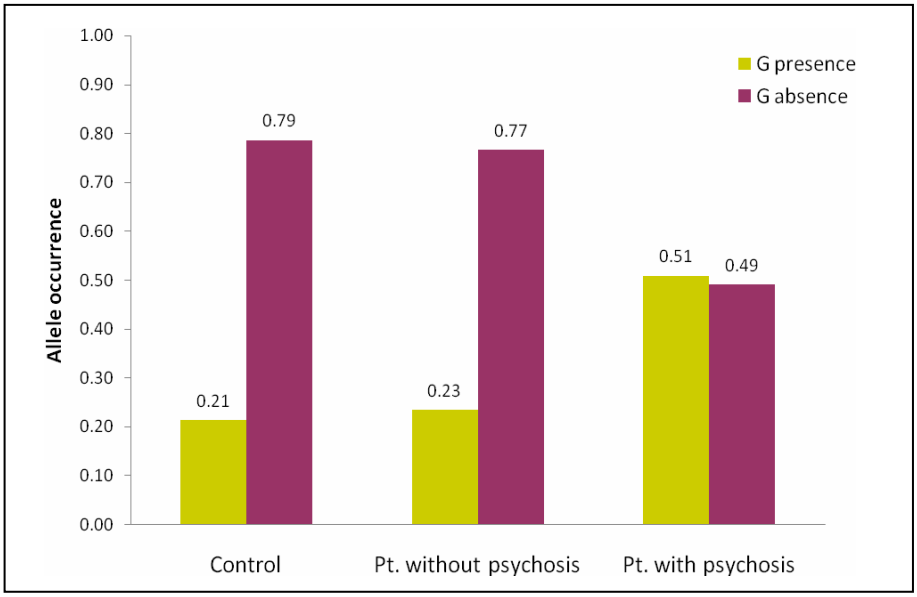

Figure 3 G allele occurrence of rs1042113 of *SLC1A2* in METH dependent patients with- and without psychosis and controls

Table 3 Genotype and allele frequencies of rs1923295, rs4756224, rs1923298 SNPs in *SLC1A2*

Polymorphisms	**n**	**Genotype count (frequency)**			***P*-value**[a]	**Allele (frequency)**		***P*-value**[a]	**HWE *P*-value**[b]
rs1923295		C/C	C/T	T/T		C	T		
Patients	48	15(31%)	25(52%)	8(17%)	0.852	55(57%)	41(43%)	1.00	0.770
with psychosis	4	3(75%)	0(0%)	1(25%)	NA	3(60%)	2(40%)	NA	
without psychosis	44	12(27%)	25(57%)	7(16%)	NA	49((56%)	39(44%)	NA	
Controls	29	8(28%)	17(59%)	4(14%)		33(57%)	25(43%)		0.450
rs4756224		A/A	A/G	G/G		A	G		
Patients	50	29(58%)	19(38%)	2(4%)	0.614	77(77%)	23(23%)	0.552	1.00
with psychosis	4	2(50%)	2(50%)	0(0%)	NA	6(75%)	2(25%)	NA	
without psychosis	46	27(59%)	17(37%)	2(4%)	NA	71(77%)	21(23%)	NA	
Controls	31	20(65%)	11(35%)	0(0%)		51(82%)	11(18%)		0.550
rs1923298		T/T	T/C	C/C		T	C		
Patients	50	32(64%)	17(34%)	1(2%)	0.762	81(81%)	19(19%)	0.392	0.670
with psychosis	4	2(50%)	2(50%)	0(0%)	NA	6(75%)	2(25%)	NA	
without psychosis	46	30(65%)	15(33%)	1(2%)	NA	75(82%)	17(18%)	NA	
Controls	30	22(73%)	8(27%)	0(0%)		52(87%)	8(17%)		1.00

[a] Fisher's exact test [b] Hardy-Weinberg equilibrium (HWE) test NA; not applicable

Table 4 Genotype and allele frequencies of rs752949 and rs 1042113 SNPs in *SLC1A2*

Polymorphism	**n**	**Genotype count (frequency)**			***P*-value** [a]	**Allele (frequency)**		***P*-value** [a]	**HWE *P*-value**
rs752949		A/A	A/G	G/G		A	G		
Patients	100	53(53%)	44(44%)	3(3%)	**0.002****	150(75%)	50(25%)	**0.014***	0.110
with psychosis	53	30(57%)	22(42%)	1(1%)	**0.009****	82(77%)	24(23%)	**0.014***	
without psychosis	47	23(49%)	22(47%)	2(4%)	0.065	68(72%)	26(28%)	0.128	
Controls	75	33(44%)	28(37%)	14(19%)		94(65%)	56(35%)		0.09
rs1042113		A/A	A/G	G/G		A	G		
Patients	100	61(61%)	37(37%)	2(2%)	**0.015***	159(8%)	41(2%)	**0.019***	0.230
with psychosis	53	26(49%)	27(51%)	0(0%)	**0.000*****	79(89%)	27(11%)	**0.002****	
without psychosis	47	35(75%)	10(21%)	2(4%)	0.615	80(85%)	14(15%)	0.329	
Controls	75	60(80%)	14(19%)	1(1%)		134(89%)	16(11%)		0.590

[a] Fisher's exact test [b] Hardy-Weinberg equilibrium (HWE) test

Significant value * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ in comparison with control

Table 5 Genotype and allele frequencies of rs4755404 SNP in *SLC1A2*

Subjects	n	Genotype frequency				Allele frequency			G Allele occurrence			C Allele occurrence		
		GG	CG	CC	*P*[a]	G	C	*P*[b]	CC	CG/GG	*P*[a]	GG	CG/CC	*P*[a]
Control	102	22 (21.6%)	54 (52.9%)	26 (25.5%)		98 (48.0%)	106 (52.0%)		26 (25.5%)	76 (74.5%)		22 (21.6%)	80 (78.4%)	
AMPH	100	20 (20.0%)	43 (43.0%)	37 (37.0%)	0.198	83 (41.5%)	101 (58.5%)	0.611	37 (37.0%)	63 (63.0%)	**0.077**[#]	20 (20.0%)	80 (80.0%)	0.784
AMPH with psychosis	53	11 (20.8%)	23 (43.4%)	19 (35.8%)	0.379	45 (42.5%)	61 (57.5%)	0.401	19 (35.8%)	34 (64.2%)	0.178	11 (20.8%)	42 (79.2%)	0.907
AMPH with mental disorder	59	12 (20.3%)	27 (45.8%)	20 (33.9%)	0.513	51 (43.2%)	67 (56.8%)	0.419	20 (33.9%)	39 (66.1%)	0.255	12 (20.3%)	47 (79.7%)	0.854
AMPH with abuse	79	16 (20.3%)	35 (44.3%)	28 (35.4%)	0.334	67 (42.4%)	91 (57.6%)	0.290	28 (35.4%)	51 (64.6%)	0.147	16 (20.3%)	63 (79.7%)	0.829
AMPH with tolerance	75	13 (17.3%)	31 (41.3%)	31 (41.3%)	**0.083**[#]	57 (38.0%)	93 (62.0%)	**0.066**[#]	31 (41.3%)	44 (58.7%)	***0.026**	13 (17.3%)	62 (82.7%)	0.485
AMPH with withdrawal	87	18 (20.7%)	38 (43.7%)	31 (35.6%)	0.294	74 (42.5%)	100 (57.5%)	0.301	31 (35.6%)	56 (64.4%)	0.130	18 (20.7%)	69 (79.3%)	0.833
AMPH with no awareness	86	16 (18.6%)	37 (43.0%)	33 (38.4%)	0.164	69 (40.1%)	103 (59.9%)	0.145	33 (38.4%)	53 (61.6%)	**0.058**[#]	16 (18.6%)	70 (81.4%)	0.614
AMPH with decrease of social activity	60	10 (16.7%)	25 (41.7%)	25 (41.7%)	0.101	45 (37.5%)	75 (62.5%)	**0.082**[#]	25 (41.7%)	35 (58.3%)	**0.032***	10 (16.7%)	50 (83.3%)	0.449
AMPH with physiological dependence	64	11 (17.2%)	27 (42.2%)	26 (40.6%)	0.123	49 (38.3%)	79 (61.7%)	**0.089**[#]	26 (40.6%)	38 (59.4%)	**0.041***	11 (17.2%)	53 (82.8%)	0.491

[a] Chi-square test

[b] Fischer's Exact test

Significant value [*] $P < 0.05$; [**] $P < 0.01$; [#] $P < 0.1$ in comparison with control

Table 6 Genotype and allele frequencies of rs1885343 SNP in *SLC1A2*

Subjects	n	Genotype frequency				Allele frequency			A Allele occurrence			G Allele occurrence		
		AA	AG	GG	P^a	A	G	P^b	GG	AG/AA	P^a	AA	AG/GG	P^a
Control	102	34 (33.3%)	56 (54.9%)	12 (11.8%)		124 (60.8%)	80 (39.2%)		12 (11.8%)	90 (88.2%)		34 (33.3%)	68 (66.7%)	
AMPH	100	33 (33.0%)	41 (41.0%)	26 (26.0%)	**0.024***	107 (53.5%)	93 (46.5%)	0.159	26 (26.0%)	74 (74.0%)	**0.010***	33 (33.0%)	67 (67.0%)	0.960
AMPH with psychosis	53	19 (35.8%)	18 (34.0%)	16 (30.2%)	**0.007****	56 (52.8%)	50 (47.2%)	0.184	16 (30.2%)	37 (69.8%)	**0.005****	19 (35.8%)	34 (64.2%)	0.754
AMPH with mental disorder	59	22 (37.3%)	20 (33.9%)	17 (28.8%)	**0.008****	64 (54.2%)	54 (45.8%)	0.291	17 (28.8%)	42 (71.2%)	**0.007****	22 (37.3%)	37 (62.7%)	0.612
AMPH with abuse	79	24 (30.4%)	36 (45.6%)	19 (24.1%)	0.090	84 (53.2%)	74 (46.8%)	0.164	19 (24.1%)	60 (75.9%)	**0.030***	24 (30.4%)	55 (69.6%)	0.673
AMPH with tolerance	75	26 (34.7%)	27 (36.0%)	22 (29.3%)	**0.006****	79 (52.7%)	71 (47.3%)	0.130	22 (29.3%)	53 (70.7%)	**0.003****	26 (34.7%)	49 (65.3%)	0.853
AMPH with no awareness	86	30 (34.9%)	36 (41.9%)	20 (23.3%)	0.072**#**	96 (55.8%)	76 (44.2%)	0.346	20 (23.3%)	66 (76.7%)	**0.037***	30 (34.9%)	56 (65.1%)	0.823
AMPH with withdrawal	87	28 (32.2%)	35 (40.2%)	24 (27.6%)	**0.016***	91 (52.3%)	83 (47.7%)	0.118	24 (27.6%)	63 (72.4%)	**0.006****	28 (32.2%)	59 (67.8%)	0.867
AMPH with decrease of social activity	60	22 (36.7%)	22 (36.7%)	16 (26.7%)	**0.023***	66 (55.0%)	54 (45.0%)	0.350	16 (26.7%)	44 (73.3%)	**0.015***	22 (36.7%)	38 (63.3%)	0.667
AMPH with physiological dependence	64	22 (34.4%)	22 (34.4%)	20 (31.3%)	**0.004****	66 (51.6%)	62 (48.4%)	0.111	20 (31.3%)	44 (68.8%)	**0.002****	22 (34.4%)	42 (65.6%)	0.890

[a] Chi-square test

[b] Fischer's Exact test

Significant value * $P < 0.05$; ** $P < 0.01$; # $P < 0.1$ in comparison with control

Animal Studies

Expression of glutamate NMDA receptor after METH administration

Immunostaining for NMDAR1 consistently demonstrated an immunoreactive band corresponding to approximately 116 kDa and β-actin immunoreactive band was approximately shown 43 kDa. Densitometric analysis was performed to quantify the alteration of NMDAR1 expression relative to β-actin immunoreactivity. ANOVA demonstrated that NMDAR1-IR was significantly increased above control in the striatum in both acute (p=0.025) and sub-acute (p=0.023) METH administration, and reached about 196% and 198% of control group, respectively (Figure 4). Moreover, a significant increase of NMDAR1-IR (508% over control) was also observed in the frontal cortex in sub-acute METH (p=0.036), but not in acute group (p=0.580) (Figure 5). However, there were no significant differences of NMDAR1 levels in hippocampal formation either in acute (p=0.839) or sub-acute (p=0.711) METH groups when compared with control group (Figure 6).

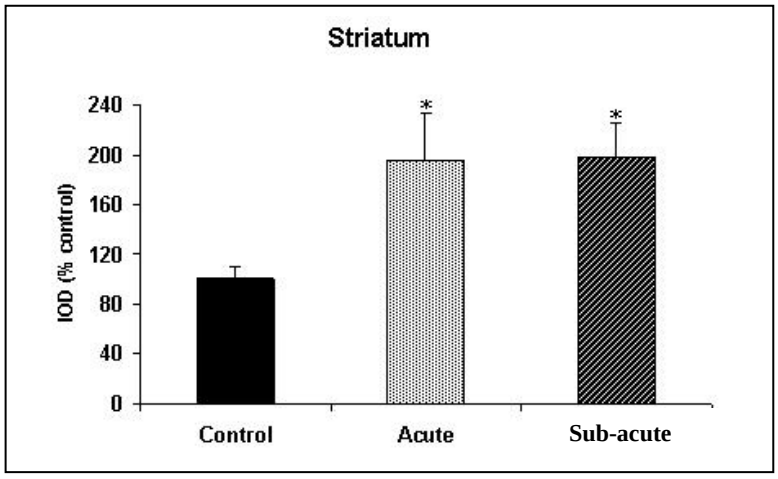

Figure 4. Expression of NMDAR1 in striatum. Data are integrated optical density of NMDAR1 immunoreactivity band, normalized to β-actin levels and expressed as percentage of the control group. Values represent Mean ± S.E.M (n=5-7). *p<0.05 in comparison with the control group by one-way ANOVA with Dunnett post hoc test.

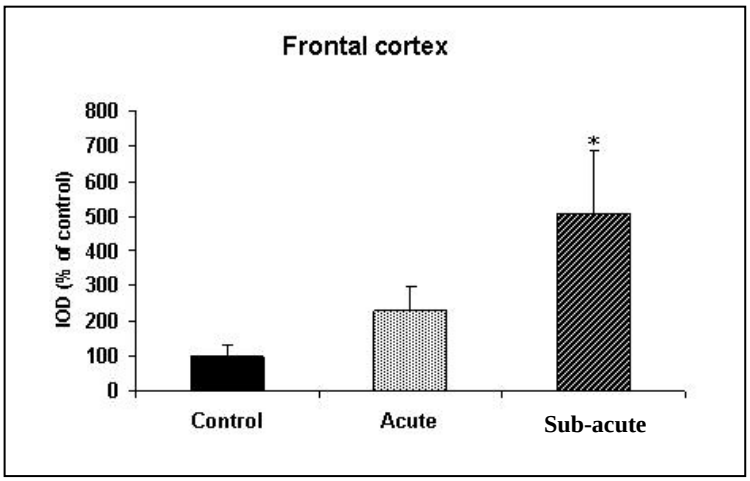

Figure 5. Expression of NMDAR1 in frontal cortex. Data are integrated optical density of NMDAR1 immunoreactivity band, normalized to β-actin levels and expressed as percentage of the control group. Values represent Mean ± S.E.M (n=5-7). **p*<0.05 in comparison with the control group by one-way ANOVA with Dunnett post hoc test.

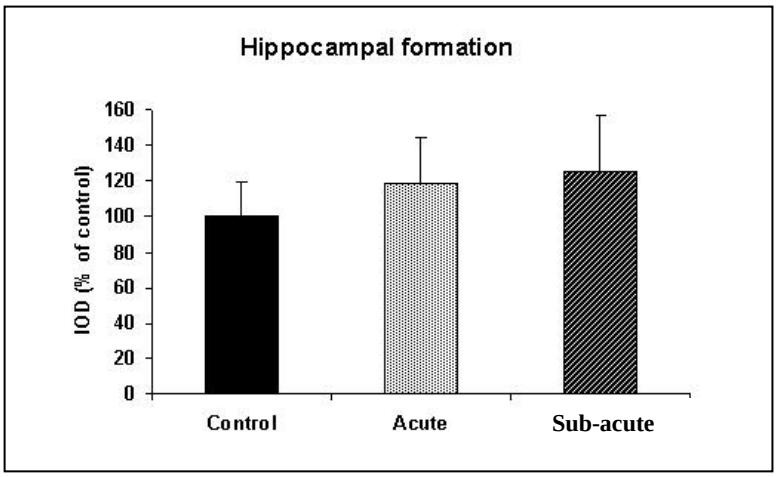

Figure 6. Expression of NMDAR1 in hippocampal formation. Data are integrated optical density of NMDAR1 immunoreactivity band, normalized to β-actin levels and expressed as percentage of the control group. Values represent Mean ± S.E.M (n=5-7).

Expression of glutamate transporter EAAT3 after METH administration

Immunostaining for EAAT3 consistently demonstrated an immunoreactive band corresponding to approximately 70 kDa and a β-actin immunoreactive band at approximately 43 kDa.. Densitometric analysis was performed to quantify the alteration of EAAT3 expression relative to β-actin immunoreactivity. ANOVA demonstrated a significant effect of METH treatment on EAAT3-IR in the hippocampal formation (F=12.253, *p*=0.001), striatum (F=11.994, *p*=0.002) and frontal cortex (F=5.469, *p*=0.0.021). A significant increase in EAAT3-IR above the control values was found in hippocampal formation (*p*=0.001) in the sub-acute group but not in the acute group (*p*=0.886) (Figure 7). On the other hand, a significant decrease in EAAT3-IR was found in striatum in both the acute (*p*=0.002) and sub-acute (*p*=0.011) groups (Figure 8). A significant decrease in EAAT-IR was also found in frontal cortex in the sub-acute group (p=0.014) and a decrease of EAAT3-IR was observed in the acute group but which did not reach statistical significance (*p*=0.070) (Figure 9).

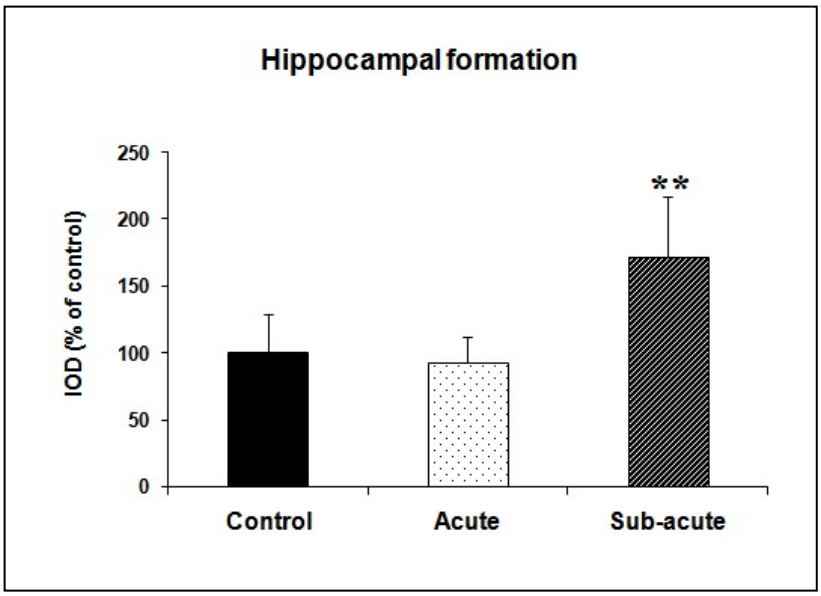

Figure 7. Expression of EAAT3 in hippocampal formation. Data are integrated optical density of EAAT3 immunoreactivity band, normalized to β-actin levels and expressed as percentage of the control group. Values represent Mean ± S.E.M (n=5-7). ***p*<0.01 in comparison with the control group by one-way ANOVA with Dunnett post hoc test.

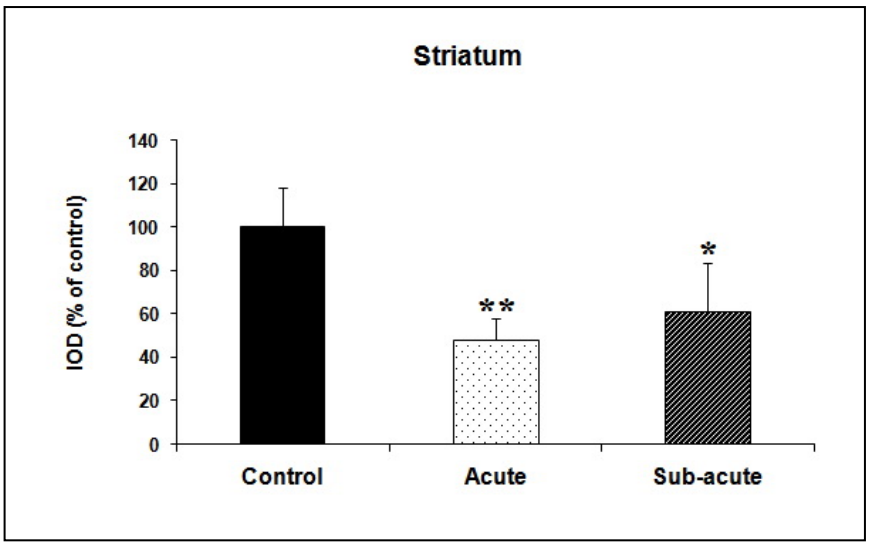

Figure 8. Expression of EAAT3 in striatum. Data are integrated optical density of NMDAR1 immunoreactivity band, normalized to β-actin levels and expressed as percentage of the control group. Values represent Mean ± S.E.M (n=5-7). *p<0.05, **p<0.01 in comparison with the control group by one-way ANOVA with Dunnett post hoc test

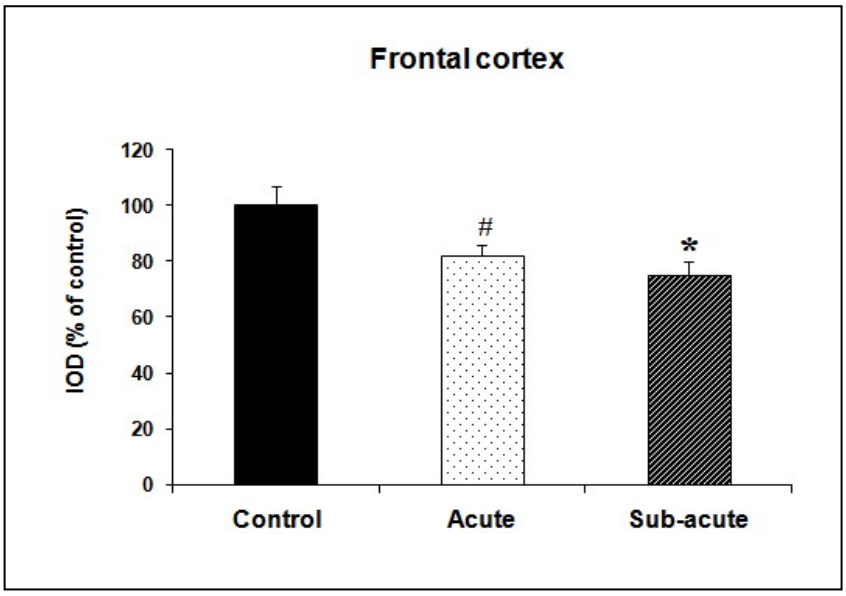

Figure 9. Expression of EAAT3 in frontal cortex. Data are integrated optical density of NMDAR1 immunoreactivity band, normalized to β-actin levels and expressed as percentage of the control group. Values represent Mean ± S.E.M (n=5-7). *p<0.05 #p<0.1 in comparison with the control group by one-way ANOVA with Dunnett post hoc test

Sperm morphology

Percentage of normal sperm morphology was decreased in animals treated with METH. It was significantly lower ($p<0.001$) in acute treated group (71.33 ± 7.86 %) when compared with untreated animals (94.50 ± 2.17 %) (Figure 10).

Sperm concentration

Total number of cauda epididimal sperm counts were significantly decreased in acute group (106.67 ± 41.01 × 10^6 cells/ml) and chronic group (122.33 ± 13.71 × 10^6 cells/ml) when compared with control group (185.00 ± 9.67 × 10^6 cells/ml) (Figure 11).

Apoptotic cell studies

The present study showed that METH administration can induce apoptotic cell activities within seminiferous tubules. The appearance of TUNEL-positive cells in seminiferous tubules in animals treated with METH were detected in almost every stage of sperm development especially in the spermatogonia lining along the basement membrane of the tubules in animals treated with methamphetamine in both acute and chronic groups (Figure 12). Qualitatively, the proportions of apoptotic cells in the tubules of animal treated with METH were larger than the control group.

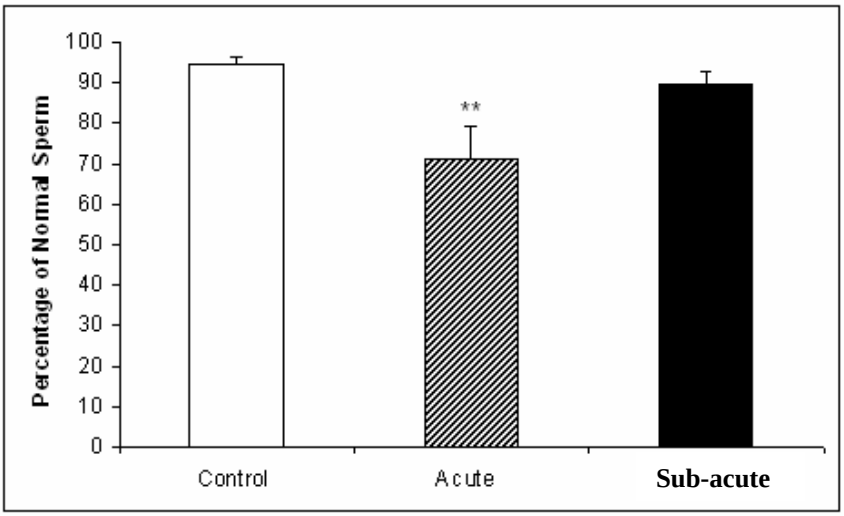

Figure 10. Percentage of normal sperm morphology of male rats after treated with various doses of methamphetamine compared with the control group. Values are means and SD, N = 6. ** Significantly different from the control group at $p<0.001$ by ANOVA post hoc Dunnett test.

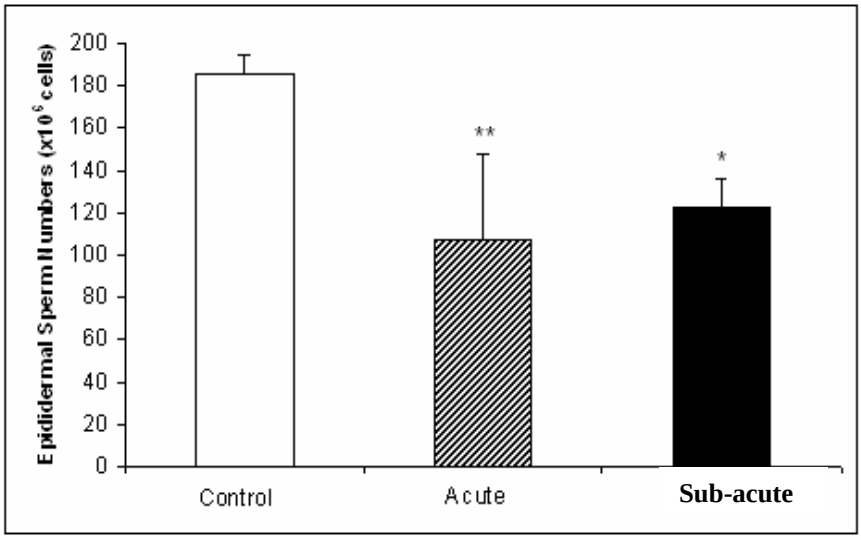

Figure 11. Cauda epididymal sperm number (x10^6 cells/ml) of male rats after treated with various doses of methamphetamine compared with the control group. Values are means and SD, N = 6. ** Significantly different from the control group at p<0.001, and * significantly different from the control group at p= 0.001 by ANOVA post hoc Dunnett test.

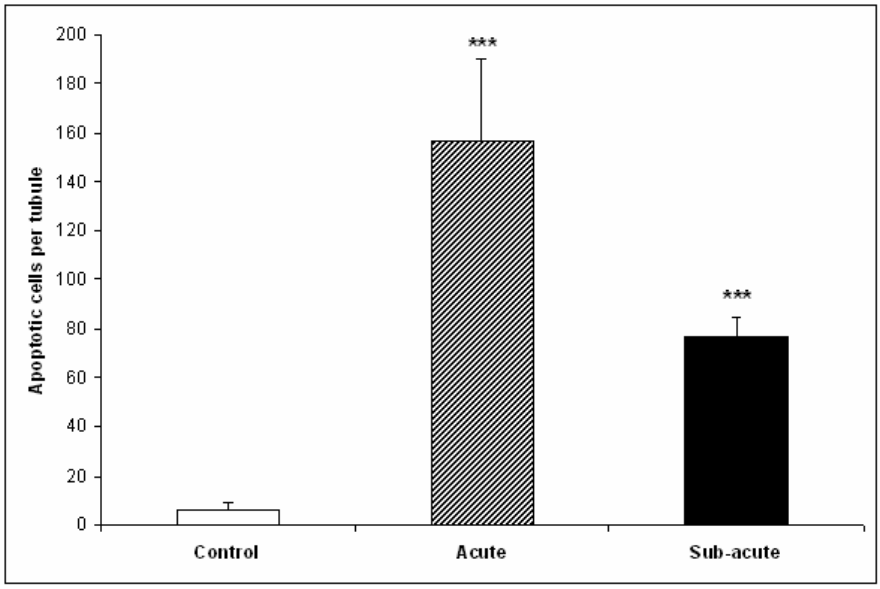

Figure 12. Apoptotic index (number of apoptotic cells per tubule) of male rats after treated with various doses of methamphetamine compared with the control group. Values are means and SD, N = 6. ** Significantly different from the control group at p<0.001 by ANOVA post hoc Dunnett test.

Discussion

Association between glutamate transporter EAAT2 gene (*SLC1A2*) polymorphisms and METH dependence

The results of this study demonstrated that the glutamate transporter EAAT2 gene (*SLC1A2*) polymorphisms were associated with vulnerability to METH dependence. The genetic variation of *SLC1A2* in the present study included seven candidate SNPs: rs1923295 (near the 5' end of the gene), rs4755404 (intron), rs4756224 (intron), rs1885343 (intron), rs1923298 (intron), rs752949 (exon5) and rs1042113 (exon8), which has been mapped to 11p13-p12 (Deng et al., 2004). Previous studies of the *SLC1A2* genetic polymorphism and drug addiction have demonstrated in alcoholics (Sander et al., 2000 and Foley et al., 2004). Our finding also provide a strong support the association of polymorphisms in *SLC1A2* in drug dependence especially METH dependence.

The findings of the present study also revealed that the glutamate transporter EAAT2 gene (*SLC1A2*) polymorphisms such as rs752949, rs1042113, rs1885343 were associated with METH dependence with psychosis. The prevalence of A allele of rs752949 was found to be elevated in METH dependence with psychosis. The result suggests that the presence of A allele of rs752949 may be a risk factor for drug addiction with susceptibility to psychosis. The G allele of rs1042113 also showed an apparently risk for METH dependence with psychosis. The A allele occurrence of rs1885343 was additionally shown to be a risk factor for METH dependence with psychosis.

The present study indicated that there was no association between rs1923295, rs4756224, and rs1923298 in *SLC1A2* and METH dependence. The result is consistent with previous studies which found no association of those three SNPs with schizophrenic patients (Deng et al., 2004 and Nagai et al., 2009). Together with our findings, it is possible that a single SNP of *SLC1A2* may be not associated with the disease but there may be an association between multiple SNPs in *SLC1A2* and the disease. Therefore, we employed a haplotype analysis (data not shown) in this study. The present study found a significant haplotype association of rs1923295- rs4756224 -rs1923298 (CGC) and rs752949- rs1042113 (AG) in METH dependent patients which is notable higher than controls. The results provide evidence to support that a combination of SNPs in *SLC1A2* might be possible to be the susceptibility locus for pathogenesis of drug dependence.

In summary, our results indicate an association of polymorphisms in *SLC1A2* in METH dependence. Moreover, the difference of allele occurrence may be a risk factor for METH

dependence with psychosis. The results of the present study provide evidence to support the hypothesis of association of *SLC1A2* genetic polymorphisms in drug dependence.

Alteration of Glutamate NMDA receptor subunit 1 expression after METH administration

The present study revealed that METH administration induced a significant increase of NMDAR1-IR in the cortical and subcortical regions of rat brain. The effect was observed in the frontal cortex after sub-acute METH. Moreover, the results showed an upregulation of NMDAR1 in the striatum in both acute and sub-acute METH administrations. Our findings demonstrate a significant response to METH-induced glutamate release in the striatum (Mark et al., 2004; Mark et al., 2007) and cerebral cortex (Shoblock et al., 2003). As METH is an abused psychostimulant, our results provide a support of psychostimulant effects on glutamatergic system. Dysfunctions of glutamatergic system may be derived from changes in the functions of dopaminergic system which are induced by psychostimulant effects (Baker et al., 2003; Karler et al, 2003). Moreover, an impairment of monoamine neurotransmission such as dopamine after repeated METH produces changes in amino acid homeostasis, probably leading to an overstimulation of NMDA receptor (Bustamante et al., 2002).

The results of this study are in addition in agreement with the previous reports on a raise in NMDA receptor binding in cerebral cortex following psychostimulants either METH (Eisch et al., 1996) or cocaine (Itzhak, 1994), suggesting the mechanisms of NMDA receptor involved in the development of drug addiction.

Supporting a hypothesis of glutamatergic dysfunction in cortical and subcortical regions following psychostimulant drug exposure, we previously observed an upregulation of NMDAR1 density in dentate gyrus following pseudoephedrine, a drug which has been considered to be a psychostimulant, administration (Nudmamud-Thanoi et al., 2006). Moreover, increased metabotropic glutamate receptor (mGluR5) protein and gene expression have been reported in the cultured cortical and hippocampal neurons after amphetamine administration (Yu et al., 2001). An increase in vesicular glutamate transporter (VGLUT1), an important component of glutamatergic neurons that regulates glutamate release, has been observed in striatal and cortical regions after METH administration, supporting an evidence which of METH-induced an elevation of glutamate release in striatum and cortex (Mark et al., 2007).

Although no alteration in NMDAR1-IR in the hippocampal formation was observed in the present study after METH treatment, the finding is consistent with previous study that has revealed no change and a slightly decrease of NMDAR1 mRNA expression in dentate gyrus after exposure to morphine and cocaine, respectively (Turchan et al., 2003). Moreover, no

differences in the NMDAR1, 2A and 2B immunoreactivities were observed in the hippocampus of METH-sensitized rats (Yamamoto et al., 1999). Taken together, our observations provide further support for a regional specific of glutamatergic dysfunction after METH administration. However, this result needs to be repeated in subregions of hippocampal formation as it is known that the hippocampal formation composed of three compartments including dentate gyrus, hippocampus proper (CA1-CA3), and subiculum, which can communicate and form interconnections within hippocampal formation on with other brain regions (Witter, 2003).

In summary, our results indicate a compensatory up-regulation of NMDA receptor expression reflecting reduced glutamatergic function. The alterations in NMDAR1 receptor found in the striatum and frontal cortex suggest that other components of glutamate synapse may be abnormal in these regions in METH abuse. Further work is still needed to study other components of glutamatergic neurotransmission in order to investigate abnormalities of glutamatergic system in METH dependence.

Alteration of Glutamate transporter EAAT3 expression after METH administration

The present study revealed different alterations of EAAT3-IR in hippocampal formation, striatum and frontal cortex after acute and chronic METH administration. It was shown that acute and sub-acute METH administration decreased EAAT3-IR in the striatum, although a reduction seen in frontal cortex did not reach statistical significance compared with control. In contrast, an increase in EAAT3 protein level following repeated administration of METH was observed in hippocampal formation. Similar with the present study, the differential changes in glutamatergic neurotransmission has been reported in the hippocampus and striatum in the METH-sensitized rats (Yamamoto et al., 1999).

Our findings showed an upregulation of EAAT3 protein following repeated administration of METH in hippocampal formation which may contribute to an elevation in extracellular glutamate level in hippocampal formation via the action in the reverse direction (Rocher and Gardier, 2001; Raudensky and Yamamoto, 2006). It is well known that the hippocampal formation consist of three compartments including dentate gurus, hippocampus proper (CA1-CA3), and subiculum. These compartments form connections within hippocampal formation which receive major cortical inputs from the entorhinal cortex and send dense direct projection or indirect reciprocity to the prefrontal cortex (Witter, 2003; Kelly, 2004). Therefore, the upregulation of EAAT3 in hippocampal formation may reflect adaptive changes in neuronal glutamate transporter activity within each of these compartments of this brain region after exposure to METH.

Decreased EAAT3 levels in striatum and frontal cortex after METH administration in this study may be a response of excitotoxicity induced neuronal cell death (Deng and Cadet, 2000; Deng et al., 2001;Xu et al., 2005; Zhu, 2006). Moreover, this finding may be supported by recent report that effect of METH can produce an increase in glutamate release in the corticostriatal pathway which can lead to exitotoxicity (Mark et al., 2004). Therefore, these findings provide further support for glutamatergic dysfunction in METH dependence, with abnormalities involving a transporter which contributes to maintaining appropriate synaptic glutamate levels.

In conclusion, the present results provide evidence to support the hypothesis of glutamatergic system involvement in the drug dependence. Moreover, our findings suggest deficits of cortico-striatal innervation and may contribute to the cognitive dysfunction related to hippocampus in drug dependence. However, further studies of other glutamatergic markers would be valuable to determine if these changes of EAAT3 expression in striatum and frontal cortex reflect deficits in glutamatergic cortico-striatal neurons.

METH induce abnormal sperm morphology

METH can induce abnormal sperm morphology, decrease sperm concentration and induce apoptotic cell activities in the seminiferous tubules in male rats. The effect of METH in sperm morphology and sperm concentration seems to be dose dependent as seen in animals treated with high dose acutely. The result is consistent with the previous report that percentage of apoptotic tubules increased with dose dependent (Yamamoto et al. 2002). Decreased normal sperm morphology and low sperm concentrations after treated with METH may be involved in its effect on inducing apoptotic activity in seminiferous tubules. The finding of the present study indicates that METH administration induce apoptosis within the seminiferous tubules of male rats after acute and sub-acute treatments. It has been reported that METH induces apoptosis in seminiferous tubules in male mice testis 24 hours after a single treatment with 5, 10, and 15 mg/kg METH, genetoxic effect of METH seems to be involved especially in spermatogenesis (Yamamoto et al. 2002). Recently, repeated METH administration can cause a decrease in cell proliferation, induce apoptosis and reduce the proliferation/apoptosis ratio in male rat germ cells (Alavi et al. 2008). In addition, it has been suggested that serum testosterone concentration seemed to be changed during the time course of METH treatment (Shin et al 1999). It has also been suggested that amphetamine had an effect in reducing the release of testosterone from the testis (Tsai et al. 1996). It has also been reported that apoptosis was increased in both spermatogonia and primary

spermatocytes after repeated administration of METH (Alavi et al. 2008). Qualitatively, the results of the present study also indicated apoptotic activity in the nucleus of leydig cell in rats treated with METH. Apoptosis in leydig cells may influence the changes of testosterone concentration in the testis leading to apoptosis of spermatogenic cells. Testosterone has been reported to suppress apoptosis in human seminiferous tubule (Erkkila et al. 1997). A decrease in testosterone concentration can induce an increase in the number of apoptotic germ cells in most stages of the cycle of the seminiferous epithelium in rats (Henriksen et al. 1995). In addition, dihydrotestosterone, an end metabolite of testosterone, can inhibit testicular apoptosis in human (Pentikainen et al. 2006).

In conclusion, METH can induce abnormal sperm morphology, low sperm concentration and apoptosis in seminiferous tubules of rats after acute and sub-acute administrations. The results suggest that methamphetamine not only works as drug of abuse in CNS, but also genotoxic in gametogenesis. The changes in testosterone concentration during that time course may play some role in abnormalities of sperm that occurred during spermatogenesis, and may also play a function in triggering the apoptosis in the spermatogenic cells. This study thus provides valuable information for further investigation of the mechanism of METH in reproductive toxicity during spermatogenesis.

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Output ที่ได้จากโครงการ

1. **ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ** (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) พร้อมแจ้งสถานะของการตีพิมพ์ เช่น submitted, accepted, in press, published

   1.1 Kerdsan W, Thanoi S and **Nudmamud-Thanoi S.** Changes in glutamate/NMDA receptor subunit 1 expression in rat brain after acute and subacute exposure to methamphetamine. Journal of Biomedicine and Biotechnology. 2009; 2009 (329631) 4 pages. (ภาคผนวก เอกสารแนบหมายเลข 1)

   1.2 **Nudmamud-Thanoi S** and Thanoi S. Methamphetamine induces abnormal sperm morphology, low sperm concentration and apoptosis in the testis of male rats Andrologia (In press) (ภาคผนวก เอกสารแนบหมายเลข 2)

   1.3 Kerdsan W, Thanoi S and **Nudmamud-Thanoi S.** Changes in Neuronal Glutamate Transporter EAAT3 Expression in Rat Brain after Exposure to Methamphetamine**.** Neuroscience Letters (revised manuscript submitted for publication) (ภาคผนวก เอกสารแนบหมายเลข 3)

2. **การนำผลงานวิจัยไปใช้ประโยชน์**

   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
   จากผลงานวิจัยสามารถสร้างความร่วมมือกับผู้ทรงคุณวุฒิต่างประเทศ โดยได้รับทุนวิจัยจาก British Council PMI2 Connect Research Co-operation Award ร่วมกับ Professor Gavin Reynolds ((ภาคผนวก เอกสารแนบหมายเลข 4)

   - **เชิงวิชาการ** (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

   จากความต่อเนื่องของผลงานวิจัยสามารถนำมาพัฒนาการเรียนการสอนและการวิจัยในระดับปริญญาโท-เอก สาขากายวิภาคศาสตร์ และวิทยาศาสตร์การแพทย์ โดยได้รับการคัดเลือกให้รับ

   - ทุนปริญญาเอกในประเทศเพื่อพัฒนาอาจารย์ที่ปรึกษา โครงการเครือข่ายเชิงกลยุทธ์เพื่อการผลิตและพัฒนาอาจารย์ในสถาบันอุดมศึกษา หลักสูตรปริญญาเอกในประเทศ ประจำปี 2550
   - ทุนโครงการปริญญาเอกกาญจนาภิเษก (คปก.) รุ่นที่ 11 และ รุ่นที่12

3. อื่น ๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

Thanoi S, Adthapanyawanich K, **Nudmamud-Thanoi S**. Methamphetamine Can Induce Apoptosis in Seminiferous Tubules, but not in Ovarian Follicles. The 29th Annual Meeting of the Society of Anatomy of Thailand, 2006; p31.

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Nudmamud-Thanoi S, Kerdsan W, Watiktinkorn P, Thanoi S. Association between the excitatory amino acid transporter 2 gene polymorphism and amphetamine-like dependence TRF Meeting (for Young Scientist), 2009.