BEHAVIORAL AND MOLECULAR STUDY OF METHAMPHETAMINE USE AND ADDICTION IN A RAT MODEL

SIM MAW SHIN

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
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SIM MAW SHIN

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ABSTRACT

Methamphetamine (METH) is a highly addictive psycho-stimulant that induces behavioral changes, most likely due to high level of METH-induced dopamine in the brain. Nucleus accumbens (NAc) and hippocampus are the critical part of the brain in which the changes occur in drug addiction. However, little is known about the underlying molecular mechanisms of METH-induced addiction on miRNA, transcriptome and protein expression. The main objectives of this study are to study the behavioral changes that occur with use and addiction to METH and to determine the global miRNA, transcriptome and protein profiling in the NAc and hippocampus of METH-addicted rats, and also to identify miRNAs, genes and proteins which are associated with METH exposure and addiction. The experimental rats were divided into 3 groups of 6 each: a control group, a single dose METH (5 mg/kg, i.p.) treatment group and a continuous 15 alternate days METH (0.25, 0.5, 1, 2, 3, 4, 5 mg/kg) treatment group. Addiction behavior in rats was determined using Conditioned Place Preference (CPP) task. The analysis of the miRNA and transcriptome profiling in the NAc and hippocampus was performed using Affymetric microarray GeneChip® System, while the protein profiling was performed using Thermo Scientific LTQ Orbitrap mass spectrometer. The results of the study showed that the continuous 15 alternate days METH treatment rats showed a preference for the drug-paired compartment of the CPP. However, a one-time acute treatment with 5 mg/kg METH did not show any significant difference in preference when compared with the control group. Addiction behavior was only seen when rats consumed increasing doses of METH over a continuous exposure period. We also found that the continuous exposure with administration of increasing doses may reduce the learning and memory ability in rats. Differential molecular profiling indicated that 170 miRNAs, 3 genes and 15 proteins were up-regulated, while

4 miRNAs, 26 genes and 27 proteins were down-regulated in the NAc when the continuous METH treatment group was compared with the controls. In comparing the METH addiction group (continuous treatment) with the non-addiction group (acute treatment), 38 miRNAs, 6 genes and 25 proteins were shown to be up-regulated and 4 miRNAs, 32 genes and 15 proteins were down-regulated. In the hippocampus, 180 miRNAs, 8 genes and 28 proteins were up-regulated, while 10 miRNAs, 13 genes and 5 proteins were down-regulated when the continuous treatment group was compared with the controls. In comparing the addiction group with those without addiction, 104 miRNAs, 10 genes and 33 proteins were shown to be up-regulated and 5 miRNAs, 20 genes and 20 proteins were down-regulated. The level of significance applied was when changes were more than two fold change and with ANOVA and FDR test with $p<0.05$. Our results suggest that the rat model that has been developed is adequate as an addiction behavior model for METH, and that dynamic changes occur in the expression of mRNAs, miRNAs and proteins with METH exposure and addiction.

ABSTRAK

Metamfetamin (METH) merupakan sejenis ubat psiko-perangsang yang menimbulkan ketagihan dan penggunaannya boleh menyebabkan perubahan tingkah laku yang kemungkinan besar disebabkan oleh peningkatan dopamin dalam otak. Nukleus akamben (NAc) dan hipokampus adalah bahagian otak yang penting di mana perubahan berlaku dalam keadaan penagihan dadah. Walau bagaimanapun, tidak banyak yang diketahui mengenai mekanisme molekul asas bagi ketagihan METH dalam hal berkaitan ekspresi miRNA, transkriptom dan protein. Objektif kajian ini adalah untuk mengkaji perubahan tingkah laku dalam penggunaan and ketagihan METH dan menentukan profil global miRNA, transkriptom dan protein dalam NAc dan hipokampus tikus yang mengalami ketagihan METH, dan juga untuk mengenalpasti hubungan antara miRNA, gen dan protein dengan penggunaan dan ketagihan metamfetamin. Tikus telah dibahagikan kepada 3 kumpulan dan terdapat 6 ekor tikus dalam setiap kumpulan: kumpulan kawalan, kumpulan yang menerima dos tunggal metamfetamin (5 mg/kg, i.p.) dan kumpulan yang menerima metamfetamin (0.25, 0.5, 1, 2, 3, 4, 5 mg/kg) yang berterusan selama 15 hari selang hari. Simptom ketagihan pada tikus telah ditentukan dengan menggunakan kaedah Conditioned Place Preference (CPP). Analisis profil miRNA dan transkriptom dalam NAc dan hipokampus telah dilakukan dengan menggunakan sistem Affymetrix GeneChip® microarray, manakala profil protein ditentukan dengan menggunakan Thermo Scientific LTQ Orbitrap mass spectrometer. Keputusan kajian menunjukkan bahawa tikus yang menerima rawatan METH secara berterusan selama 15 hari selang hari menunjukkan keutamaan untuk petak CPP yang diberi METH. Walau bagaimanapun, rawatan tunggal dengan METH 5 mg/kg tidak menunjukkan sebarang perbezaan yang signifikan dalam CPP berbanding dengan kumpulan kawalan. Gejala ketagihan hanya diperhati apabila tikus diberi dos

METH yang meningkat dalam tempoh pendedahan yang berterusan. Kami juga mendapati bahawa pendedahan yang berterusan bersama dengan pemberian METH dalam dos yang meningkat boleh mengurangkan keupayaan pembelajaran dan ingatan pada tikus. Perbezaan profil yang dijalankan menunjukkan bahawa 170 miRNAs, 3 gen dan 15 protein mempunyai peningkatan regulasi, manakala 4 miRNAs, 26 gen dan 27 protein menunjukkan penurunan regulasi pada NAc apabila berbanding kumpulan rawatan berterusan METH dengan kumpulan kawalan. Apabila membandingkan kumpulan ketagihan METH (rawatan berterusan) dengan kumpulan tiada ketagihan (rawatan tunggal), 38 miRNA, 6 gen dan 25 protein menunjukkan peningkatan regulasi, manakala 4 miRNA, 32 gen dan 15 protein menunjukan penurunan regulasi. Dalam hipokampus, 180 miRNA, 8 gen dan 28 protein menunjukan peningkatan regulasi, manakala 10 miRNA, 13 gen dan 5 protein menunjukan penurunan regulasi apabila berbanding kumpulan rawatan berterusan dengan kumpulan kawalan. Apabila membandingkan kumpulan ketagihan dengan kumpulan yang tiada ketagihan, 104 miRNA, 10 gen dan 33 protein menunjukkan kenaikan dalam regulasi, manakala 5 miRNAs, 20 gen dan 20 protein telah menunjukkan penurunan dalam regulasi. Tahap signifikan yang diaplikasikan dalam kajian ini ialah apabila peubahannya melebihi dua kali ganda dan ujian ANOVA dan FDR adalah $p<0.05$. Keputusan kajian ini mencadangkan bahawa model tikus yang dimajukan adalah memadai sebagai model ketagihan bagi METH, dan perubahan dinamik berlaku dalam ekspresi miRNA, mRNA dan protein, dan ini mungkin boleh dikaitkan dengan penggunaan dan ketagihan METH.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	percent
˚C	:	degree Celcius
±	:	plus/minus
5-HT	:	5-hydroxytryptamine
AD	:	Alzheimer's disease
ADHD	:	attention deficit hyperactivity disorder
ALDOA	:	Aldolase A
ANOVA	:	Analysis of Variance
ATP		Adenosine triphosphate
ATS	:	Amphetamine-type stimulants
BDNF	:	brain-derived neurotrophic factor
BRIMS	:	Brain Research Institute Monash Sunway
CA	:	Canada
CCKR	:	cholecystokinin receptors
CDK5	:	Cell division protein kinase 5
cDNA	:	complementary DNA
cm	:	Centimeter
CPP	:	Conditioned place preference
CREB	:	cAMP response elements binding
CXCR4	:	C-X-C chemokine receptor type 4
DA	:	dopamine
DHA	:	Docosahexaenoic acid
DISC1	:	Disrupted in Schizophrenia
DNA	:	deoxyribonucleic acid

DNM1L	:	Dynamin-1-like
DNMT3A	:	DNA (Cytosine-5-)-Methyltransferase 3 Alpha
DPYSL2		Dihydropyrimidinase-Like 2
DRD2	:	Dopamine Receptor D2
DSM	:	Diagnostic and Statistical Manual of Mental Disorders
e.g	:	exempli gratia
EGF	:	epidermal growth factor receptor
ERK5	:	Extracellular-signal-regulated kinase 5
FAK	:	Focal adhesion kinase
FDR	:	False Discovery Rate
FGF	:	Fibroblast growth factors
FOXG1	:	Forkhead box protein G1
g	:	gram
GABA	:	gamma-aminobutyric acid
GDNF	:	Glial cell-derived neurotrophic factor
GluA2	:	Glutamate receptor 2
Glul	:	Glutamate-Ammonia Ligase
GNAI3		Guanine Nucleotide Binding Protein Alpha Inhibiting Polypeptide 3
GNRH	:	Gonadotropin-releasing hormone
GSTT2	:	glutathione S-transferase (GST) theta 2
GWAS	:	genome-wide association study
H_0	:	null hypothesis
H_1	:	research hypothesis
HTT	:	Huntinting protein
i.p.	:	Intraperitoneal
IPA®	:	Ingenuity Pathway Analysis

kg	:	kilogram
l x w x h	:	Length , width and height
LC	:	liquid chromatography
MAO	:	monoamine oxidase
MAPK	:	Mitogen-activated protein kinases
MeOH	:	methanol
METH	:	Methamphetamine
mg	:	milligram
min	:	minute
miRNA	:	microRNA
mL	:	milliliter
mm	:	Millimeter
mRNA	:	messenger ribonucleic acid
MWCO	:	molecular weight cutoff
n	:	Number
NAc	:	Nucleus accumbens
NAD		Nicotinamide adenine dinucleotide
PAK	:	p21-activated kinases
PCA	:	Principal component analysis
PCR	:	Polymerase chain reaction
PDGF	:	Platelet-derived growth factor
PKA	:	Protein kinase A
POA	:	Preoptic area
PTEN	:	Phosphatase and tensin
PUMA	:	p53 upregulated modulator of apoptosis
PVC	:	Polyvinyl chloride

qPCR	:	quantitative real-time polymerase chain reaction
RAM	:	Radial arm maze
RIN	:	RNA integrity number
RM	:	reference memory
SA	:	Self-administration
SD	:	standard deviation
SEM	:	Standard error of the mean
SIRT1	:	Sirtuin 1
SOD2	:	superoxide dismutase 2
SPSS	:	Statistical Package for the Social Science
STAT3	:	Signal transducer and activator of transcription 3
TAC	:	Transcriptome Analysis Console
TAOK1	:	TAO Kinase 1
TH	:	tyrosine hydroxylase
TRIB1		Tribbles Pseudokinase 1
TX	:	Texas
UK	:	United Kingdom
UM	:	University of Malaya
USA	:	United State of America
UTR	:	untranslated region
VEGF	:	Vascular endothelial growth factor
VTA	:	Ventral tegmental area
WM	:	working memory
µg	:	microgram

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CHAPTER 1:

INTRODUCTION

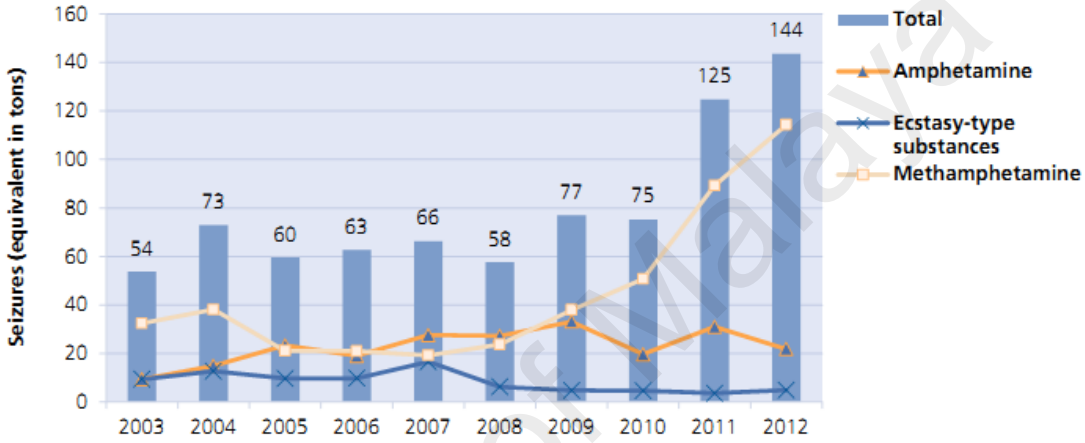
Drug abuse is a problem world-wide and Malaysia is no exception, and the impact of this abuse on society is increasing. The past decade has seen a significant increase in the popularity of methamphetamine (METH) in East Asia and the Pacific region. The National Institute on Drug Abuse (2015) reported that METH use had increased to 242,000 cases in 2013, compared with the year 2010 and it has grown to be a major illicit drug problem in the USA. Similar concerns exist in countries such as UK, Finland and Australia. According to The United Nations Office on Drugs and Crime (UNODC) (2014), the manufacturing of METH continued to rise from year to year. Production of METH was expanded with a large increase of the METH manufacturers in North America, while South-West Asia has also become a significant production zone for METH which is then supplied to other parts of Asia.

Over the last decade, dependence on METH has risen to an epidemic level worldwide (Elkashef et al., 2008). The use of METH continued to rise in East and Southeast Asia, with seizures of METH reaching a record level in the year 2012 (UNODC, 2014). METH pills are primarily used in countries such as Cambodia, the Lao People's Democratic Republic, Vietnam, Thailand and Myanmar, whereas METH in crystalline form is the commonly used form in Brunei Darussalam, Malaysia, Cambodia, Indonesia, Japan, the Philippines and in the Republic of Korea (UNODC, 2014). The proportion of heroin users among registered drug users decreased in 2012, however, the number of synthetic drug users increased more than the heroin users. As reported by UNODC (2014), there has been a large increase in the use of synthetic drugs like METH (UNODC, 2012). Over the past five years, METH seizures have almost quadrupled, from 24 tons in 2008 to 114 tons in 2012 (Figure 1.1). It is

approximately 80% of the total amphetamine-type stimulants (ATS) seized worldwide. Nearly 64% of global METH seizures occurred in North America, and 36% of seizures occurred in East and South-East Asia.

Figure 1.1: Global seizures of amphetamine-type stimulants, 2003-2012

Source: UNODC annual report questionnaire and other official sources.Note: Total ATS includes amphetamine, "ecstasy"-type substances, methamphetamine, non-specified ATS, other stimulants and prescription stimulants.



In Malaysia, the National Anti-Drug Agency (2014) reported that 4117 addicts were dependent on METH in 2014 and it was the third major drug of abuse in Malaysia, after heroin and morphine. Although the number had decreased as compared with the previous year, METH is still the 3rd major drug of abuse in Malaysia, after heroin and morphine. Although several behavioral therapies including cognitive behavioral and contingency management interventions have been clinically applied, there is no specific medication for the treatment of METH addiction (Hamamoto & Rhodus, 2009). As a result, the development of effective treatments for METH addiction has become a pressing matter.

In 2001, the American Academy of Pain Medicine, the American Pain Society, and the American Society of Addiction Medicine jointly issued a statement that

addiction is primary a chronic, neuro-biological disease that can be influenced by genetic, psycho-social, and environmental factors for its development and manifestations. It is characterized by behaviors like loss of control over drug use, compulsive use, repeated use regardless of harm, and also craving (Savage et al., 2001).

According to The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), addiction has been categorized into three stages: (a) preoccupation/anticipation, (b) binge/intoxication, and (c) withdrawal/negative affect. These stages represent (a) constant cravings and preoccupation to obtain the substance; (b) the use of more of the substance than necessary to experience the intoxicating effects; and (c) occurrence of tolerance, withdrawal symptoms and decreased motivation of normal life activities.

Drug addiction is characterized by compulsive drug use without control regardless of negative consequences and it is the most prevalent psychiatric disorders, contributing to mortality, and also physical and social morbidity. It is considered to be a brain disease because drugs can change the brain structure and its functions. The changes can be long lasting and can cause destructive behaviors to the individual who abuse the drugs (NIDA, 2007). Drug addiction is a complex trait, which is influenced by neurobiological, psychosocial and environmental factors. Among the neurobiological factors contributing to drug addiction is enhanced dopaminergic (DA) neurotransmission induced by the addictive drugs, particular within the midbrain DA system, and is a well documented and common feature among the many classes of abused drugs (White, 2002).

In general, it is known that social and psychological factors play important roles in addiction, however it is obvious that genetic factors also play contributory roles (Nestler, 2000). Some epidemiological studies reported that genetic factors contribute

around 40–60% of the risk in alcoholism (Kendler, Neale, Heath, Kessler, & Eaves, 1994). Recent studies found that the rates of heritability are similar among different types of drug addiction which include opium and cocaine (Kendler, Karkowski, Neale, & Prescott, 2000; Tsuang et al., 1998). Heavy use and dependence of the stimulant, cocaine, and of opiate are dependent only on genetic factors, with heritability of liability usually ranging from 60% to 80% (Kendler et al., 2000). Many linkage and association studies are now in progress to identify specific genetic variants that confer risk to addiction (Long et al., 1998; Reich et al., 1998) and some relatively large chromosomal regions have been identified to be involved in addiction vulnerability, although the specific genetic mechanisms are still unclear. Nowadays, biological research on addiction attempts to bridge the gap between different aspects which may cause addiction, especially when the focus of research is on the psychological phenomenon of craving, which describes the intense urge of an addicted person to re-administer the drug. They are trying to discover the modifications of cell receptor densities, gene expression rates and neuronal circuits which can be related to this phenomenon. Furthermore, epigenetic modification, such as DNA methylation, might also modify gene expression in the brain in the development of addiction (McGowan & Szyf, 2010).

According to Nestler (2000), it is more difficult to find genes that contribute to risk for addiction as compared with other psychiatric disorders and even for most of the common diseases. One of the reasons is the fact that addiction is a complex trait and every single gene may contribute a relatively small effect. It is therefore difficult to identify these genes. It is also possible that different genetic variants may promote addiction in different parentage. Furthermore, some epidemiological studies have reported that non-genetic factors like poverty, crime and delinquency, also contribute to the risk of addiction, although not much work has been done to verify this (Nestler, 2000). Animal studies have also found that environmental factors like stress can interact

with genotype to determine the final response to drugs of abuse (Nestler, Berhow, & Brodkin, 1996). From Figure 1.2, it can be seen that both genetic variations and environmental factors interact to increase the risk to drug addiction. Genetic and environmental factors, through the establishment of normal synaptic structure and function could determine an individual's inherent sensitivity to initial drug use (Nestler, 2000). It is likely that different individuals may have different inherent sensitivity to drug exposure. Individual nerve cells and the brain circuits involved will adapt over the time to chronic drug use, which can finally develop into addiction. An altered level of certain proteins or mutated proteins could alter the structure or function of specific brain circuits during neurodevelopment (Nestler, 2000). These changes may alter the responsiveness of an individual to initial drug exposure and cause adaptation to the brain after repeated drug use. At the same time, environmental stimuli combined with genetic factors could influence these neural circuits to affect addiction vulnerability in both humans and animal models.

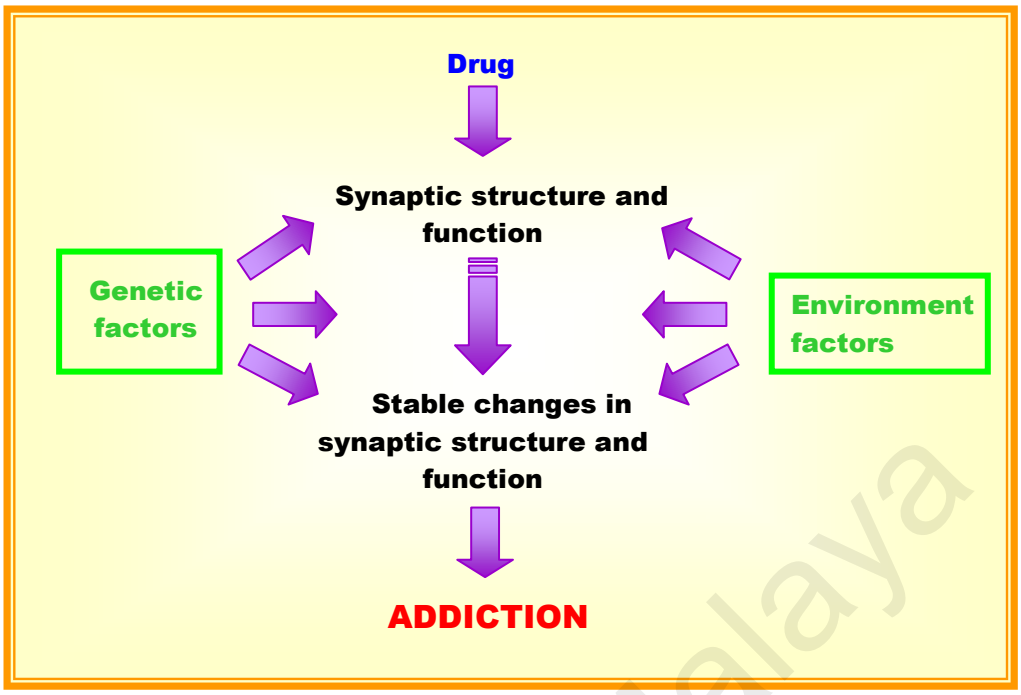


Figure 1.2: Genetic and environmental factors combined to influence the process by which repeated exposure to a drug of abuse causes addiction

(Adapted from Nestler, 2000)

It is well-known that environmental factor may play certain roles in drug addiction, however, at the current stage, we are focusing more on genetics influences in drug addiction. At the genetics level, gene expression is the most fundamental level at which the genotype gives rise to the phenotype. Such phenotypes are often expressed by the synthesis of proteins, together with the regulation of small RNA molecule (microRNA), that controls the organism's shape and behavior. Therefore, regulation of gene expression is thus critical to an organism's development.

1.1 Objectives

The primary goals of the research included in this thesis is to investigate genetic influences on METH use and addiction which could have effects on neurodevelopmental gene expression and how the disrupted genetic pathways may correlate with neurobehavioural changes consistent with METH addiction.

The specific objectives of the study are as follows:

1. To develop and optimize behavioural tests that would be appropriate for evaluation of METH addiction and METH-induced memory disturbance in a rat model.
2. To determine the effective and optimum dose of METH and the exposure duration that will induce addiction behavior in a rat model.
3. To determine whether extended access to METH would interfere with memory and learning ability using a radial arm maze.
4. To determine the global miRNA profiling in the rat nucleus accumbens and hippocampus following a METH acute exposure and continuous exposure with addiction symptom.
5. To determine the global trascriptome profiling in the rat nucleus accumbens and hippocampus following METH acute exposure and continuous exposure with addiction symptom.
6. To determine the global protein profiling in the rat nucleus accumbens and hippocampus following METH acute exposure and continuous exposure with addiction symptom.
7. To perform interaction analysis of the miRNAs, transcriptomes and proteins identified in (4), (5) and (6) in order to evaluate associations with its target moleculr and biological pathways which are relevant to METH addiction.

1.2 Justification of study

METH abuse has become epidemic in Malaysia over last decades. The growing popularity of METH was of particular concern. Therefore, studies related to METH abuse are strongly encouraged by the Malaysian government to assist and help to manage problems related to abuse of drug.

In general, miRNAs are short non-coding RNAs that regulate mRNAs expression, subsequently affecting the production of functional proteins in the cell. In this study, the findings related to global profiling of miRNA, transcriptome and protein may provide more comprehensive view of these interactions to show how their expression relates to METH addiction. Thus, the study may be able to provide insight into the molecular mechanism of METH addiction, not only in an animal model, but it may also be applicable to humans. So far, very little is known about miRNA, transcriptome, and protein expression levels or patterns in METH addiction and the exact mechanism of addiction at the molecular level. Therefore, this field of study is still a relatively novel and interesting area to explore.

Besides that, the advantage of this study is that we compared the miRNAs, transcriptome and proteins profiling between METH addicted rats with those which are exposed to a single dose METH but without being addicted, thereby eliminating biases caused by drug use. To date, most of the researches in this field have compared METH exposure or addiction with controls which were not exposed to METH. Through this profiling, it may be possible to predict which miRNAs, genes or proteins in the cell are associated with susceptibility to METH addiction. These informations could be useful to achieve more effective treatment for METH abuse and addiction in the future.

Furthermore, this study may provide some hints towards the discovery of the drugs for the treatment of patients with methamphetamine abuse and addiction because

to date, there is still no specific drug for the treatment of stimulant dependence and psychosis, especially for METH (Johnson et al., 2008) There is a great need, not only for novel treatments, but for understanding of its molecular mechanisms.

Therefore, the results from this study could make a significant contribution to the management of METH abuse and dependence, and even in its prevention in the future.

1.3 Research hypothesis

The research hypothesis (H_1):

1 The experimental rats with acute METH treatment do not show METH addiction phenotype.

2 The rats which received a relatively long period of continuous METH treatment with increasing doses show addiction to METH.

3 A continuous exposure to METH interferes with memory and learning ability in a rat model.

4 A significantly altered expression will be detected in certain miRNAs in the nucleus accumbens and hippocampus which are relevant to METH use and addiction.

5 A significantly altered expression will be detected in certain mRNAs in the nucleus accumbens and hippocampus which are relevant to METH use and addiction.

6 Certain proteins that are relevant to METH use and addiction are significantly altered in the nucleus accumbens and hippocampus.

The null hypothesis (H_0):

1 The experimental rats with acute METH treatment induce addiction phenotype to METH.

2 The rats which received a relatively long period of continuous METH treatment do not show addiction to METH.

3 A continuous exposure to METH does not interfere with the memory and learning ability in a rat model.

4 There is no significant altered expression of miRNA in the nucleus accumbens and hippocampus which are relevant to METH use and addiction.

5 There is no significantly altered expression of mRNA in the nucleus accumbens and hippocampus which are relevant to METH use and addiction.

6 There is no significantly altered expression of proteins in the nucleus accumbens and hippocampus which are relevant to METH use and addiction.

CHAPTER 2:

LITERATURE REVIEW

2.1 Methamphetamine

Methamphetamine (METH), $C_{10}H_{15}N$, commonly named *N*,α-dimethylphenethylamine, also known as desoxyephedrine, methylamphetamine, or phenylisopropylmethylamine (Logan, 2002). In Malaysia, METH is cheap and easy to obtain in the illegal drug market under the street names "batu", "chalk", "crank", "cris", "crystal", "glass", "go-fast", "ice", "meth", "shabu", "speed", and "zip" (Molitor, Truax, Ruiz, & Sun, 1998). The administration of METH can include swallowing or injecting, but because of its unpleasant taste, users are most likely to snort or smoke the drug. METH is an amphetamine derivative (Logan, 2002). It was first synthesized in Japan in 1919 by Ogata, patented in 1920, and then licensed to Burroughs Wellcome, which was then commercialized as the anorectic Methedrine® (Ogata, 1919).

METH is addictive and its withdrawal symptoms are common with heavy use and relapse is common. Psychostimulant like METH creates a neuro-chemical escalation of the pleasure and it can intensify emotions, increase energy, elevate self-esteem, and heighten sexuality, while reducing inhibitions and impairing judgment (Gawin & Ellinwood, 1988). When METH is administered, the individual may face some side effects including decreased appetite and increased energy and activity. Serious side effects of METH are not common (Nicks, 2010). Long term use of METH could show many negative health consequences, including extremely weight loss, severe dental problems and some psychologicall effects like anxiety, confusion, mood disturbances and violent behavior, while chronic METH use can display some psychotic problems, such as paranoia, visual and auditory hallucinations (Hart, Marvin, Silver, &

Smith, 2012). Nevertheless, the acute effects or a low dose of METH may show some positive effects like improvements in speed of cognitive processing, attention, concentration, working memory and psychomotor performance (Johnson et al., 2005; Mohs, Tinklenberg, Roth, & Kopell, 1978).

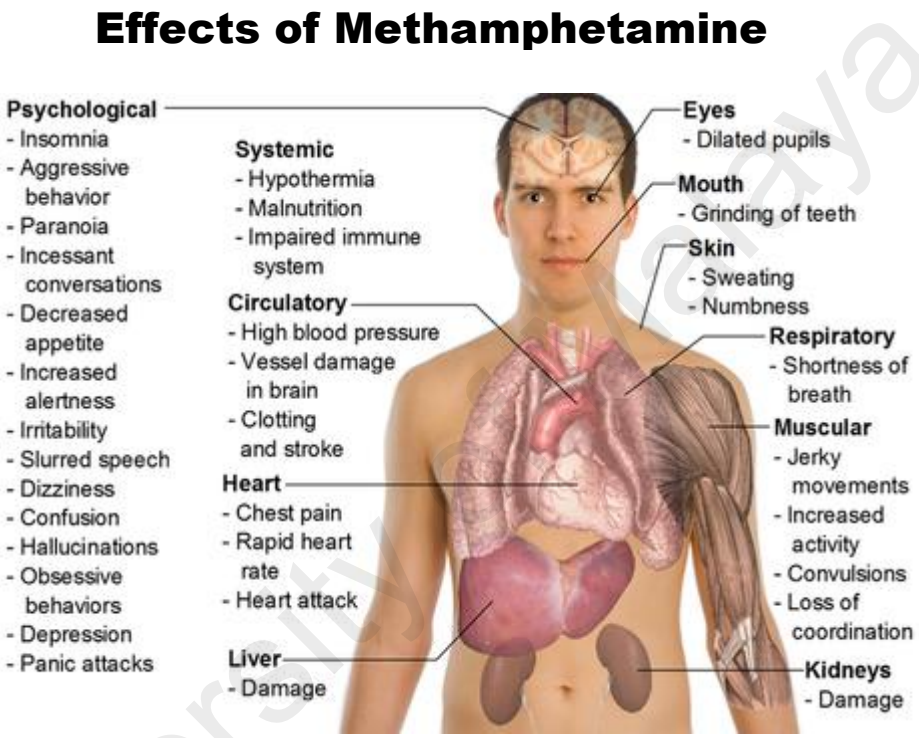


Figure 2.1: Short- and long-term physical and mental effects that may result from methamphetamine use, including rare effects

(Adapted from Drugtoxicology, 2016, https://drugtoxicology.wikispaces.com/Methamphetamine+-+Side+Effects+of+Meth+use)

METH is a central nervous system stimulant which can causes chemical reactions in the brain. The release of neurotransmitter dopamine causes increased motivation. Prolonged use of METH can disturb brain functions. However, it can increase alertness and produces euphoria, which is a feeling of great joy and excitement. Furthermore, this drug can also keep the users awake for long periods. Sleep deprivation is the most prevalent side effect of METH and leads to a feeling of tiredness and

depression. Besides that, chemical imbalances in the brain can lead to hallucinations, extreme paranoia, bizarre, violent behavior and mood disturbances (Nicks, 2010).

The pharmacology of the METH is complex and it is basically a sympathomimetic drug that has similar structure with endogenous transmitters which can interact with their receptors in the nervous system (Logan, 2002). The structure of METH is similar to the prototypical sympathetic neurotransmitters which include catecholamines, noredrenaline, dopamine, and adrenaline. METH interacts with pre-synaptic receptors by competitive antagonism, and it has been shown to have an agonist effect at postsynaptic receptors (Logan, 2002).

METH acts on the central nervous system (CNS) and causes release of monoamine neurotransmitters, including dopamine, noradrenaline and serotonin (Kuczenski, Segal, Cho, & Melega, 1995). The primary mechanisms by the amphetamine class of drugs like METH are to increase levels of monoamines, especially dopamine, from synaptic vesicles into the cytosol (Brown, Hanson, & Fleckenstein, 2001) and to reverse the transport of neurotransmitter via plasma membrane transporters (Khoshbouei, Wang, Lechleiter, Javitch, & Galli, 2003). In addition, psychostimulants can inhibit the activity of monoamine transporters (Schmitz, Lee, Schmauss, Gonon, & Sulzer, 2001), and decrease the expression of dopamine transporters at the nerve cell surface (Saunders et al., 2000). There have also been reports that METH can increase monoamines in cytosol by inhibiting the activity of monoamine oxidase (MAO), and increasing the expression of the dopamine synthesizing enzyme, namely, tyrosine hydroxylase (TH) (Mandell & Morgan, 1970). In general, Barr et al. (2006) reported that amphetamines act as a highly potent releaser of monoamines in the nerve terminal.

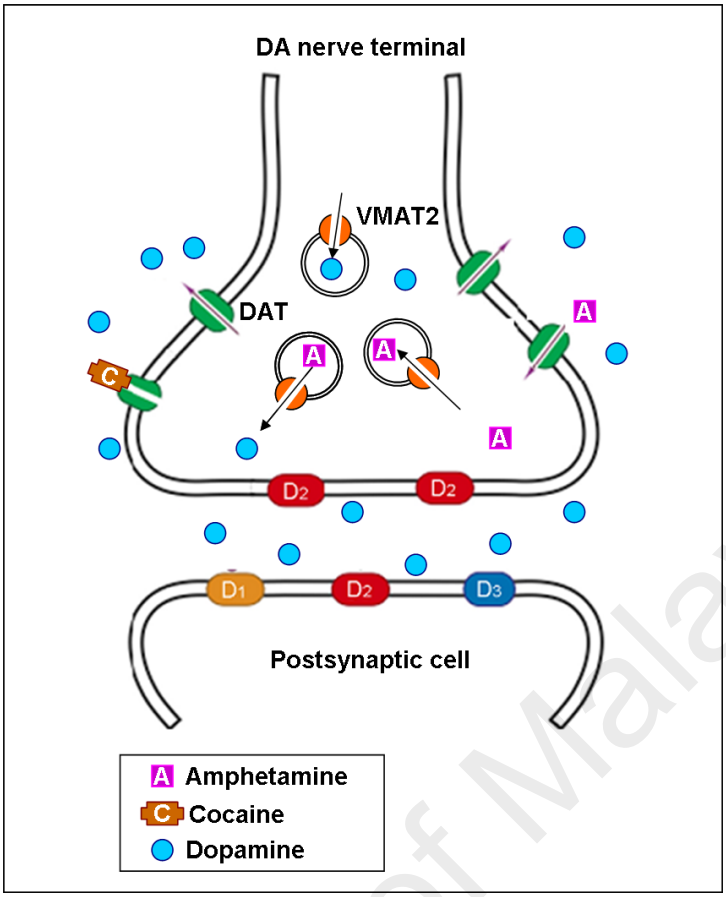


Figure 2.2: **Physiological mechanisms by which psychostimulants (amphetamine and cocaine) increase synaptic levels of monoamines in the pre- and post-synaptic terminals.**
DAT = dopamine transporter; VMAT = vesicular monoamine transporter

(Adapted from Howell & Kimmel, 2008)

Figure 2.2 represents a dopaminergic synapse in the pre- and post-synaptic terminals. Dopamine enters the vesicles in the pre-synaptic neuron through VMAT2. When dopamine is released into the synapse, it can bind to post-synaptic dopamine receptors, such as D1, D2, and D3 receptors. D2 receptors which are located at the pre-synaptic terminal, regulate the release of neurotransmitter dopamine via a feedback mechanism (Howell & Kimmel, 2008). The dopamine transporter (DAT) is located at perisynaptically and plays a role in terminating the actions of dopamine through a transport mechanism. The presence of the psychostimulants at the DAT can alter the normal function of the dopamine receptor. Generally, amphetamine like cocaine can

block DAT and inhibits re-uptake of dopamine into the pre-synaptic nerve terminal, thus extending its effects in the synapse. These psychostimulants also blocks the DAT and suppress dopamine uptake, but also induces release of dopamine from intracellular vesicles. An in-vivo study showed that psychostimulants may interact with numourous monoamine transporters in the reward mechanism (Tzschentke & Schmidt, 2000). However, it has been found that the behavioral effects by the addictive properties in the psychostimulants have been strong related to enhanced dopaminergic activity.

2.2 Addiction-relevant brain regions

The mesolimbic pathway, also named as the reward pathway, is characterized by the interaction of several areas of the brain, such as ventral tegmental area (VTA), prefrontal cortex, nucleus accumbens, hippocampus, and so on. Previous studies have been reported these parts of the brain are highly associted with rewarding experience of drug use and drug-seeking behavior (Korpi et al., 2015). The mesolimbic pathway consists of dopaminergic neurons of the medial forebrain bundle whose cell bodies are situated in a neuron cluster of the midbrain called the ventral tegmental area whose axons run to the nucleus accumbens and the hippocampus (Oades and Halliday, 1987). Evidences have suggested that the nucleus accumbens and the hippocampus play important roles in this addiction-relevant pathway (Adinoff, 2004; Salgado & Kaplitt, 2015). Thus in this study, these 2 brain regions were selected to be studied in METH addiction.

2.2.1 Nucleus accumbens

The nucleus accumbens (NAc) is located in the basal forebrain rostral to the preoptic area (POA) of the hypothalamus (Carlson, 2013). The NAc and the olfactory tubercle jointly form the ventral striatum, which is considered part of the basal ganglia (Nicola, 2007). Each cerebral hemisphere has its own NAc, which can be divided into two parts: the core and the shell. These two contiguous areas have overlapping connections, but may make different contributions to the functions of the NAc.

Dopamine (DA) is a neurotransmitter that regulates reward and pleasure. It is highly involved in drugs of abuse, in which it increases extracellular dopamine in the NAc shell and the core, while this effect is more obvious in the NAc shell. However, only amphetamine-type drugs produce significant increases in extracellular dopamine in both the shell and the core of the NAc (Pontieri, Tanda, & Di Chiara, 1995).

The NAc has an important role in the cognitive processing of aversion, motivation, pleasure, reward and reinforcement learning (Wenzel, Rauscher, Cheer, & Oleson). Besides that, it has been considered as an important structure implicated in motivational and emotional processes, the limbic-motor interface, and the effects of some psychoactive substainces. The NAc has been involved in many neurological and psychiatric disorders, including depression, obsessive-compulsive disorder, bipolar disorder, anxiety disorders, Parkinson's disease, Alzheimer's disease, Huntington's disease, obesity, and in drug use and dependence (Salgado & Kaplitt, 2015).

The most widely recognized function of the NAc is its role in the "reward circuit" of the brain (Nestler, Hyman, & Malenka, 2009). However, it has been found that it is less involved in processing fear, impulsivity, and the placebo effect (Basar et al., 2010; Schwienbacher, Fendt, Richardson, & Schnitzler, 2004; Zubieta & Stohler, 2009). For anything that is considered rewarding (e.g. eating food, sexual intercource

and taking drugs of abuse), dopamine neurons in certain region of the ventral tegmental area (VTA) are activated. When activated, it leads to an increase in dopamine levels in the NAc. Besides that, the NAc is a critical area of a major dopaminergic pathway in the brain called the mesolimbic pathway, which is stimulated during reward behavior. Although most of the drugs of abuse trigger the mesolimbic dopamine system, more studies have suggest that dopamine-independent reinforcement occurs at NAc, indicating that there is a plurality of inputs involved in the activation of crucial reinforcement circuitry in these areas (Koob, 1992; Nestler, 2005).

2.2.2 Hippocampus

The hippocampus is named because of its similarity in structure to a seahorse (Greek words "hippo" for horse and "kampos" for sea). It is a major part of the brains of humans and other vertebrates. Humans and other mammals have two parts of hippocampi which lie in the left and right sides of the brain. It is located next to the cerebral cortex. In primates, it is located in the medial temporal lobe of the brain, underneath the cortical surface (Amaral & Lavenex, 2006) and it is part of the limbic system and functions to combine the information from short-term memory to long-term memory and spatial navigation.

Over the last decade, many animal studies demonstrated that addictive drugs like cocaine and METH can lead to the disruption of neurogenesis in the hippocampus (Canales, 2007). A study revealed that impairment in the ventral subiculum of the hippocampus can affect cocaine self-administration in rats (Caine, Humby, Robbins, & Everitt, 2001). This finding has provided insights into the potential involvement of the hippocampus in drug addiction. Furthermore, hippocampus has been long recognized to be associated mainly with learning and memory (Jarrard, 1993). A recent study

suggested that declarative memory underlying the impulsive behavior is strongly implicated in drug addiction (Zhai et al., 2014). A study has also found that the craving behavior from cocaine exposure increases blood flow in the hippocampus which may be involved in several forms of memory (Kilts et al., 2001).

Addiction-relevant molecular targets in the hippocampus can be evaluated using some proteomics approaches. Generally, administration of drugs can change the protein expression. A recent study used a quantitative proteomics analysis technique to study the effects of morphine exposure on the protein expression profile at hippocampal (Moron et al., 2007). This finding shows that repeated morphine use can change the synaptic allocation of endocytic proteins in hippocampus. They found that endocytic proteins like clathrin may play important roles in morphine-induced changes at hippocampal synapses. This information is useful and provides insight into the study of the mechanisms underlying drug-induced neuroadaptation. Furthermore, this study suggests that hippocampal neuroadaptations are commonly induced by repeated drug administration and it may provide further understanding on the mechanisms relevant to the development of addiction.

2.3 Behavioral studies on drug addiction and drug-induced memory impairment

Extended daily self-administration (SA) paradigm have been proven to provide clinically relevant techniques for addiction study, because it depend on response-contingent drug delivery and is reminiscent of human drug taking behavior by an escalation of drug intake (Ahmed & Koob, 1999; Ahmed, Walker, & Koob, 2000; Kitamura, Wee, Specio, Koob, & Pulvirenti, 2006). To study the behaviors in the animal models, different kinds of tests have being developed. Conditioned place preference (CPP) paradigm has become a popular alternative to drug self-administration for assessing the rewarding effects of a variety of drugs (Schechter & Calcagnetti, 1993; Tzschentke, 1998). This technique is often used to determine the addiction potential of drugs.

Although many different designs and apparatus are applied in addiction models, the common characteristics of the CPP involve the correlation of a certain environment with drug exposure, accompanied by the relationship of a different environment with the absence of the drug (Adam, John, & John, 2009). However, the behavioral effects of drugs used in the CPP paradigm rely on species, strain, route of drug administration, time interval of drug intake, dose concentration, and the CPP apparatus used in the study. Most of the drugs of abuse develop CPP with dependence on the dose used. The CPP paradigm is able to provide a reliable indicator to study addiction of drugs. Thus it has been widely used to elucidate the effects of drugs.

METH addiction is a chronic, relapsing brain disorder that can induce psychiatric symptoms and cognitive impairments. Previous study revealed that long-term use of METH show deficits in specific cognitive domains, especially during memory tasks (Scott et al., 2007). The previous studies demonstrated that extended access to METH self-administration increased METH use, enhanced reinstatement of

drug-seeking and interrupted novel object recognition (Rogers, De Santis, & See, 2008; Schwendt et al., 2009). In the animal models, some components of episodic memory can be assessed easily. Among behavioral tests, radial arm maze (RAM) is one of the most suitable apparatus to study spatial learning and memory (Olton 1978). RAM consists of eight horizontal arms installed radially above the floor. RAM procedure has been optimized to distinguish between the spatial working memory errors (repeat entries into baited areas) and spatial reference memory errors (entering no baited arms) (Jarrard, 1978, 1983). In our study, only four maze arms are baited. This version of the task was used by Jarrard (1983) and it aims to test working and reference memory simultaneously. The same arms of the maze are baited in every test and, across sessions, hence the rats learn and remember which of these arms contain the reward. This is considered the reference memory task, and entry into a non-baited arm will be categoried as a reference memory error. Repeated entry into one of the baited arms within a training session will be categoried as a working memory error.

2.4 MicroRNA expression

MicroRNAs (miRNAs), a class of small non-coding transcripts, which play key roles in the regulation of gene expression during differentiation and neuro-development processes (Kosik, 2006). The target mRNAs are either suppressed for translation step or subjected to degradation by miRNA (Figure 2.3) (Kosik, 2006). Enriched expression of certain miRNAs in the brain might be involved in long-term potentiation and regulation of structural and functional aspects of synaptic plasticity, such as neuronal morphogenesis and activity dependent translation during synapse formation, memory and addiction (Ashraf, McLoon, Sclarsic, & Kunes, 2006; Fiore & Schratt, 2007; Huang & Li, 2009b; Kosik, 2006; Pietrzykowski et al., 2008; Schratt et al., 2006). This finding was supported by previous studies indicating that abnormal expression of some

miRNAs may lead to synaptic dysfunction, thereby contributing to the etiology of different neurological disorders (Beveridge et al., 2008; Johnson et al., 2008; Nelson et al., 2006; Niwa, Zhou, Li, & Slack, 2008; G. Wang et al., 2008; W. X. Wang et al., 2008). In the brain's reward circuitry, miRNAs have been implicated in mediating the effects of cocaine (Chandrasekar & Dreyer, 2009, 2011; Hollander et al., 2010; Im, Hollander, Bali, & Kenny, 2010), nicotine (Huang & Li, 2009a, 2009b), alcohol (Pietrzykowski et al., 2008), and other drugs of abuse (He, Yang, Kirkmire, & Wang, 2010; Zhou et al., 2009). In cocaine addiction, the up-regulation of miR-181a and the down-regulation of two other miRNAs, let-7d and miR-124a, have been identified in the addiction-related regions of the rat brain (Chandrasekar & Dreyer, 2009). Manipulating the changes of miRNAs in the NAc has been shown to be sufficient to suppress or promote drug-seeking behavior (Chandrasekar & Dreyer, 2011; Hollander et al., 2010; Im et al., 2010). In general, Saba et al. (2012) believed that even a single miRNA, is sufficient to change the expression of a number of downstream target genes that regulate the addiction-relevant neuronal mechanisms.

2.5 Transcriptome/gene expression

Transcriptome is the full range of all messenger RNA (mRNA) molecules in one cell or in an organism. Different from the genome, which has high stablity, the transcriptome actively changes and can be varied with external environmental conditions. Because it includes all mRNA transcripts, the transcriptome can reflect the genes that are being actively expressed at certain time, with the exception of mRNA degradation process like transcription attenuation.

The transcriptome can be considered as a precursor of the proteome, the entire set of proteins expressed by a genome. The whole process from gene, mRNA to protein

was described in Figure 2.3. However, though such transcriptome studies that provide information regarding comparative levels of mRNA in cells have been instructive, one must be cautious because protein expression is dynamic with respect to place and time, and there exists only moderate correlation between protein levels and its corresponding mRNA in a given cell (Anderson & Seilhamer, 1997; Gygi, Rochon, Franza, & Aebersold, 1999). The study of mRNA expression can be complicated by the fact that any relatively small alteration in mRNA expression can produce big changes at the protein expression levels in the cell.

RNA sequence mirrors the sequence of the genome from which it was transcribed. By analyzing the transcriptome, scientists can undertand when and where each gene is activated or deactivated in the cells of an organism. In most of the organisms including human, almost all of the cells contain the same set of genes, but they show different patterns of gene expression.

Nowadays, several laboratory techniques can be used to study the expression levels of transcriptome. One of the most prevalent techniques is microarray. Microarray can be applied to identify the expression of more than thousands of genes at the same time, and is able to provide gene expression profiles, which describe changes in the transcriptome in response to a particular diseases or treatments (Anderson & Seilhamer, 1997).

2.6 Protein expression

METH is addictive and repeated METH exposure can enhance behavioral response to subsequent drug intake (Robinson & Berridge, 1993). This phenomenon is called behavioral sensitization. Sensitization is a persistent phenomenon, caused by chronic alteration of neuronal plasticity in the CNS. The behavioral sensitization is considered to be associated with the psycho-pathology of METH addiction, METH psychosis, and schizophrenia (Stam, Bruijnzeel, & Wiegant, 2000; Stewart & Badiani, 1993; Vanderschuren & Kalivas, 2000). Thus, protein expression profiles of METH esxposure are important to be investigated in order to understand the underlying mechanisms of the alteration in neuronal plasticity. High doses of METH exposure can cause neuronal toxicity in dopaminergic neurons, including dopaminergic terminal degeneration, dopamine depletion, and reduction of the DAT in the cell (Frey, Kilbourn, & Robinson, 1997; Ricaurte, Guillery, Seiden, Schuster, & Moore, 1982; Wagner, Seiden, & Schuster, 1979). Moreover, previous studies have indicated that high dose of METH exposure also leads to the alteration of the mRNA and protein expression (Cadet, Jayanthi, & Deng, 2003; Davidson, Gow, Lee, & Ellinwood, 2001; Kita, Wagner, & Nakashima, 2003). Another study demonstrated that the rats which had been treated with high doses of METH showed significant association with Alzheimer's disease (AD) (Liao, Kuo, Hsu, Cherng, & Yu, 2005). However, a low dose of METH use does not cause neurotoxic effects. A previous study reported that 20 mg or higher dose of METH usually induced neurotoxicity on neostriatam in rats (Fukumura, Cappon, Pu, Broening, & Vorhees, 1998). Thus, low dose of METH is normally used to elucidate protein expression changes, because it might be more relevant to the neuroadaptive processes involved in chronic METH psychosis and schizophrenia (Yamada, Nagai, & Nabeshima, 2005). Some of the studies reported that mRNA and protein expression are altered after a single low dose of METH exposure (Chen & Chen,

2005; Funada, Zhou, Satoh, & Wada, 2004; Ujike, 2002; Wang, Smith, & McGinty, 1995). However, the mechanism of how METH influences neuronal plasticity via the alteration in protein expression after a single low dose METH use is still unclear. Proteomics is widely used as a valuable tool to study expression changes in complex biological systems, especially in psychiatric research. Compared to other diseases, the application of the proteomics is still relatively new in the study of psychiatric disorders, especially in drug addiction.

Nowadays, many methods have been used to study protein expression, such as Western blot, immunohistochemistry, protein array and immunofluorescence, but these methods should pass several validation tests (Kononen et al., 1998; Nilsson et al., 2005; Uhlen & Ponten, 2005). Therefore, importance of data reliability requires more sensitive techniques to confirm specificity of antibodies to avoid any false data. Mass spectrometry receives increasing popularity in proteomics as method of choice and it has been welcomed by researchers in this area for its incredible sensitivity for identification, characterization or quantitation of proteins in sophisticated biological samples. Many different mass spectrometry instruments have been launched and one of the latest versions is Orbitrap Mass Analyzer in which ions derived from the sample orbit by an electrostatic force around a central electrode while centrifugal force of another electrode (encapsulating the central electrode) influences them. Ions' oscillations frequencies are measured by a detector and m/z ratio of ions can be read with high accuracy. Method of choice for peptide identification is usually bottom-up procedure in which purified sample is digested in order to obtain smaller peptides, Orbitrap instrument is able to detect peptide of 1ppm concentration accurately (Scigelova & Makarov, 2006). Because of its high accuracy and sensitivity, this method has been used as a validation method in several of the protein studies (Dias et al., 2013; Zybailov, Sun, & van Wijk, 2009).

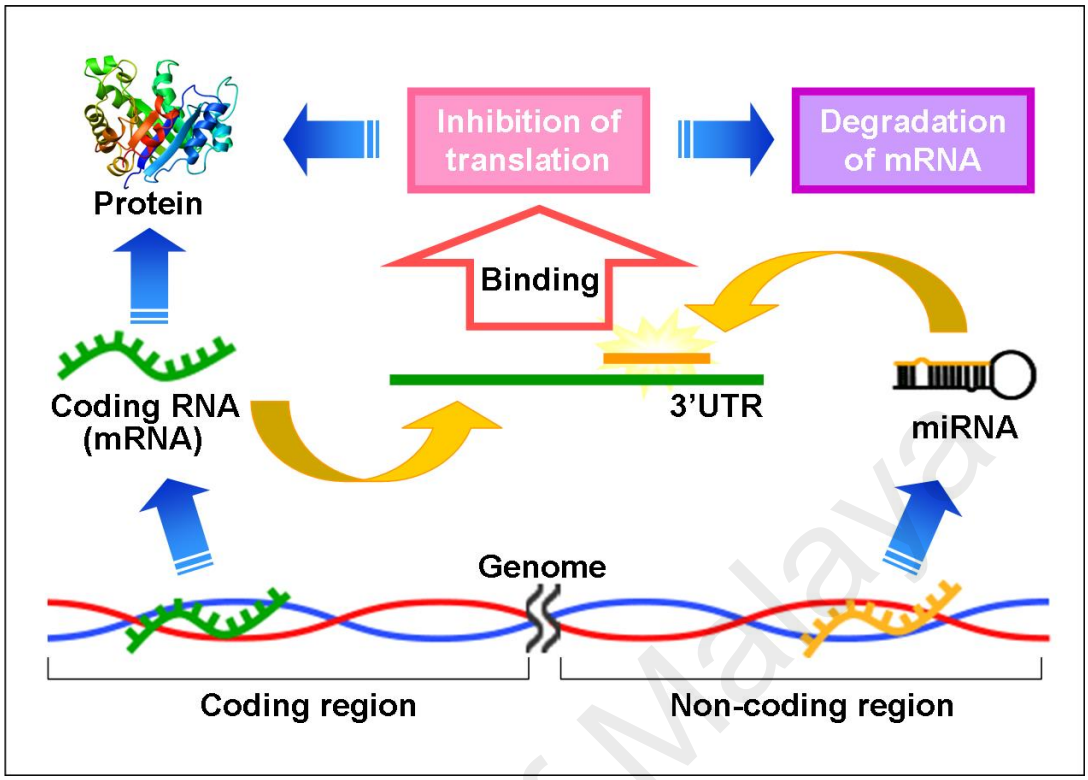


Figure 2.3: Correlation between miRNA, mRNA and protein expression.
A miRNA regulates many mRNAs, and conversely, single mRNA is regulated by several miRNAs. These miRNA are short post-transcriptional regulators that bind to target messenger RNA, most commonly resulting in gene-silencing and results in suppression of protein translation.

(Adapted from Toray Industries Inc (2005), http://www.3d-gene.com/en/service/analysis/ana_004.html)

CHAPTER 3:

MATERIALS AND METHODS

3.1 Experimental animal

Male Wistar rats weighing approximately 250-350 g were habituated approximately one week prior to treatment in a specific pathogen free animal facility (Brain Research Institute, Monash University Malaysia). The rats were divided into 3 groups: a control group that was administrated with saline (n = 9), a single dose acute METH treatment (5 mg/kg, i.p.) group (n = 9) and a continuous 15 alternate days METH treatment group with increasing doses (0.25, 0.5, 1, 2, 3, 4, 5 mg/kg) (n = 9). All drug solutions were prepared in saline and injected intraperitoneally in a volume of 150 µl.

Animals were housed in polyethylene cages with stainless-steel wire lids containing hardwood bedding in a humidity- and temperature-controlled room with a 12 hour light: dark cycle. All experiments were carried out during the dark cycle. Animals were given rat chow and water *ad libitum*. All the animal behavioral tests were conducted in dim light. Experimental protocols were approved by the Monash University Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (MARP/2013/065) (Apendix D).

3.2 Behavioral studies

3.2.1 Conditioned Place Preference (CPP) paradigm

The CPP test is a Pavlovian classical conditioning method which indicates drug use condition is paired with a neutral environment. Black and white colors are applied as cues for the experimental animals to reconigze and differentiate between each compartment of the CPP box. In our study, place preference conditioning was conducted in Plexiglass boxes (90×22×30 cm) (l×w×h) which were divided into two chambers (40×22×30 cm) by insertion of two liftable Plexiglass doors and a small grey zone (10×22×30 cm) is located at the middle of the chamber. Both the chambers acquired visual and tactile cues: one region of the chamber had black walls with white horizontal stripes and black color floor while the another region had white walls with vertical black lines and white color floor.

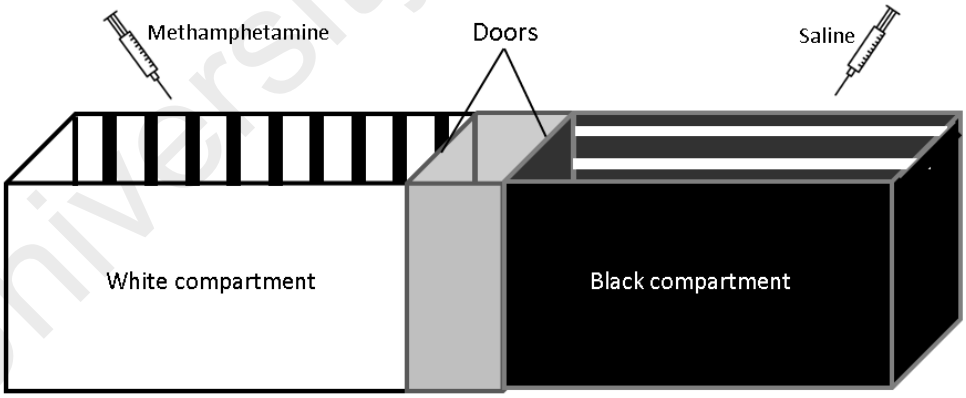


Figure 3.1: Schematic diagram of the Conditioned Place Preference apparatus.

In addition, a small white light was attached to the white compartment of the CPP apparatus. Detachable dividers, complementing the chambers walls, were used to close-off each chamber. The total time consumed in the drug-paired condition of the CPP is considered as a measurement of the positive reinforcing or rewarding effects of the drug.

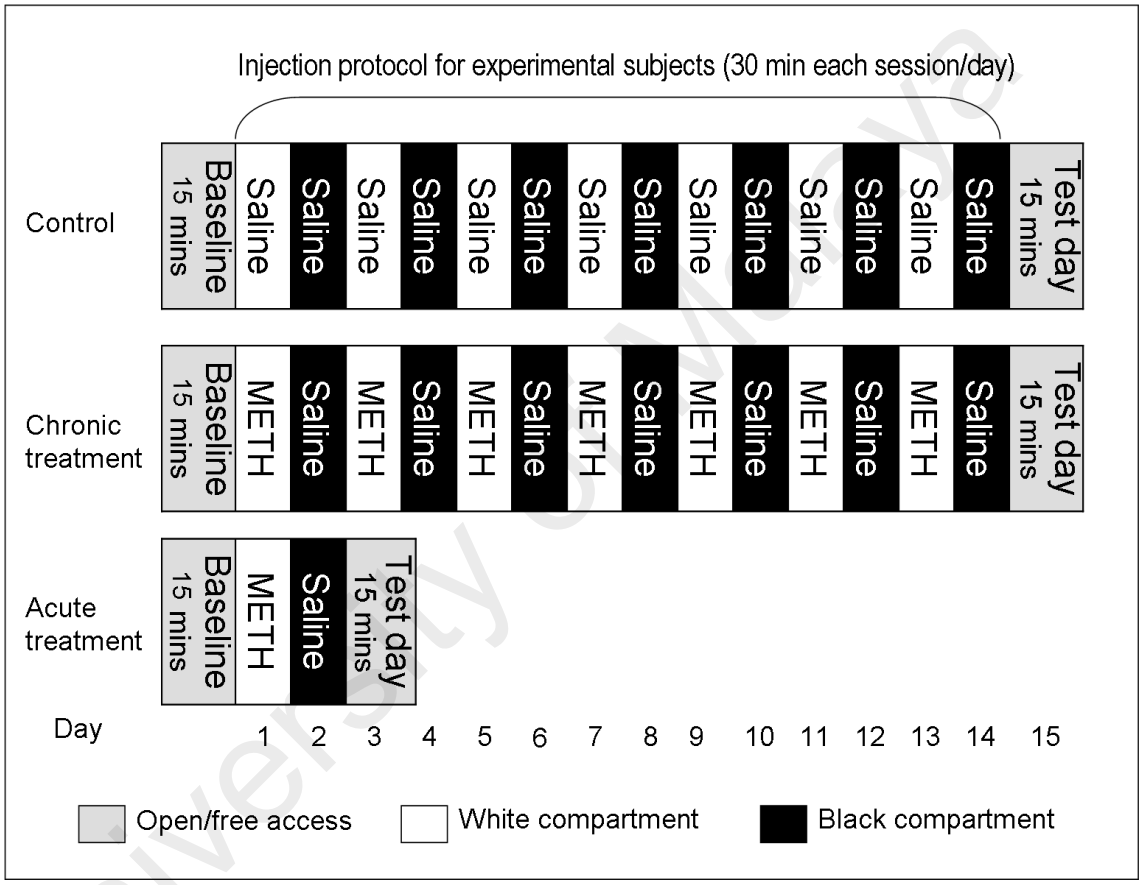


Figure 3.2: Experimental design and injection protocol for the CPP test.

I. Pre-conditioning phase

In the pre-conditioning condition, animals were allowed free access to both compartments of the CPP box for 15 minutes and the time used in each compartment were recorded. The purpose of the pre-conditioning is to determine the natural preference of the experimental animal for either one of the CPP compartments.

II. Conditioning phase

During the conditioning phase, the animals were restricted to either the white or black compartment. The animals designated to the METH groups were intraperitoneally injected with METH after 10 minutes in the drug-paired compartment and a further 35 minutes will be given to stay in the same compartment after drug injection (Adam, John, & John, 2009). For METH-conditioned group, METH was given with increasing doses (0.25, 0.5, 1, 2, 3, 4, 5 mg/kg) on days 1, 3, 5, 7, 9, 11 and 13 respectively or with saline on days 2, 4, 6, 8, 10, 12 and 14. While for acute METH treatment group with 5 mg/kg dose, METH was given on day 1 and saline on day 2. A total period of 45 minutes was provided for the drug to take affect. However, control rats received equivalent saline treatment and were also being confined in the drug-paired compartment of the CPP box.

III. Post-conditioning or test phase

On day 16, the CPP test was conducted 24 h after the last injection, with no drug treatment on the test day. The rats were again given free access to both compartments of the CPP box for 15 minutes, and the total time consumed in each compartment was recorded for further analyses by using a video camera. In addition, the difference in the amount of time spent was compared between the case and the control experimental rats.

3.2.2 Radial arm maze

The radial maze apparatus is designed with eight horizontal arms (Figure 3.2). The maze, 25 cm tall and 46 cm in diameter, was constructed of black PVC wall and floor. Different items were placed around the maze and remained in the same place for whole test periods. A small (4.5 cm-diameter and 0.9 cm-deep) translucent plastic dish was mounted as a food cup at the end of each arm. The tests were conducted in a quiet and dim light environment. To avoid the interruption by odors cues, the maze was completely wiped with ethanol and the maze was rotated for every section of the test. Maze arms were baited by placing one 45 to 50 mg pellets in the food cup. One of the arms was selected to be a start point with a stopper at the entrance of the arm. A video camera was attached above the central point of the maze to record and monitor the behavior of the rats in the maze.

I. Training procedure

During the first 2 days, all rats were allowed to explore the apparatus for 30 min each day. No food was present on the maze during the adaptation. The rats were given a restricted diet and their weights were monitored throughout the whole experiments so that they could be maintained at 85%–95% of free-feeding weight (Crusio & Schwegler, 2005).

For the next 6 days, the rats were given one trial per day. The time to completion of the task and the sequence of arms visited were recorded. Prior to each test session, three 45-mg food pellets were placed in the food cup at the end of three selected arms. The session ended when the rats had either obtained all the food or when 20 minutes had elapsed.

The rat was then placed at the end of a selected arm as a starting point and a timer was started when the door was opened. A choice was defined as having occurred when the rat had placed all four feet upon an arm. This was to train the rats to remember the location of the foods before the treatment of METH.

II. Actual test phase

Prior to each trial, three baited arms were selected as baited using the same procedure as in the free choice training trials. The experimenter recorded the sequence of arms chosen. One trial per day was conducted for 8 days, at intervals between the METH injection days. The variables used for the analysis of the performance are (a) the index which shows the number of repeated entries for each test (enter into an arm that has been visited before) and the total number of repeated entries each test, (b) the index which indicates the number of correct entries among the eight arm entries of each test, and the total number of entries within each test (c) the time spent to visit each arm (total time to complete the test divided by the total number of arm entries) (Liu & Bilkey, 1999, 2002; Liu, Rushaidhi, Collie, Leong, & Zhang, 2008).

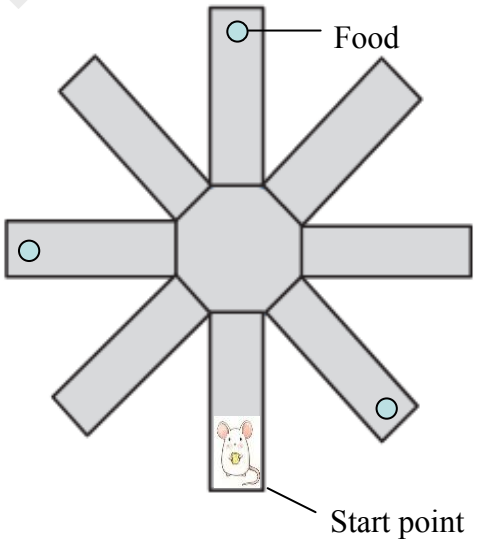


Figure 3.3: Schematic diagram of the eight-arm radial maze.

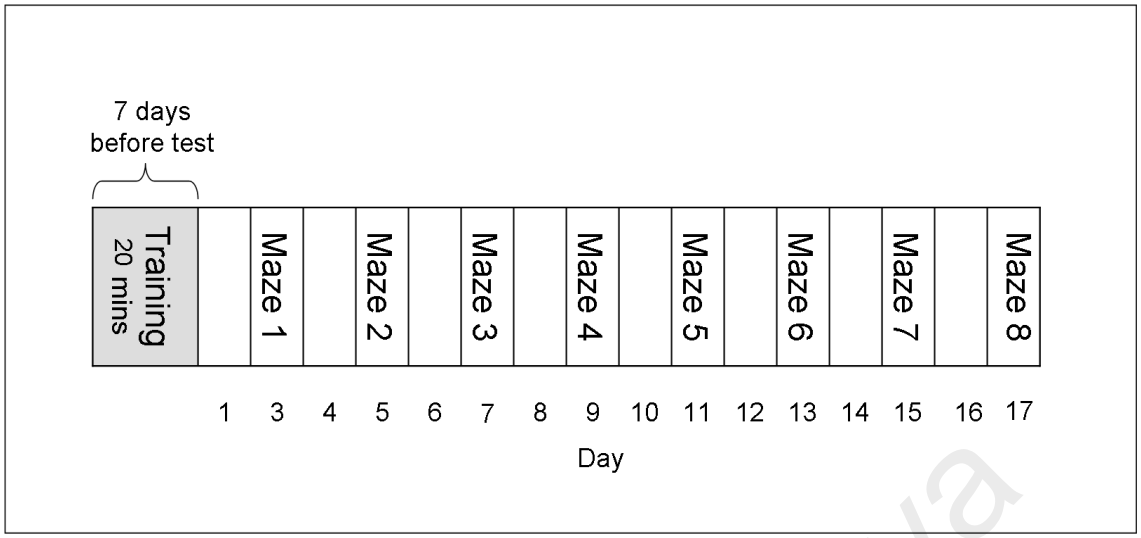


Figure 3.4: Experimental design and for the radial arm maze test.

3.2.3 Statistic analysis for behavioral study

For addiction behavior study, statistical analyses were performed by using the Statistical Package for Social Science (SPSS, Chicago). The results of the behavioral study were evaluated by Student's t test and analyses of variance (ANOVA). Differences were considered significant at $p < 0.05$. In cases of multiple comparisons, the Bonferroni correction was performed.

3.3 Tissue dissection

After the final CPP test was completed, rats were briefly anesthetized with CO_2 asphyxiation and sacrificed immediately, and following that, the whole brain was removed and frozen on dry ice. NAc and hippocampus tissues were dissected according to the rat brain atlas (Paxinos & Watson, 2006). NAc tissue (bregma +1.2 to +2.5 mm) and hippocampus tissue (bregma -1.92 to -4.20) were dissected by coronal sections using cryostat. All tissues were kept frozen at -80°C until use.

3.4 Global miRNA profiling

3.4.1 Total RNA isolation

A total of 18 male rats (6 rats in each from the controls, acute METH treatment and METH continuous treatment group) were being selected to perform global miRNA profiling. Total RNA that included miRNAs and other small RNAs were extracted from the rat's nucleus accumbens and hippocampal tissues using TRIzol reagent, followed by column-based purification, as described in the manufacturer's protocol for TRIzol® Plus RNA Purification Kit (Ambion, Austin, TX). The RNA was eluted in 30 μL of RNase-free water. RNA concentration was determined with a spectrophotometer (NanoDrop 2000 Spectrophotometer; Nanodrop, Thermo Scientific, USA), and RNA integrity was verified with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto CA).

3.4.2 MiRNA array methods and analyses

MiRNA profiling was performed with the nucleus accumbens and hippocampal tissue. Total RNA (500 ng) was 3' labeled with the FlashTag Biotin HSR RNA Labeling Kit (Affymetrix, Santa Clara, CA), according to the manufacturer's protocol. The Enzyme Linked Oligosorbent Assay (ELOSA) was performed to confirm that the FlashTag Biotin HSR labeling Kit has performed appropriately as a biotin labeling process. After passed the ELOSA quality control, samples were then hybridized onto Genechip miRNA 4.0 arrays for 16 hours using GeneChip Hybridization, Wash, and Stain Kit (Affymetrix) and the corresponding protocol. After washing, the GeneChips were scanned in an Affymetrix GeneChip Scanner 3000 run with Affymetrix GeneChip Command Console Software and read out as cell intensity (CEL) files. The normalized signal intensity was $\log_2$ transformed, and data analysis was performed with Expression Console Software and Transcriptome Analysis Console (TAC) Software (Affymetrix).

The Statistical Analysis of Microarrays was used, and miRNAs with ≥2-fold expression change with ANOVA test of $p < 0.05$ and a false-discovery rate (FDR) of ≤5% were selected and considered significantly altered. Global sample variation was assessed by principal components analysis. Ingenuity® Pathway Analysis (IPA®) (Qiagen, Ontario, Canada) was used to identify potential gene targets and the most significantly affected pathways from Ingenuity Expert Findings, TarBase, TargetScan and miRecords databases.

3.4.3 Poly-A RNA exogenous positive controls

The obtained cell intensity (CEL) files were quality assessed using Affymetrix quality control pipeline in Expression Console software to monitor the entire target preparation. The pipeline consisted of the following items and their complementary probes:

- Detection of spiked-in sample preparation probes: Lysine (Lys), Phenylalanine (Phe), Threonine (Thr), and Diaminopimelic acid (Dap) should be detected in the following intensity order: Lys < Phe < Thr < Dap.
- Detection of spiked-in hybridisation probes: The enzymes BioB, BioC, and BioD from the biotin synthesis pathway of Escherichia coli as well as the creX gene from bacteriophage P1 should be detected in the following intensity order: BioB < BioC < BioD < creX.

3.4.4 Validation of miRNA array findings by qPCR

Gene-specific PCR forward primers and a universal PCR reverse primer were designed according to the miRNA sequences. The U6 small nuclear RNA (NR_003027) was applied as an internal control (Schmittgen, Jiang, Liu, & Yang, 2004). The expression of the miRNA precursors was determined using quantitative real-time PCR according to the protocol provided by GeneCopoeia, USA. Quantitative real-time PCR was conducted using Applied Biosystems StepOnePlus^{TM} Real-Time PCR machine. Unless indicated, the qPCR was performed using All-in-one miRNA Detection kit (GeneCopoeia, USA). Single strain cDNA conversion was performed at 37°C for 60 minutes, followed by 85°C for 5 minutes. Cycling parameters for qPCR reaction were 95°C for 5 min to denaturing, then 40 cycles of 95°C for 10 s, and 60°C for 20 s, with a final recording step of 75°C for 20 s. Melting curves were conducted to comfirm that only a single product was amplified. All reactions including negative controls were duplicated. The relative expression level for each miRNA was analysed by the comparative CT method ($2^{-\Delta\Delta Ct}$).

3.5 Global transcriptome profiling

3.5.1 Rat gene array methods and analyses

Transcriptome profiling was performed with the nucleus accumbens and hippocampus tissue. Total RNA (500 ng) was 3' labeled with the FlashTag Biotin HSR RNA Labeling Kit (Affymetrix, Santa Clara, CA), according to the manufacturer's protocol. Poly-A RNA exogenous positive controls was performed as described in section 3.4.2 to monitor the labeling process independently from the quality of the starting RNA samples. After the labeling process, samples were then hybridized onto Rat Gene 2.0 arrays for 16 hours (Affymetrix). Hybridization images were then scanned and digitized with the Genechip Scanner 3000 (Affymetrix). The normalized signal intensity was $\log_2$ transformed, and data analysis was performed with Expression Console Software and Transcriptome Analysis Console (TAC) Software (Affymetrix). After we used the Statistical Analysis of Microarrays, miRNAs with ≥2-fold expression change with ANOVA test of $p < 0.05$ and a false-discovery rate (FDR) of ≤10% were selected and considered significantly altered. Global sample variation was assessed by principal components analysis.

Ingenuity® Pathway Analysis (IPA®) (Qiagen, Ontario, Canada) was used to identify potential gene targets and the most significantly affected pathways from Ingenuity Expert Findings, TarBase, TargetScan and miRecords databases.

3.5.2 Validation of transcriptome array findings by qPCR

Gene-specific PCR forward primers and a universal PCR reverse primer were designed according to the mRNA sequences. The expression of the housekeeping genes GAPDH was applied as an internal control (Tsolakidou et al., 2010). The expression of the mRNA precursors was determined using qPCR according to the protocol provided by GeneCopoeia, USA. Quantitative real-time PCR (qPCR) was conducted using Applied Biosystems StepOne plus real-time PCR machine. Unless indicated, the qPCR was performed using All-in-one mRNA Detection kit (GeneCopoeia, USA). Single strain cDNA conversion was performed at 37°C for 60 minutes, followed by 85°C for 5 minutes. Cycling parameters for qPCR reaction were 95°C for 5 min to denaturing, then 40 cycles of 95°C for 10 s, and 60°C for 20 s, with a final recording step of 75°C for 15 s. Melting curves were conducted to comfirm that only a single product was amplified. All reactions including negative controls were duplicated. The relative expression level for each miRNA was analysed by the comparative CT method ($2^{-\Delta\Delta Ct}$).

3.6 Global protein profiling

3.6.1 Sample preparation

The rats' brain tissues were collected and total proteins were extracted from the rat's nucleus accumbens and hippocampal tissues using AllPrep Protein Kit (Qiagen, Hilden, Germany) according the protocol provided. After that, all samples were desalted and buffer exchanged to 10 mM Tris pH 8.0. The concentration was determined using ToPA Protein Assay Kit (ITSI-Biosciences, USA). 25 µg of depleted and undepleted samples were reduced with Dithiothreitol and alkylated with iodoacetamide. In-solution digestion was performed by using trypsin as the enzyme. Samples were dried after overnight trypsinization and resuspended in 10 mM potassium phosphate in 25 % acetonitrile. After that, the protein samples were treated with 40 mL of a 10% MeOH (1% acetic acid) solution and centrifuged at 4000 x g for 30 min. to eliminate cell debris. From the stock solution, all different samples were prepared by a two-step centrifugal separation using 150kDa and 9 kDa molecular weight cutoff filter (MWCO) (Thermo Fisher Scientific, MA USA). Sample volumes of 20, 40, and 80 mL were used for each replicate. The high- and low-MW samples were digested and analyzed. A portion of the low-MW sample was kept for intact analysis. Each sample was spiked with the PRTC kit (Thermo Fisher Scientific) prior to LC-MS analysis.

3.6.2 Liquid Chromatography

For 1st dimension liquid chromatography (LC) separation, peptides were fractionated in a stepwise manner by Strong Cation eXchange using 200, 300, 400, and 500mM ammonium acetate adjusted to pH 3.0 with formic acid. Peptides were reconstituted in 100 µl 0.1% formic acid and desalted using ziptip, dried in a speed vac and reconstituted in a solution containing 2% acetonitrile and 0.1% formic acid. For the 2nd dimension LC separation, samples were loaded onto a PicoFrit C18 nanospray

column (New Objective) using a Thermo Scientific Surveyor Autosampler operated in the no waste injection mode.

3.6.3 Mass Spectrometry

Peptides were eluted from the column using a linear acetonitrile gradient from 2 to 35% acetonitrile over 210 minutes followed by high and low organic washes for another 20 minutes into an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, MA USA) via a nano spray source with the spray voltage set to 1.8kV and the ion transfer capillary set at 180 degrees Celsius. A data-dependent Top 7 method was used where a full MS scan from m/z 350-1700 was followed by MS/MS scans on the three most abundant ions.

All the protein data were analyzed using software, named Proteome Discoverer 1.4 (Thermo Scientific) and the SEQUEST algorithm which consists of the updated species-specific database for rat from NCBI. Proteins were identified when there are more than one peptides had X-correlation scores greater than 1.5, 2.0, and 2.5 for respective charge states of +1, +2, and +3. Trypsin was used because this enzyme was able to provide up to two missed cleavages each peptide; carbamidomethyl was also be applied as a static modifications and oxidation of methionine as a variable modification. Label free quantitation was performed with Sieve 2.1 software. Default parameters were used except that the frames threshold was changed to 8000. Precursor area / height from each peptide will be obtained from Sieve 2.0 software (Thermo Scientific).

3.6.4 Statistic analysis for global protein profiling

The expression of proteins which were ≥2-fold altered and ANOVA test of $p < 0.05$ were selected and considered significantly up-regulated, while protein expression with ≤2-fold altered and $p < 0.05$ were be considered significantly down-regulated. The fold change within -2 to 2 was considered as unchanged.

3.7 Target prediction and network analysis

The Ingenuity® Pathway Analysis (IPA®) Software (Ingenuity Systems, Redwood City, CA, USA; www.ingenuity.com) was used to analyze the list of predicted miRNA targets and its related genetic pathway. The miRNAs which were significantly associated with METH addiction behavior was analyzed to identify the miRNA targets and genetic pathways which are related to addiction. Filtering was performed to remove duplicates and genes with no annotation in the IPA® system. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Networks of genes involved in neurological and psychological disorders were then algorithmically generated. Furthermore, the interaction between the METH-addiction miRNAs with METH-addiction genes and proteins from transcriptome and protein profiling was also analyzed to identify their targets and the relevant pathways which are related to METH addiction.

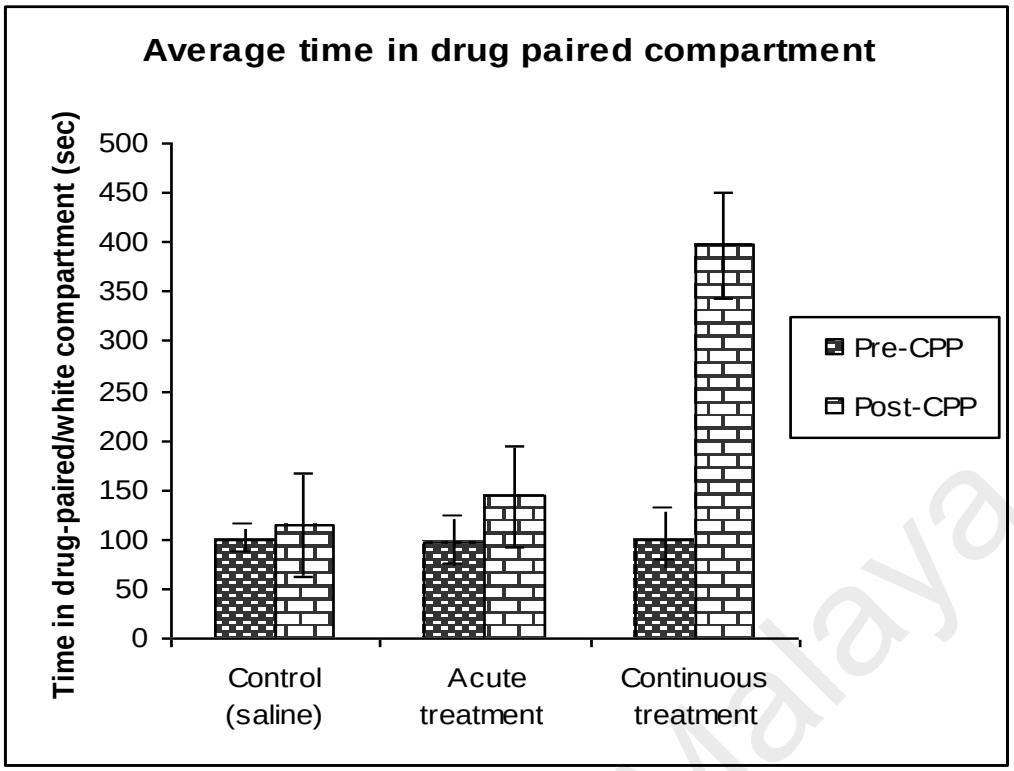
CHAPTER 4:

RESULTS

4.1 Animal behavioral studies

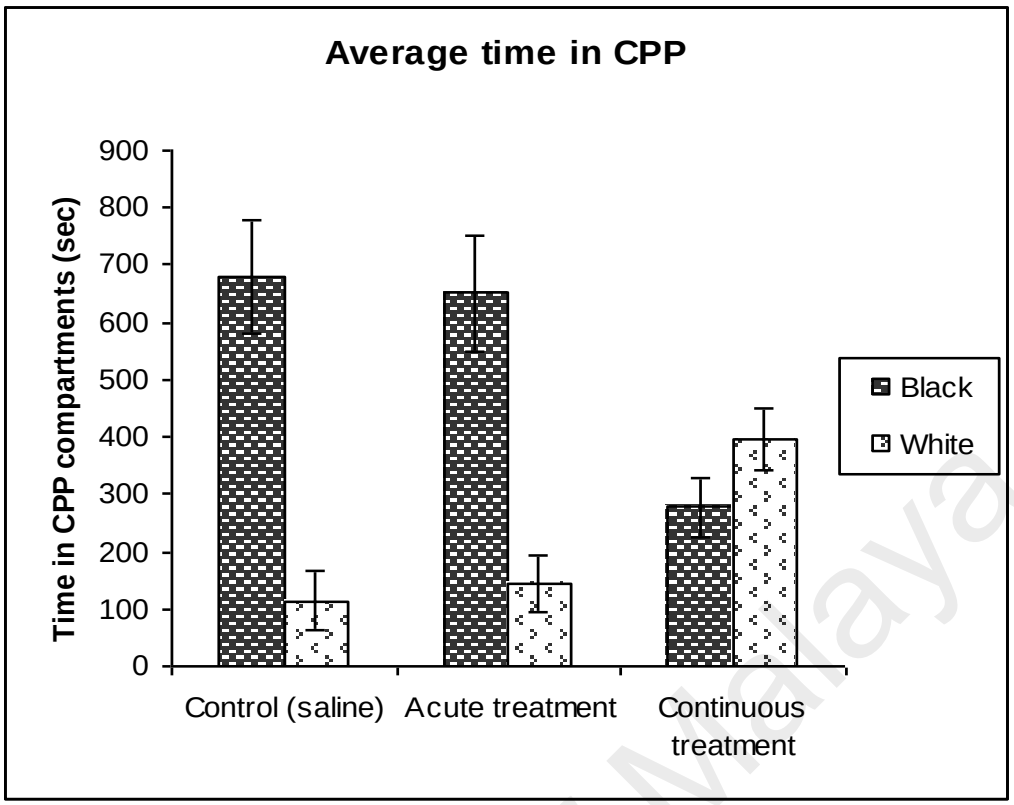
4.1.1 Conditioned Place Preference (CPP) paradigm

Animals which were subjected to saline conditioning spent less time in the conditioning compartment, indicating a preference for the opposite compartment of the CPP when compared to animals exposed to METH conditioning. However, a continuous 15 alternate days METH treatment rats showed a preference for the white color drug-paired compartment of the place preference apparatus (Figure 4.1 & 4.2). However, a one time acute treatment with 5 mg/kg METH did not show a preference for the drug-paired compartment (Figure 4.1 & 4.2). The average time spent for the continuous METH treatment rats in the drug-paired compartment was 396.89±54 sec while the acute METH treatment and the saline treatment rats are 143.56±51 sec and 114.67±51 sec, respectively. When compared with pre-treatment test, the rats exposed to a 15 days continuous METH treatment exhibited a significant preference to drug-paired compartment in comparison to the acute METH treatment and saline conditioned animals (Figure 4.1). The administration of METH (0.25, 0.5, 1, 2, 3, 4, 5 mg/kg) for 15 alternate days either in the black or the white compartment of the CPP cage resulted in strong significant preference for the METH-paired compartment compared with the time spent in the same compartment before drug administration ($F = 125.23$, $p < 0.001$) (Figure 4.1). The single dose (5 mg/kg) acute METH treatment and saline injection alone did not produce any significant change in compartment preference. It's showed that there is not addiction behavior although one time injection of high dose METH is given.



Treatment	Pre-CPP		Post-CPP		ANOVA		
	Mean	SD	Mean	SD	F	P value	Bonferroni
Control (saline)	98.8	12.93	114.67	51.49	0.44	0.518	p>0.017
Acute treatment	97	25.71	143.56	50.81	3.59	0.083	p>0.017
Continuous treatment	99	29.91	396.89	54.49	125.23	**<0.001**	**p<0.017**

Figure 4.1: Average time spent by METH-administrated rats in drug paired compartment of CPP (post-CPP vs pre-CPP).
Comparison of the time spent between pre-conditioning and post-conditioning in drug paired compartment. The 15 alternate days continuous METH treatment resulted in strong significant preference for the METH-paired compartment compared with the time spent in the same compartment before drug administration. However, there is no significant difference between the acute METH treatment group and the controls.



Treatment	Black		White		ANOVA		
	Mean	SD	Mean	SD	F	P value	Bonferroni
Control (saline)	679.44	98.96	114.67	51.49	-	-	-
Acute treatment	650.56	99.8	143.56	50.81	1.44	0.248	p>0.017
Continuous treatment	277.56	52.43	396.89	54.49	127.54	**<0.001**	**p<0.017**

Figure 4.2: Average time spent by METH-administrated rats in drug paired compartment of CPP (white vs black).
Comparison of the time spent between pre-conditioning and post-conditioning in drug paired compartment. The 15 alternate days continuous METH treatment resulted in strongly significant preference for the METH-paired compartment compared with the time spent in the same compartment before drug administration. However, there is no significant difference between the acute METH treatment group and the controls.

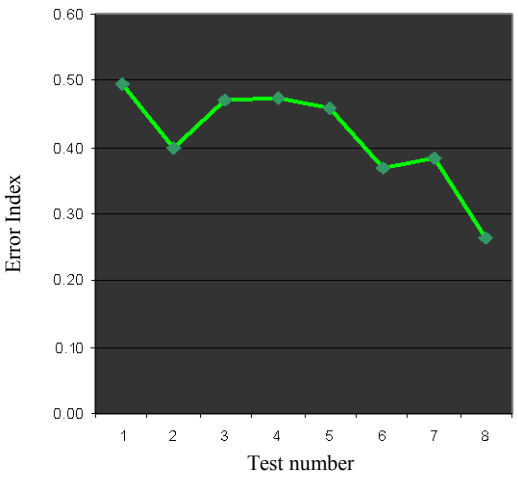
4.1.2 Radial arm maze test

Four categories of the radial maze study have been tested; these are arms error, entries error, repeated entry and latency test. We found that there was a strong significant difference for the arms error test and entries error test when comparing between the control group and the METH group at the dose of 2, 4 and 5 mg/kg (Figure 4.3a, b & Table 4.1). However, only the trial with 5 mg/kg was shown to be significantly different after adjustment by Bonferroni correction test. When comparing repeated entry index between METH group and the controls, it was only significantly different at trial with 4 and 5 mg/kg METH, either adjusted or not adjusted by Bonferroni test (Figure 4.3c). For the latency test, we found that it was only significantly different at the final dose, 5 mg/kg METH (Figure 4.3d).

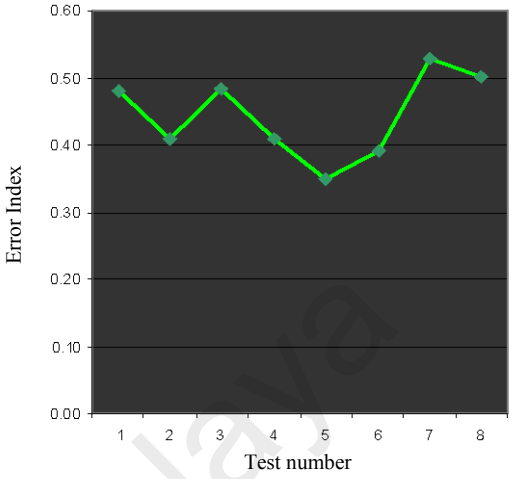
At the beginning of the low dose of METH use, our results showed that the METH group has similar chart pattern with the control. However, a turning point came when there was increased number of the errors at 2 mg/kg of METH.

a) Arm errors

Saline treatment

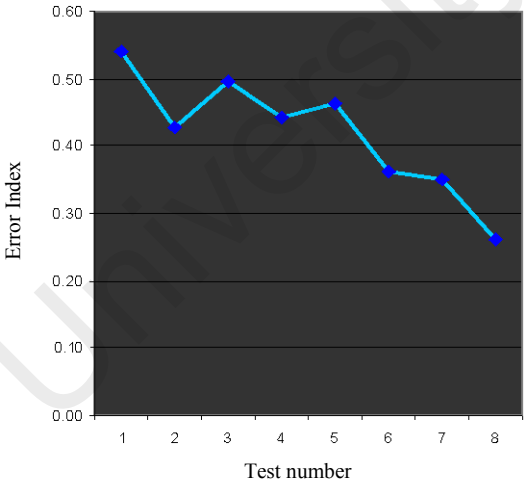


METH treatment



b) Entry errors

Saline treatment



METH treatment

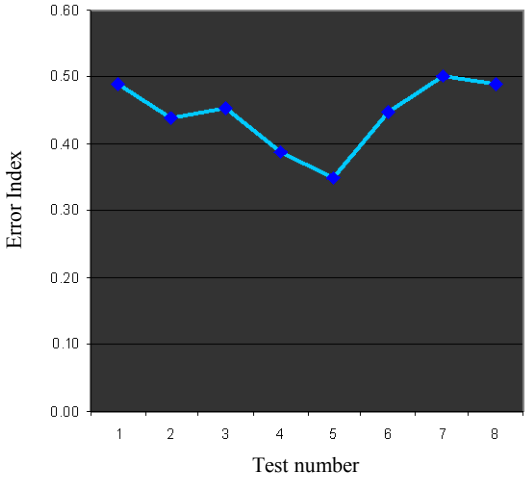
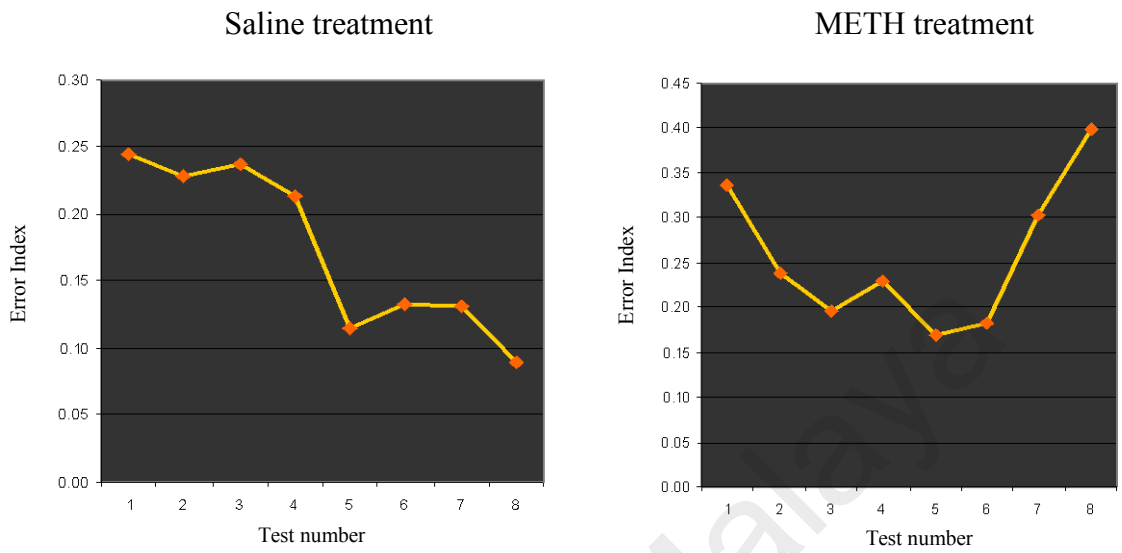


Figure 4.3, continued.

c) Repeated entry



d) Latency test

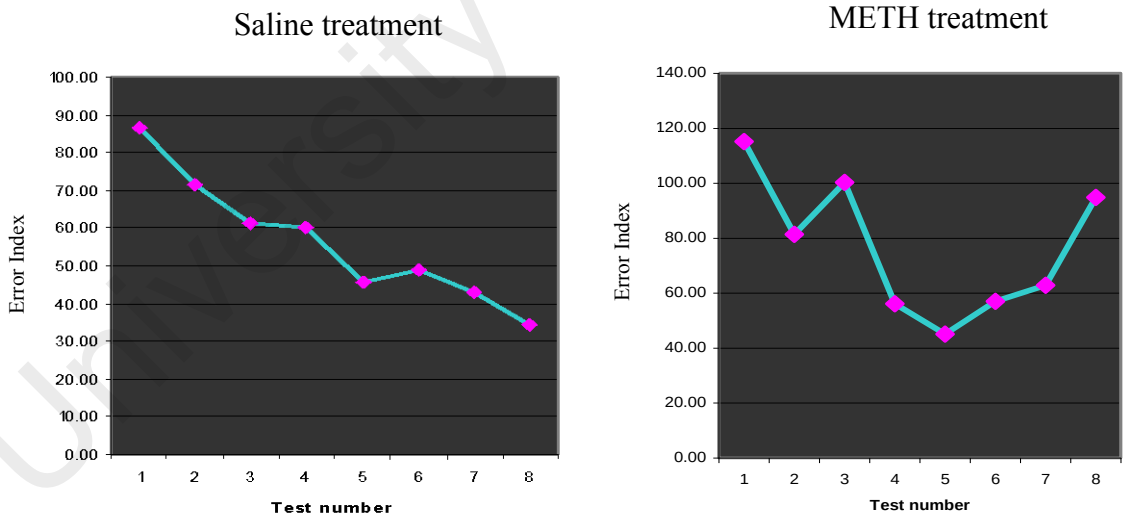


Figure 4.3: The effects of the methamphetamine abuse to memory and learning. Legend: Test 1: no drug treatment; Test 2: 0.25 mg/kg METH; Test 3: 0.5 mg/kg METH; Test 4: 1 mg/kg METH; Test 5: 2 mg/kg METH; Test 6: 3mg/kg METH; Test 7: 4 mg/kg METH and Test 8: 5 mg/kg METH.

Table 4.1: The effects on the learning and memory following METH exposure

Test	Trials	Control		Methamphetamine			T-test	p-value
		Mean	SD	Dose (mg/kg)	Mean	SD		
Error arm	1	0.49	0.06	0	0.48	0.11	-0.239	0.814
	2	0.40	0.18	0.25	0.41	0.18	0.118	0.908
	3	0.47	0.07	0.5	0.48	0.07	0.303	0.766
	4	0.47	0.11	1	0.41	0.13	1.057	0.306
	5	0.46	0.08	2	0.35	0.12	2.288	**0.036**
	6	0.37	0.16	3	0.39	0.15	-0.274	0.788
	7	0.38	0.11	4	0.53	0.06	-3.591	**0.002***
	Final	0.26	0.13	5	0.50	0.08	-4.717	**<0.001***
Error enter	1	0.54	0.09	0	0.49	0.14	0.901	0.381
	2	0.43	0.19	0.25	0.44	0.18	-0.115	0.910
	3	0.50	0.12	0.5	0.45	0.08	1.040	0.314
	4	0.44	0.11	1	0.39	0.16	0.773	0.451
	5	0.46	0.08	2	0.35	0.12	2.288	**0.036**
	6	0.36	0.18	3	0.45	0.13	-1.216	0.242
	7	0.35	0.11	4	0.50	0.08	-3.308	**0.004***
	Final	0.26	0.12	5	0.49	0.10	-4.417	**<0.001***
Repeat enter	1	0.24	0.11	0	0.34	0.20	-1.314	0.207
	2	0.23	0.20	0.25	0.24	0.21	-0.103	0.919
	3	0.24	0.19	0.5	0.20	0.20	0.435	0.669
	4	0.21	0.14	1	0.23	0.20	-0.246	0.809
	5	0.11	0.17	2	0.17	0.18	-0.727	0.478
	6	0.13	0.14	3	0.18	0.11	-0.842	0.412
	7	0.13	0.10	4	0.30	0.13	-3.110	**0.007***
	Final	0.09	0.09	5	0.40	0.13	-5.882	**<0.001***
Latency (in sec)	1	86.44	41.00	0	115.11	43.11	-1.446	0.168
	2	71.56	41.30	0.25	81.33	33.93	-0.548	0.591
	3	61.44	34.00	0.5	100.33	54.11	-1.826	0.087
	4	60.22	6.78	1	56.11	20.49	0.571	0.576
	5	45.44	14.33	2	45.11	18.91	0.042	0.967
	6	49.00	21.34	3	57.00	23.02	-0.765	0.456
	7	43.00	23.15	4	62.89	26.49	-1.696	0.109
	Final	34.67	15.59	5	94.78	46.59	-3.671	**0.002***

* Significant after Bonferroni test.

4.2 Global miRNA profiling analysis

The global profiles of miRNAs in two rat brain tissues, nucleus accumbens and hippocampus, were studied by using Affymetrix Genechip miRNA 4.0 arrays which are specific for the rat species.

4.2.1 Nuclues accumben tissue

4.2.1.1 Global miRNA microarray analysis

The quality of the RNA samples was tested by using gel electrophoresis and a bioanalyzer, both of which indicated that the quality of the RNA was good (RIN $\geq$ 8). In the preliminary array screening, we observed an altered expression of 170 miRNAs in the METH addiction group which was subjected to a continuous 15 alternate days METH treatment, compared to the controls (Figure 4.6). Out of 170 miRNAs, 166 miRNAs were significantly up-regulated (fold-change $\geq$ 2, $p < 0.05$, FDR $\leq$ 5%) and 4 down-regulated (fold-change $\leq$ -2, $p < 0.05$, FDR $\leq$ 5%). Upon comparison between acute METH treatment without addiction group and the control group, there were 162 miRNAs which were significantly up-regulated and 2 were down-regulated (Figure 4.6).

Further stratification among the acute METH treatment without addiction group and METH addiction group which was subjected to a continuous 15 alternate days METH treatment, revealed that 132 miRNAs were simultaneously up-regulated in both of the groups when compared with controls (green area in Figure 4.6). A total of 70 miRNAs showed significant difference between these two groups with the control group. However, among the 70 miRNAs, altered expressions of 32 miRNAs (30 up-regulations and 2 down-regulations) were observed in the acute METH treatment without addiction group, while another 38 miRNAs (34 up-regulations and 4 down-regulations) were observed in METH addiction group (blue and yellow area in Figure

4.6). However, expressions of 59 miRNAs (52 up-regulations and 7 down-regulations) were statistically significantly altered when compared between the METH addiction group and non-addiction group (Table 4.2).

Although there are 132 miRNAs which were simultaneously up-regulated in the addiction group and without addiction groups, we also found that 28 miRNA were increased more than hundred percent and 3 was decreased more that fifty percent when compared the fold change of the miRNA expression between the group before addiction and after addiction (Table 4.3).

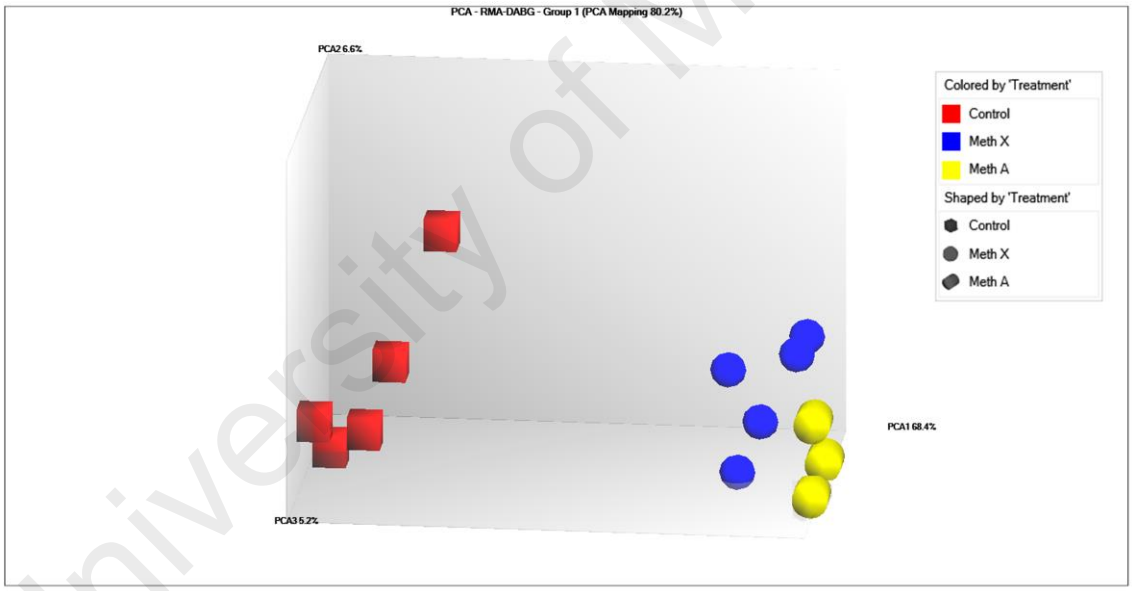


Figure 4.4: PCA mapping shows that the samples clustered in 3 different groups.

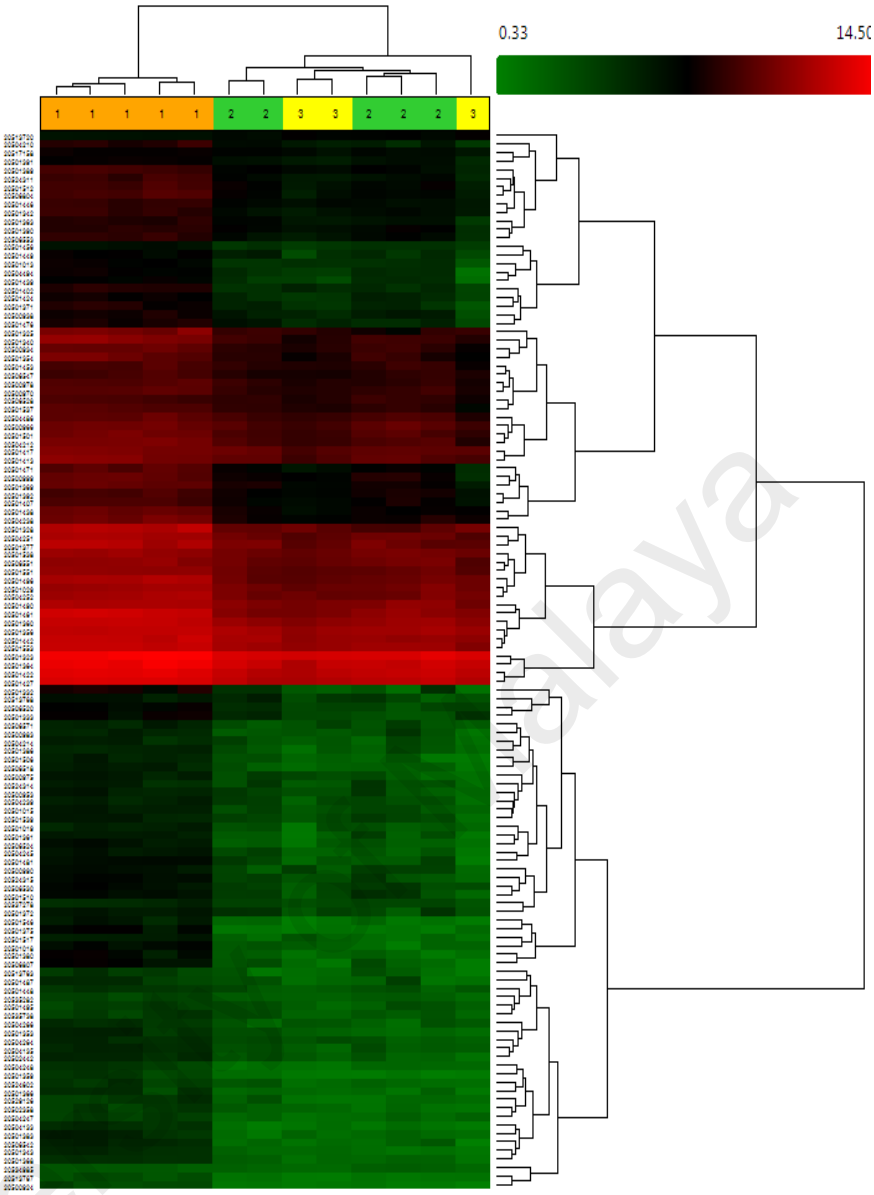


Figure 4.5: A miRNA expression map shows differentiated genes.
Red indicates high expression and green indicates low expression, relative to the median.

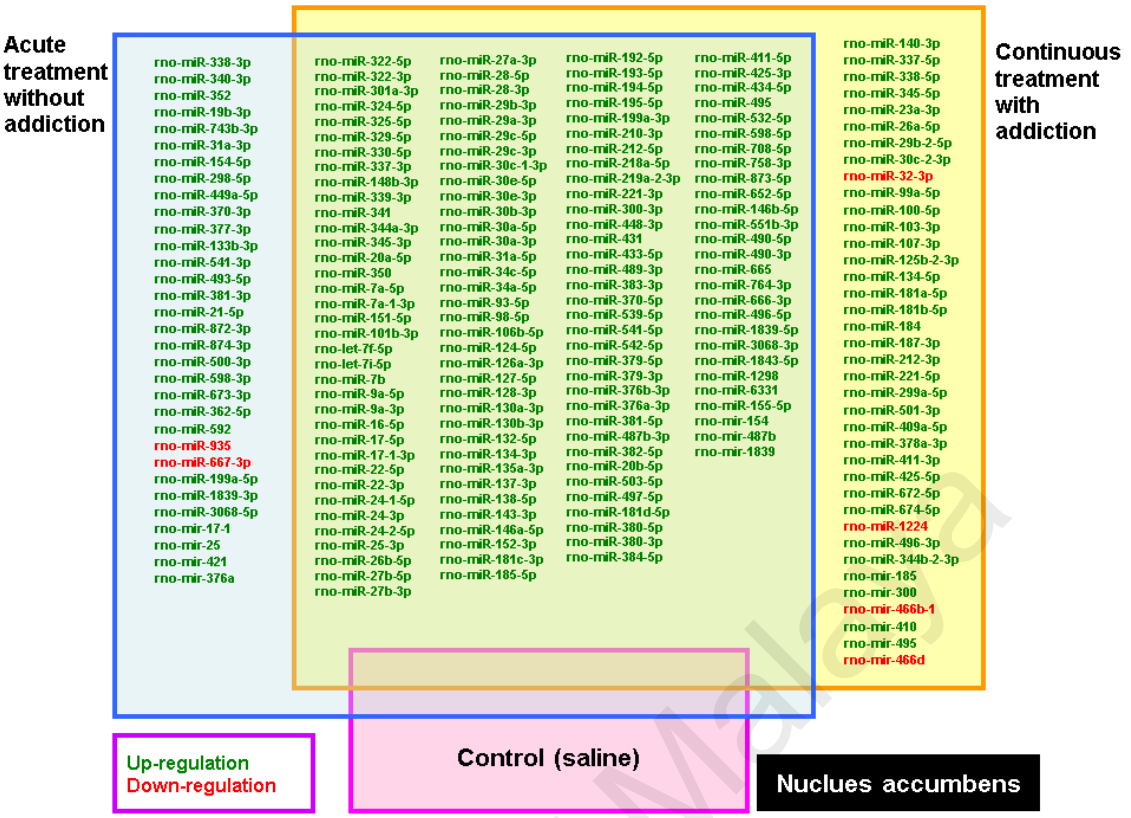


Figure 4.6: The significantly altered expression of miRNA in the acute treatment group without addiction and the continuous treatment group with addiction when compared with the control group.

Table 4.2: The significantly altered expression of miRNA in nucleus accumbens which are associated with METH addiction by comparing the continuous treatment group with acute treatment group without addiction.

miRNA ID	Fold change	ANOVA p-value
rno-miR-496-3p	13.13	<0.001
rno-miR-194-5p	5.40	0.035
rno-miR-299a-5p	4.46	0.006
rno-miR-132-3p	4.44	0.033
rno-miR-127-5p	4.29	0.016
rno-miR-411-3p	3.84	0.008
rno-miR-30c-5p	3.67	<0.001
rno-miR-221-5p	3.67	0.002
rno-miR-501-3p	3.60	<0.001
rno-miR-551b-3p	3.47	0.021
rno-miR-409a-5p	3.43	0.001
rno-miR-184	3.36	0.004
rno-miR-187-3p	2.98	<0.001
rno-miR-99a-5p	2.91	<0.001
rno-miR-337-5p	2.73	<0.001
rno-miR-181a-5p	2.64	0.040
rno-miR-345-5p	2.63	0.006
rno-miR-29b-2-5p	2.63	0.004
rno-miR-124-5p	2.60	0.035
rno-miR-125b-2-3p	2.57	0.005
rno-mir-185	2.53	0.007
rno-mir-410	2.50	0.006
rno-miR-338-5p	2.45	<0.001
rno-miR-181b-5p	2.45	<0.001
rno-miR-344b-2-3p	2.43	0.007
rno-miR-140-3p	2.39	<0.001
rno-miR-143-3p	2.37	0.011
rno-miR-674-5p	2.32	<0.001
rno-mir-495	2.31	0.008
rno-miR-212-3p	2.30	<0.001
rno-miR-425-3p	2.29	<0.001
rno-miR-146b-5p	2.29	0.006
rno-miR-107-3p	2.27	<0.001
rno-miR-100-5p	2.25	<0.001
rno-miR-195-5p	2.25	0.004
rno-miR-672-5p	2.23	0.001
rno-miR-30b-5p	2.22	0.036
rno-miR-134-3p	2.22	<0.001
rno-miR-106b-5p	2.20	0.010
rno-miR-425-5p	2.19	0.037
rno-mir-300	2.16	0.007
rno-miR-103-3p	2.14	<0.001
rno-miR-378a-3p	2.11	<0.001
rno-miR-708-5p	2.11	0.020
rno-miR-497-5p	2.10	0.032

Table 4.2 continued

miRNA ID	Fold change	ANOVA p-value
rno-miR-465-5p	2.09	0.027
rno-miR-27a-3p	2.08	0.042
rno-miR-376b-3p	2.07	0.039
rno-miR-23a-3p	2.05	<0.001
rno-miR-26a-5p	2.05	0.001
rno-miR-551b-5p	2.04	0.023
rno-mir-543	2.02	0.044
rno-mir-466b-1	-2.04	0.005
rno-miR-1224	-2.27	<0.001
rno-mir-466d	-2.43	0.004
rno-miR-32-3p	-2.62	0.002
rno-miR-628	-2.73	0.017
rno-miR-881-3p	-3.42	0.029
rno-miR-200b-5p	-4.71	0.011

Table 4.3: The changes of the miRNA expression in nucleus accumbens which are significant in both addiction and non-addiction group when compared with the controls.

Mirbase ID	Fold change		Changes (%)
	Before addiction	After addiction	
rno-miR-9a-5p	11.15	74.62	569%
rno-miR-28-5p	2.97	11.93	302%
rno-miR-24-2-5p	10.85	42.30	290%
rno-miR-34c-5p	12.62	39.44	213%
rno-miR-152-3p	11.70	36.51	212%
rno-miR-379-3p	4.59	10.68	133%
rno-miR-193-5p	2.41	5.48	127%
rno-miR-181c-3p	5.06	11.27	123%
rno-miR-25-3p	3.07	6.81	122%
rno-miR-490-3p	5.10	11.25	121%
rno-miR-350	6.02	12.57	109%
rno-miR-30b-3p	8.03	16.11	101%
rno-miR-341	9.39	18.79	100%
rno-miR-9a-3p	31.14	15.47	–50%

4.2.1.2 Validation with quantitative PCR (qPCR)

The differential expression of the 6 miRNAs and 9 samples (3 samples from each of the METH addicted, METH treatment without addicted and the control groups, respectively), were further validated by SYBR Green qPCR. The miRNAs were selected for validation according to high fold change, low p-value, and low FDR. We observed consistent results for all miRNAs. In our qPCR findings, miR-9a-3p shows significant up-regulation in both of the treatment groups (acute treatment: $\Delta\Delta Ct = -4.246$, 18.97 fold changes; continuous treatment: $\Delta\Delta Ct = -1.577$, 2.98 fold changes), miR-29b-3p shows only significant up-regulation in acute treatment group (acute treatment: $\Delta\Delta Ct = -1.13$, 2.18 fold changes; continuous treatment: $\Delta\Delta Ct = -0.28$, 1.21 fold changes), miR-218a-5p shows significant up-regulation in both treatment groups (acute treatment: $\Delta\Delta Ct = -4.833$, 33.2 fold changes; continuous treatment: $\Delta\Delta Ct = -3.382$, 10.42 fold changes), miR-7a-3p, miR-199a-3p and miR-322-3p also show significant up-regulation in both treatment groups (miR-7a-3p: acute treatment: $\Delta\Delta Ct = -3.637$, 12.44 fold changes; continuous treatment: $\Delta\Delta Ct = -1.285$, 2.44 fold changes), (miR-199a-3p: acute treatment: $\Delta\Delta Ct = -1.759$, 3.39 fold changes; continuous treatment: $\Delta\Delta Ct = -4.085$, 16.97 fold changes), and (miR-322-3p: acute treatment: $\Delta\Delta Ct = -1.404$, 2.65 fold changes; continuous treatment: $\Delta\Delta Ct = -1.908$, 3.75 fold changes).

4.2.1.3 Enrichment pathway analysis and target gene prediction

The pathway analysis software, IPA, defined significant networks in nucleus accumbens, main functions and canonical pathways related with the differentially expressed genes for each comparison analyzed. From the profiling carried out, among the addiction-associated miRNAs, we found that 24 miRNAs were highly involved in neurological and psychological disorder pathway following filtering by the IPA system. A total of 1046 gene targets which have been reported to be involved in METH addiction phenotype were identified. From the analysis, miR-30c-5p showed the highest number of gene targets, in which 162 gene targets are related to the addiction pathway (Figure 4.7). This is followed by miR-27a-3p, miR-181a-1-3p and miR-23a-3p, which indicated 146, 126 and 109 gene targets, respectively. Similarly, in this comparison, these miRNAs and the related gene targets are highly associated with 20 canonical pathways which are related to neurological and psychological disorder, e.g.: MAPK Signaling, CREB Signaling, G-Protein Coupled Receptor Signaling (GPCRs), GnRH Signaling; cAMP-mediated signaling; and G Beta Gamma Signaling (Table 4.4).

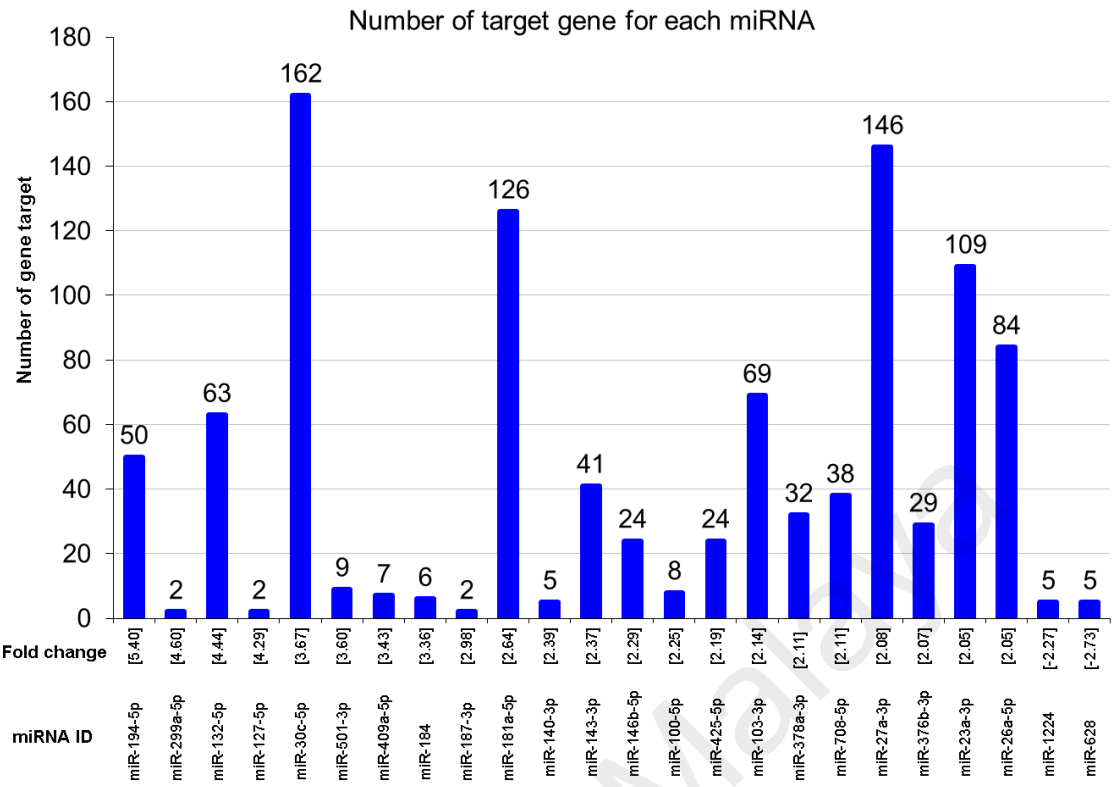


Figure 4.7: The number of miRNA gene targets which are associated with methamphetamine addiction.

Table 4.4: The relationship between the addiction-related miRNAs and the enriched synaptic signaling pathways in nucleus accumbens which may be associated with METH addiction.

Synaptic pathways	Addiction-related miRNAs
Glutamate Receptor Signaling	miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-140-3p, miR-181a-5p, miR-184, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-425-5p
G Beta Gamma Signaling	miR-100-5p, miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-140-3p, miR-181a-5p, miR-184, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-409-5p
CREB Signaling in Neurons	miR-100-5p, miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-140-3p, miR-181a-5p, miR-184, miR-187-3p, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-409-5p, miR-425-5p, miR-1224-5p
GABA Receptor Signaling	miR-100-5p, miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-181a-5p, miR-187-3p, miR-23a-3p, miR-378a-3p, miR-425-5p
G-Protein Coupled Receptor Signaling	miR-100-5p, miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-140-3p, miR-181a-5p, miR-184, miR-187-3p, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-425-5p, miR-1224-5p
Serotonin Receptor Signaling	miR-103-3p, miR-132-3p, miR-23a-3p, miR-26a-5p
Dopamine Receptor Signaling	miR-100-5p, miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-181a-5p, miR-187-3p, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-425-5p
GNRH Signaling	miR-100-5p, miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-140-3p, miR-181a-5p, miR-187-3p, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-425-5p, miR-1224-5p
CXCR4 Signaling	miR-100-5p, miR-103-3p, miR-212-3p, miR-26a-5p, miR-140-3p, miR-181a-5p, miR-184, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-409-5p, miR-425-5p
cAMP-mediated signaling	miR-100-5p, miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-140-3p, miR-181a-5p, miR-187-3p, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-425-5p, miR-1224-5p
Protein Kinase A (PKA) Signaling	miR-100-5p, miR-103-3p, miR-212-3p, miR-26a-5p, miR-140-3p, miR-181a-5p, miR-184, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-409-5p, miR-425-5p
Neuregulin Signaling	miR-100-5p, miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-181a-5p, miR-23a-3p, miR-378a-3p
Calcium Signaling	miR-100-5p, miR-103-3p, miR-212-3p, miR-26a-5p, miR-181a-5p, miR-184, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-425-5p
MAPK Signaling	miR-100-5p, miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-140-3p, miR-181a-5p, miR-184, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-425-5p, miR-1224-5p, miR-125b-2-3p, miR-672-5p
Rac Signaling	miR-100-5p, miR-103-3p, miR-212-3p, miR-26a-5p, miR-181a-5p, miR-23a-3p, miR-134-5p, miR-425-5p
Synaptic Long Term Potentiation	miR-100-5p, miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-181a-5p, miR-23a-3p, miR-134-5p
Gap Junction Signaling	miR-100-5p, miR-103-3p, miR-212-3p, miR-26a-5p, miR-140-3p, miR-181a-5p, miR-184, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-425-5p
GDNF Family Ligand-Receptor Interactions	miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-181a-5p, miR-23a-3p, miR-134-5p, miR-378a-3p, miR-425-5p, miR-1224-5p
PDGF Signaling	miR-103-3p, miR-132-3p, miR-212-3p, miR-26a-5p, miR-181a-5p, miR-184, miR-187-3p, miR-23a-3p
PAK Signaling	miR-103-3p, miR-212-3p, miR-26a-5p, miR-181a-5p, miR-184, miR-23a-3p, miR-134-5p, miR-409-5p, miR-425-5p

4.2.2 Hippocampal tissue

4.2.2.1 Global miRNA microarray analysis

In the preliminary array screening, a total of 276 miRNAs were detected. The results showed that a significantly altered expression of 190 miRNAs in the METH addiction group which experienced continuous 15 alternate days METH treatment compared to those of controls, whereby 180 miRNAs were significantly up-regulated (fold-change ≥ 2, $p < 0.05$, FDR $\leq 5\%$) and 10 miRNAs were down-regulated (fold-change ≤ -2, $p < 0.05$, FDR $\leq 5\%$) (yellow box in Figure 4.10). For comparing between acute METH treatment without addiction group and the controls, there were 78 miRNAs that were significantly up-regulated and 8 were down-regulated (blue box in Figure 4.10).

Further stratification among the acute METH treatment without addiction group and METH addiction group which was subjected to continuous 15 alternate days METH treatment revealed that 76 miRNAs were simultaneously up-regulated and 5 were down-regulated in both of the groups when compared with controls (green area in Figure 4.10). The expression of 114 miRNAs was significantly different between these two groups. However, among the 114 miRNAs, an altered expression of 5 miRNAs (2 up-regulations and 3 down-regulations) presented in acute METH treatment without addiction group, while another 109 miRNAs (104 up-regulations and 5 down-regulations) presented in METH addicted group (Figure 4.10 & Table 4.5).

Although there are 76 miRNAs and 5 miRNAs that have been simultaneously up-regulated and down-regulated, respectively, in both addiction group and without addiction groups, we noticed that 11 miRNA were increased more than 50 percent while 4 were decreased approximately 40 percent when comparing between the fold change of the miRNA expression in the group before addiction and after addiction (Table 4.6).

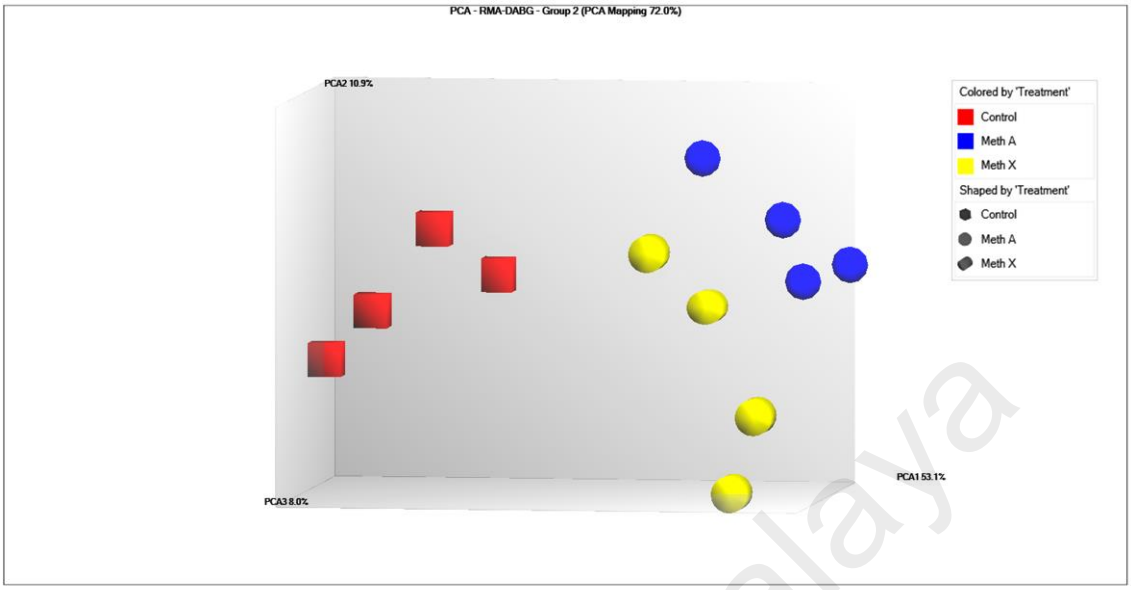


Figure 4.8: PCA mapping the miRNA microarray data shows that the samples clustered in 3 different groups.

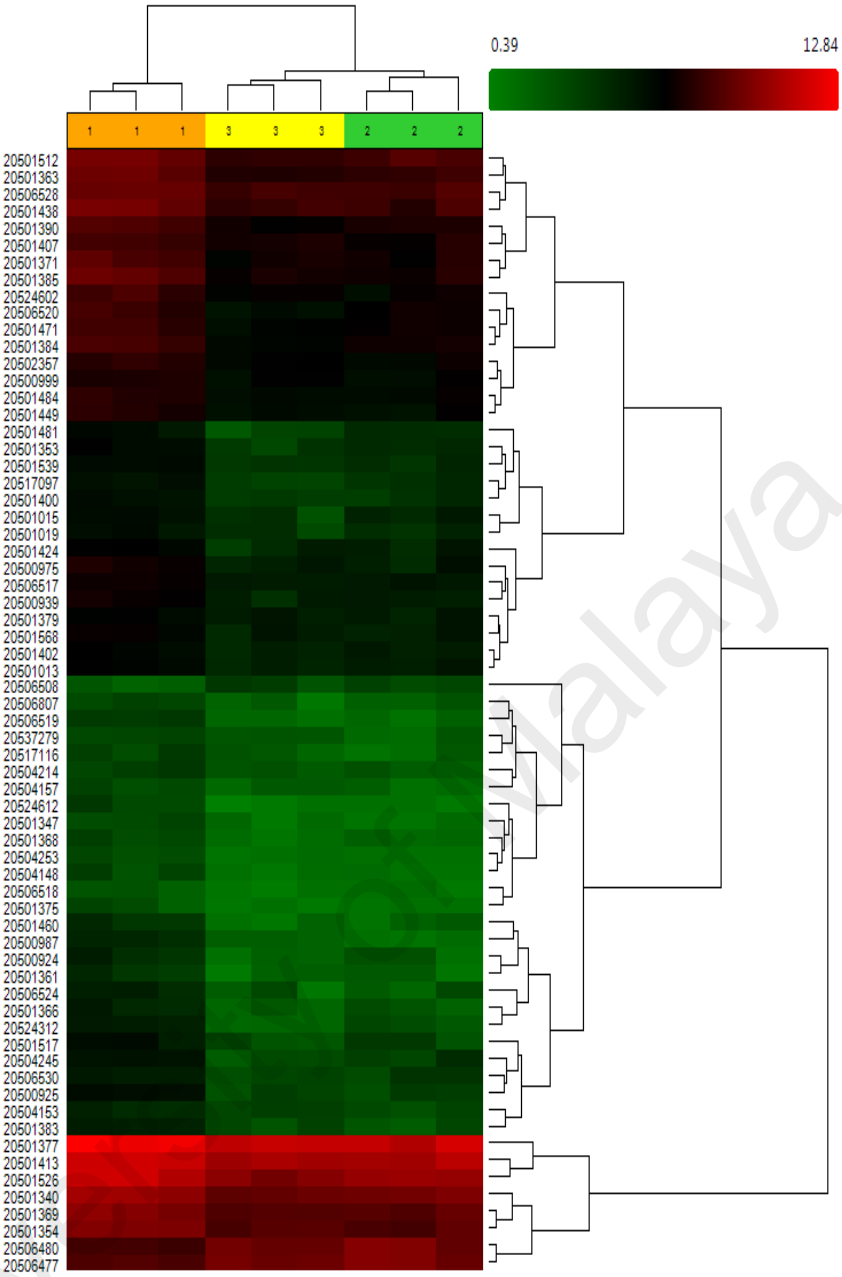


Figure 4.9: A miRNA expression map showing differentiated genes.
Red indicates low expression while green indicates high expression, relative to the median.

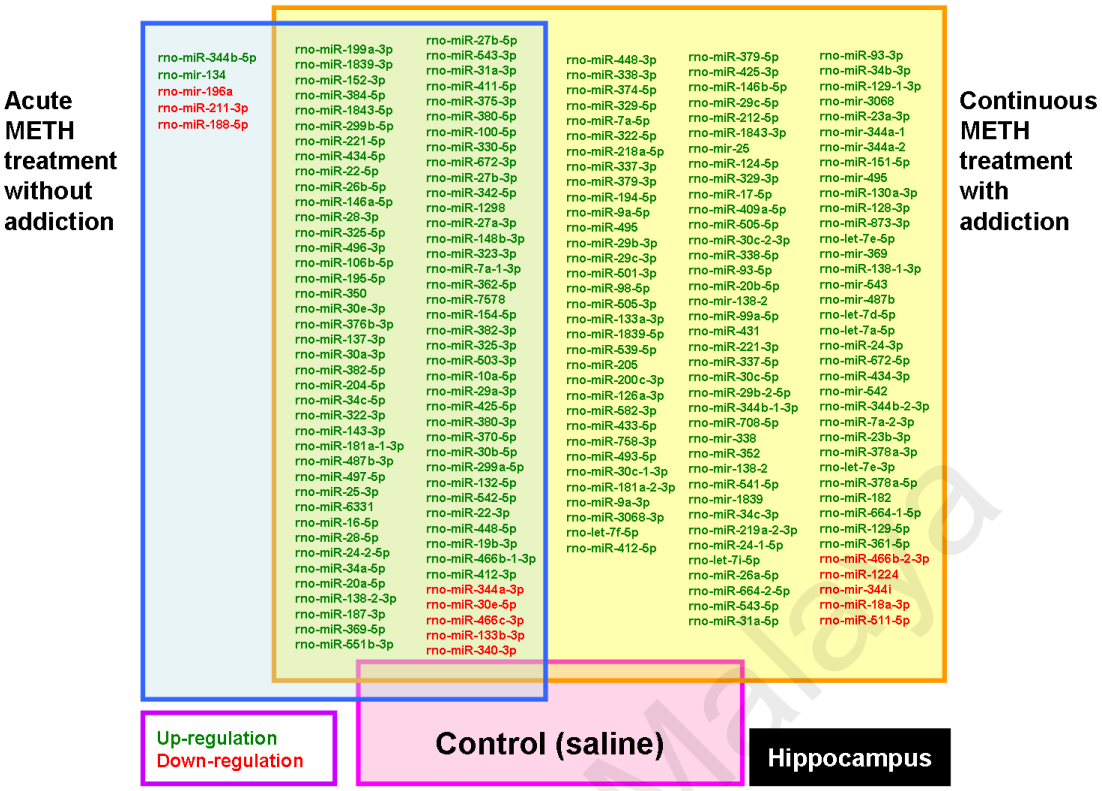


Figure 4.10: The significantly altered expression of miRNA in the acute treatment group without addiction and the continuous treatment group with addiction as compared with the control group.

Table 4.5: **The significantly altered expression of miRNA in hippocampus which are associated with METH addiction by comparing the continuous treatment group with acute treatment group without addiction.**

miRNA ID	Fold change	ANOVA p-value
rno-miR-448-3p	12.53	0.002
rno-miR-374-5p	7.75	0.037
rno-miR-329-5p	6.91	0.008
rno-miR-7a-5p	6.22	0.028
rno-miR-322-5p	5.77	0.003
rno-miR-218a-5p	4.94	0.003
rno-miR-337-3p	4.77	0.003
rno-miR-379-3p	4.72	0.006
rno-miR-194-5p	4.30	<0.001
rno-miR-9a-5p	4.16	0.016
rno-miR-29b-3p	4.06	0.016
rno-miR-29c-3p	3.96	0.001
rno-miR-338-3p	3.66	0.001
rno-miR-501-3p	3.64	0.004
rno-miR-98-5p	3.57	0.023
rno-miR-505-3p	3.54	0.006
rno-miR-133a-3p	3.44	0.026
rno-miR-1839-5p	3.14	0.002
rno-miR-539-5p	3.12	0.003
rno-miR-205	3.11	0.012
rno-miR-200c-3p	3.08	0.035
rno-miR-126a-3p	3.05	0.005
rno-miR-582-3p	3.03	<0.001
rno-miR-433-5p	3.01	0.01
rno-miR-758-3p	2.96	0.039
rno-miR-493-5p	2.94	0.034
rno-miR-30c-1-3p	2.82	0.021
rno-miR-9a-3p	2.71	0.007
rno-miR-3068-3p	2.64	0.002
rno-miR-379-3p	2.61	0.006
rno-let-7f-5p	2.55	0.007
rno-miR-412-5p	2.55	0.003
rno-miR-379-5p	2.51	0.005
rno-miR-425-3p	2.48	0.019
rno-miR-29c-5p	2.35	0.009
rno-miR-1839-3p	2.33	0.004
rno-miR-152-3p	2.27	0.003
rno-miR-212-5p	2.25	0.007

Table 4.5 continued

miRNA ID	Fold change	ANOVA p-value
rno-mir-25	2.25	0.039
rno-miR-124-5p	2.22	0.002
rno-miR-17-5p	2.20	0.024
rno-miR-409a-5p	2.20	0.003
rno-miR-505-5p	2.19	0.003
rno-miR-30c-2-3p	2.16	<0.001
rno-miR-505-3p	2.12	0.006
rno-miR-384-5p	2.12	0.005
rno-miR-338-5p	2.12	0.002
rno-miR-93-5p	2.12	0.002
rno-mir-211	2.11	0.049
rno-miR-20b-5p	2.11	0.038
rno-mir-138-2	2.07	0.009
rno-miR-99a-5p	2.06	0.001
rno-miR-431	2.03	0.022
rno-miR-299b-5p	2.01	0.018
rno-miR-221-3p	2.01	0.002
rno-miR-30c-5p	2.01	<0.001
rno-miR-495	2.01	0.036
rno-miR-29b-2-5p	2.01	<0.001
rno-miR-344b-1-3p	2.01	0.012
rno-miR-708-5p	2.00	0.027
rno-mir-338	2.00	0.009
rno-miR-352	2.00	0.019
rno-miR-329-3p	2.00	0.007
rno-miR-340-3p	-2.01	0.006
rno-miR-3558-5p	-2.01	0.015
rno-miR-511-5p	-2.29	0.031

Table 4.6: The changes of the miRNA expression in hippocampus which are significant in both addiction and non-addiction group when compared with the controls.

Mirbase ID	Fold change		Changes (%)
	Before addiction	After addiction	
rno-miR-199a-3p	4.58	12.31	169%
rno-miR-1843-5p	1.79	3.7	107%
rno-miR-221-5p	1.78	3.53	98%
rno-miR-434-5p	2.14	4.13	93%
rno-miR-26b-5p	4.41	7.95	80%
rno-miR-28-3p	1.86	3.22	73%
rno-miR-496-3p	1.85	2.99	62%
rno-miR-106b-5p	2.72	4.22	55%
rno-miR-350	2.16	3.3	53%
rno-miR-376b-3p	4.55	6.9	52%
rno-miR-137-3p	4.36	6.6	51%
rno-miR-344a-3p	2.77	1.62	-42%
rno-miR-30e-5p	6.2	3.59	-42%
rno-miR-466c-3p	-2.98	-1.58	-47%
rno-miR-133b-3p	3.24	1.6	-51%

4.2.2.2 Validation with quantitative PCR (qPCR)

For the rat hippocampus tissue, the differential expression of the 6 miRNAs for 9 samples (3 samples each from METH addicted, acute METH treatment without addicted and control groups, respectively) were further validated by SYBR Green qPCR. The miRNAs were selected for validation according to high fold change, low p-value, and low FDR. We observed consistent results for all miRNAs, except miR-199a-3p. In our qPCR findings, miR-340-3p shows significant up-regulation in both treatment groups (acute treatment: $\Delta\Delta Ct = -2.379$, 5.2 fold changes; continuous treatment: $\Delta\Delta Ct = -1.20$, 2.3 fold changes), miR-1839-5p shows only significant up-regulation in the continuous treatment group (acute treatment: $\Delta\Delta Ct = -0.427$, 1.34 fold changes; continuous treatment: $\Delta\Delta Ct = -1.42$, 2.68 fold changes), miR-143-3p shows significant up-regulated in both treatment group (acute treatment: $\Delta\Delta Ct = -1.17$, 2.25 fold changes; continuous treatment: $\Delta\Delta Ct = -1.51$, 2.83 fold changes), miR-322-3p also shows significant up-regulation in both treatment group (acute treatment: $\Delta\Delta Ct =$

−1.112, 2.16 fold changes; continuous treatment: ΔΔCt = −1.24, 2.36 fold changes), miR-218a-5p (acute treatment: ΔΔCt = −0.841, 1.79 fold changes; continuous treatment: ΔΔCt = −3.44, 10.8 fold changes). However, the expression of the miR-199a-3p in the acute treatment as indicated by the qPCR result is in contradiction with the microarray result. In qPCR finding, its expression was not significantly different in acute treatment when compared with the controls (ΔΔCt = -0.102, 1.07 fold changes), while it was significantly different by 4.6 fold changes in microarray result. However, expression of miR-199a-3p in the continuous treatment group was consistent with the qPCR result which showed significant up-regulation (ΔΔCt = -1.934, 3.8 fold changes).

4.2.2.3 Enrichment pathway analysis and target gene prediction

The IPA defined significant networks in the hippocampus, the main functions and the canonical pathways related with the differentially expressed genes for each comparison analyzed. From the profiling carried out, among the addiction-associated miRNAs, we found that 28 miRNAs which were highly involved in neurological and psychological disorder pathway following filtering by the IPA system. A total of 191 gene targets which have been reported to be involved in the METH addiction phenotype were identified. From the analysis, let-7a-5p showed the highest number of gene targets, in which 34 gene targets are related to the addiction pathway (Figure 4.11). This is followed by miR-30c-5p which indicated 25 gene targets, while miR-29b-3p and miR-146a-5p indicated 13 gene targets each. Similarly, in this comparison, these miRNAs and the related gene targets are highly associated with 44 canonical pathways which are related to neurological and psychological disorder, e.g.: AMPK Signaling; CREB Signaling; Axonal Guidance Signaling, Pi3K/AKT Signaling, CXCR4 Signaling and so on (Table 4.7).

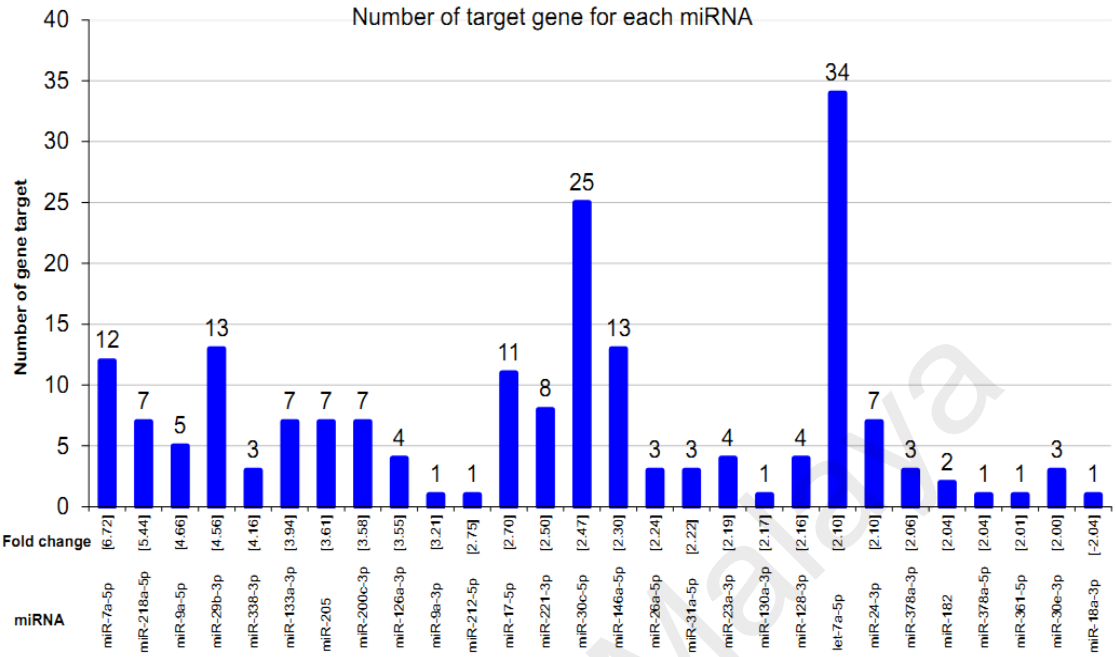


Figure 4.11: The number of miRNA gene targets which are associated with methamphetamine addiction.
MiR-181a-5p showed the highest number of gene targets, in which 188 gene targets are related to the addiction pathway. This is followed by miR-23a-3p and miR-26a-5p, which indicated 176 and 138 gene targets, respectively.

Table 4.7: The relationship between the addiction-related miRNAs and the enriched synaptic signaling pathways in hippocampus which may be associated with METH addiction

Synaptic pathways	Addicted-associated miRNAs
AMPK Signaling	miR-221-3p, miR-24-3p, miR-31-5p, miR-126a-3p, let-7a-5p, miR-17-5p, miR-9a-5p, miR-218-5p, miR-7a-5p, miR-29b-3p
Aryl Hydrocarbon Receptor Signaling	miR-221-3p, let-7a-5p, miR-17-5p, miR-218-5p, miR-29b-3p
Axonal Guidance Signaling	miR-221-3p, miR-128-3p, miR-24-3p, miR-31-5p, miR-181a-5p, miR-126a-3p, let-7a-5p, miR-17-5p, miR-26a-5p, miR-30c-5p, miR-218-5p, miR-7a-5p, miR-29b-3p
cAMP-mediated signaling	miR-17-5p, miR-30c-5p, miR-7a-5p
CD40 Signaling	miR-221-3p, miR-24-3p, miR-146a-5p, miR-126a-3p, miR-17-5p, miR-218-5p, miR-29b-3p
CDK5 Signaling	miR-221-3p, miR-24-3p, miR-31-5p, miR-181a-5p, let-7a-5p, miR-7a-5p, miR-29b-3p
Cell Cycle: G1/S Checkpoint Regulation	miR-221-3p, let-7a-5p, miR-17-5p, miR-9a-5p, miR-29b-3p
Chemokine Signaling	miR-221-3p, miR-24-3p, miR-181a-5p, let-7a-5p, miR-30c-5p, miR-218-5p, miR-7a-5p
CREB Signaling in Neurons	miR-221-3p, miR-181a-5p, miR-126a-3p, let-7a-5p, miR-17-5p, miR-30c-5p, miR-218-5p, miR-7a-5p, miR-29b-3p
CXCR4 Signaling	miR-221-3p, miR-181a-5p, miR-126a-3p, let-7a-5p, miR-17-5p, miR-30c-5p, miR-218-5p, miR-7a-5p, miR-29b-3p
Cyclins and Cell Cycle Regulation	miR-221-3p, miR-31-5p, let-7a-5p, miR-17-5p, miR-7a-5p, miR-29b-3p
Docosahexaenoic Acid (DHA) Signaling	miR-221-3p, miR-181a-5p, miR-126a-3p, let-7a-5p, miR-17-5p, miR-9a-5p, miR-218-5p, miR-29b-3p
Dopamine-DARPP32 Feedback in cAMP Signaling	miR-221-3p, miR-31-5p, miR-17-5p, miR-30c-5p, miR-218-5p, miR-7a-5p, miR-29b-3p
eNOS Signaling	miR-221-3p, miR-126a-3p, let-7a-5p, miR-17-5p, miR-218-5p, miR-29b-3p
ERK5 Signaling	miR-221-3p, miR-181a-5p, let-7a-5p, miR-17-5p, miR-7a-5p
FAK Signaling	miR-17-5p, miR-26a-5p, miR-30c-5p, miR-218-5p, miR-7a-5p, miR-29b-3p
G Beta Gamma Signaling	miR-30c-5p, miR-218-5p, miR-7a-5p
GABA Receptor Signaling	miR-30c-5p, miR-29b-3p
Gap Junction Signaling	miR-30c-5p, miR-218-5p, miR-7a-5p, miR-29b-3p
GDNF Family Ligand-Receptor Interactions	miR-221-3p, miR-126a-3p, miR-17-5p, miR-218-5p, miR-7a-5p
Glutamate Receptor Signaling	miR-181a-5p, let-7a-5p, miR-30c-5p, miR-7a-5p

Table 4.7 continued

Synaptic pathways	Addicted-associated miRNAs
GNRH Signaling	miR-221-3p, miR-24-3p, miR-17-5p, miR-30c-5p, miR-218-5p, miR-7a-5p, miR-29b-3p
G-Protein Coupled Receptor Signaling	miR-30c-5p, miR-146a-5p, let-7a-5p, miR-17-5p, miR-9a-5p
HMGB1 Signaling	miR-221-3p, miR-24-3p, miR-218-5p, miR-29b-3p
JAK/Stat Signaling	miR-221-3p, let-7a-5p, miR-17-5p
mTOR Signaling	miR-221-3p, miR-31-5p, miR-126a-3p, miR-17-5p, miR-7a-5p
Neuregulin Signaling	miR-221-3p, miR-17-5p, miR-26a-5p, miR-7a-5p, miR-29b-3p
Neurotrophin/TRK Signaling	miR-128-3p, miR-17-5p, miR-9a-5p, miR-29b-3p
NF-κB Signaling	miR-128-3p, miR-133a-3p, miR-146a-5p, miR-30c-5p, miR-218-5p, miR-7a-5p
P2Y Purigenic Receptor Signaling Pathway	let-7a-5p, miR-17-5p, miR-30c-5p
PAK Signaling	miR-31-5p, let-7a-5p, miR-17-5p
Parkinson's Signaling	miR-133a-3p, let-7a-5p, miR-17-5p, miR-29b-3p
PDGF Signaling	miR-24-3p, miR-133a-3p, miR-146a-5p, let-7a-5p, miR-17-5p
Phospholipase C Signaling	miR-17-5p, miR-30c-5p, miR-29b-3p
PI3K/AKT Signaling	miR-221-3p, miR-31-5p, miR-181a-5p, let-7a-5p, miR-17-5p, miR-9a-5p, miR-26a-5p, miR-7a-5p, miR-29b-3p
Prolactin Signaling	miR-126a-3p, miR-17-5p, miR-218-5p, miR-7a-5p, miR-29b-3p
Protein Kinase A Signaling	miR-17-5p, miR-126a-3p, miR-30c-5p, miR-29b-3p
PTEN Signaling	miR-221-3p, miR-128-3p, miR-133a-3p, miR-181a-5p, let-7a-5p, miR-17-5p, miR-9a-5p, miR-26a-5p, miR-7a-5p, miR-29b-3p
Reelin Signaling in Neurons	miR-128-3p, let-7a-5p, miR-17-5p, miR-30c-5p
SAPK/JNK Signaling	miR-126a-3p, miR-17-5p, miR-30c-5p, miR-7a-5p
STAT3 Pathway	miR-128-3p, miR-133a-3p, miR-181a-5p, miR-17-5p, miR-7a-5p
Synaptic Long Term Depression	miR-221-3p, miR-133a-3p, miR-31-5p, miR-181a-5p
Tight Junction Signaling	miR-221-3p, miR-128-3p, miR-31-5p, miR-30c-5p
VEGF Signaling	miR-181a-5p, let-7a-5p, miR-17-5p, miR-9a-5p

4.3 Global transcriptome profiling analysis

The global transcriptome profiling was conducted by using Affymetrix GeneChip® Rat Gene 2.0 ST array in two parts of the rat brain tissues, nucleus accumbens and hippocampus. This array is able to detect a total of 28,407 RefSeq transcripts and 23,586 RefSeq (Entrez) gene count in the rat species.

4.3.1 Nucleus accumbens

4.3.1.1 Global transcriptome microarray analysis

In the preliminary array screening, a total of 22,260 gene transcripts were detected. The results showed that significantly altered expression of 92 gene transcripts in the METH addiction group which have been exposed to a continuous 15 alternate days METH treatment compared to those of controls, in which 6 transcripts in 3 genes were significantly up-regulated (fold-change ≥ 1.5, $p < 0.05$, FDR $\leq 10\%$) and 86 transcripts in 23 genes were down-regulated (fold-change ≤ -1.5, $p < 0.05$, FDR $\leq 10\%$) (Table 4.10). For comparison between the acute METH treatment without addiction group and the control group, there were 15 transcripts in 10 genes which were significantly up-regulated and 28 transcripts in 9 genes were down-regulated (Table 4.9). However, we observed a significantly altered expression of 38 genes in the METH addiction group which are experienced a continuous 15 alternate days METH treatment compared with the acute METH treatment without addiction group (Table 4.8). From the 38 genes, six genes were significantly up-regulated while 32 genes showed down-regulation. Further stratification shows that none of the genes or gene transcripts has simultaneously significantly altered expression between the addiction and the non-addiction group.

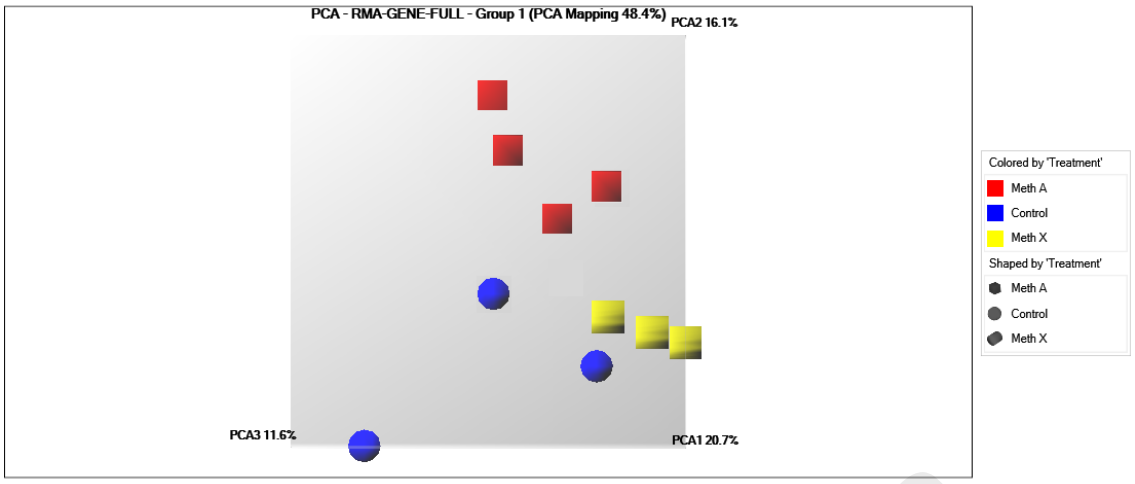


Figure 4.12: Transcriptomic microarray PCA map shows that the samples are clustered in 3 different groups in the nucleus accumbens.

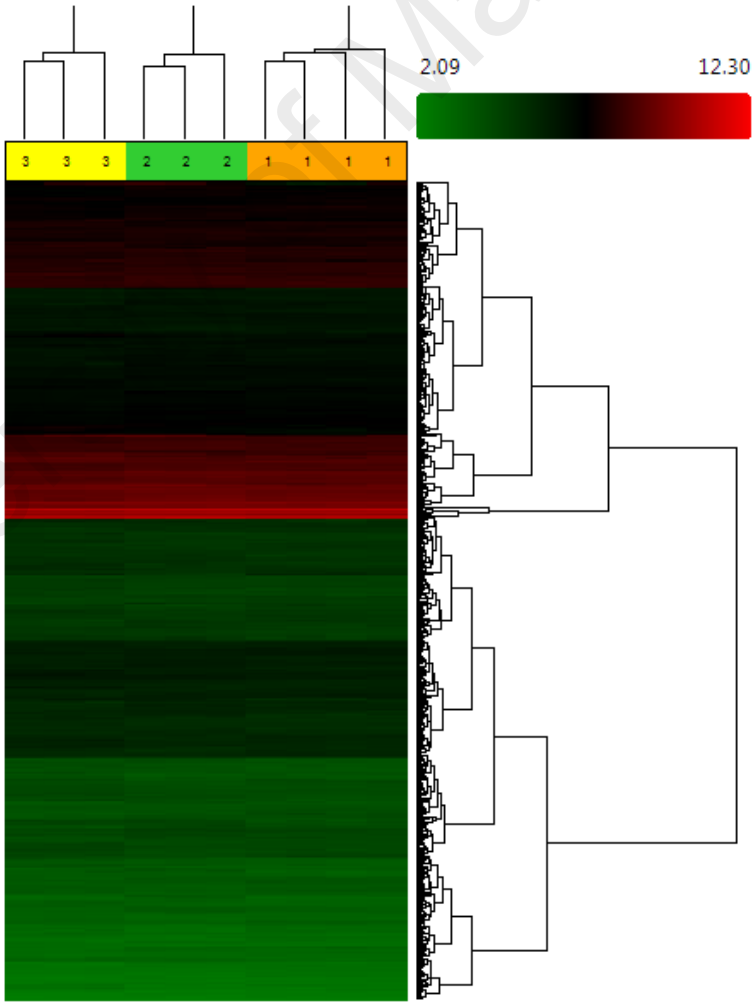


Figure 4.13: Gene transcript expression map shows differentiated genes in the nucleus accumbens.

Table 4.8: The significantly altered gene expression in the nucleus accumbens of the addiction group when compared with the non-addiction group

Gene Symbol	Fold Change
Tpt1	2.36
LOC688875	1.67
LOC680579	1.64
LOC102557159	1.56
Zbtb8a	1.53
LOC102554516; RGD1311300	1.52
Defa9	-1.45
LOC102546709	-1.46
LOC365499	-1.46
LOC102549465; LOC102546376	-1.47
Olr202	-1.47
Vom2r62	-1.48
RT1-O1	-1.49
Mina	-1.5
LOC302228; LOC681107; LOC100910078	-1.51
Olr531	-1.52
RGD1565478; LOC100362092; Igha	-1.54
LOC689453; LOC501467; LOC685989; LOC686719; LOC690467	-1.56
Igha	-1.57
LOC100909409; LOC679730; LOC501467; LOC689453; LOC688649	-1.57
LOC685226; LOC501467; LOC685989; LOC686719; LOC689453; LOC501317; LOC100364452	-1.57
Hist2h4a; Hist1h4a; Hist1h4b; Hist1h4m; Hist2h4	-1.58
RGD1560580	-1.58
Vom1r53; Vom1r54	-1.58
LOC501467; LOC689453; LOC501317; LOC685989; LOC686719	-1.59
LOC501317; LOC501467; LOC691712; LOC679730; LOC689453; LOC681364;	-1.6
LOC501467; LOC679730; LOC689453; LOC685989; LOC686719	-1.61
LOC691718; LOC100911906; LOC100912547	-1.62
LOC689453; LOC501467; LOC501317; LOC690467; LOC685989; LOC686719	-1.65
LOC501467; LOC679730; LOC689453; LOC501317; LOC100909409; LOC102548412	-1.67
LOC100360169; LOC691718	-1.68
LOC501467; LOC691712; LOC679730; LOC689453; LOC681364; LOC685183;	-1.68
LOC100909409	-1.69
LOC501467; LOC685989; LOC686719; LOC685226; LOC691712; LOC679730;	-1.72
LOC685226; LOC501467; LOC685989; LOC686719	-1.74
LOC679711	-1.78
RGD1565478; LOC100362092	-1.82
RGD1562953	-1.85

Table 4.9: The significantly altered gene expression in the nucleus accumbens of the acute treatment group without addiction as compared with the control group

Gene Symbol	Fold Change	ANOVA p-value
rno-mir-466b-2	1.74	<0.001
LOC691715	1.68	0.005
Hps5; Saa4	1.57	0.028
Gbp5	1.54	0.035
Hist2h4a; Hist1h4a;Hist1h4b; Hist1h4m; Hist2h4	1.53	0.040
Fezf2	1.46	0.018
Pla2g7	1.46	0.027
Ptgs2	1.46	0.033
Commd7	1.45	0.026
Slc17a7	1.45	0.029
LOC102548535	-1.46	0.020
Olr1002; Olr956	-1.47	0.005
Mir3065; rno-mir-3065	-1.51	0.046
Rnf135	-1.51	0.029
Gstm6	-1.52	0.012
LOC363337	-1.54	0.049
RGD1563818	-1.58	0.010
LOC680342; LOC680190	-1.60	0.006
Olr1454	-1.72	0.002

Table 4.10: The significantly altered gene expression in the nucleus accumbens of the continuous treatment group with addiction as compared with control group

Gene Symbol	Fold Change	ANOVA p-value
LOC688875	1.67	0.012
LOC680579	1.64	0.034
LOC102557159	1.56	0.019
LOC102549465; LOC102546376	-1.50	0.015
Olr202	-1.50	0.007
Vom2r62	-1.50	0.041
RT1-O1	-1.50	0.047
Mina	-1.51	0.033
LOC302228; LOC681107; LOC100910078	-1.51	0.029
Olr531	-1.52	0.021
LOC689453; LOC501467; LOC685989	-1.56	0.048
LOC100909409; LOC679730; LOC50146	-1.57	0.047
LOC685226; LOC501467; LOC685989; LOC686719	-1.57	0.041
RGD1560580	-1.58	0.016
Vom1r53; Vom1r54	-1.58	0.018
LOC501467; LOC689453; LOC501317; LOC685989	-1.59	0.041
LOC501317; LOC501467; LOC691712; LOC679730	-1.60	0.043
LOC501467; LOC679730; LOC689453; LOC685989	-1.61	0.042
LOC689453; LOC501467; LOC501317; LOC690467	-1.65	0.039
LOC363337	-1.66	0.017
LOC501467; LOC679730; LOC689453; LOC501317	-1.67	0.045
LOC501467; LOC691712; LOC679730; LOC689453	-1.68	0.046
LOC100909409	-1.69	0.036
LOC501467; LOC685989; LOC686719; LOC685226	-1.72	0.037
LOC685226; LOC501467; LOC685989; LOC686719	-1.74	0.030
LOC679711	-1.78	0.048

4.3.1.2 Validation with quantitative PCR (qPCR)

For the rat nucleus accumbens tissue, the differential expression of the 5 gene transcripts for 9 samples (3 samples each from METH addicted, acute METH treatment without addicted and control groups, respectively) were further validated by SYBR Green qPCR. The genes were selected for validation according to fold change ≥ ±1.50, low p-value, low FDR, and the availability of the primers sequence. We observed consistent results for all transcripts. In our qPCR findings, TPT1 shows significant up-regulation in the continuous treatment compared with the controls (acute treatment: ΔΔCt = −0.403, 1.3 fold change; continuous treatment: ΔΔCt = −1.12, 2.16 fold change), MINA shows down-regulation in both treatment group, however in microarray data only continuous treatment shows significant down-regulation (acute treatment: ΔΔCt = 0.184, 0.88 fold change; continuous treatment: ΔΔCt = 0.773, 0.585 fold change), GPB5 shows significant up-regulation in continuous treatment campared to the controls (acute treatment: ΔΔCt = −1.56, 2.95 fold change; continuous treatment: ΔΔCt = −0.48, 1.39 fold change), OLR1454 shows significant down-regulated in acute treatment group compared with controls (acute treatment: ΔΔCt = 0.37, 0.78 fold change; continuous treatment: ΔΔCt = -0.25, 1.19 fold change) and LOC363337 shows significant down-regulation in both METH treatment group as compared with controls (acute treatment: ΔΔCt = 0.66, 0.63 fold change; continuous treatment: ΔΔCt = 0.91, 0.53 fold change)

4.3.2 Hippocampus

4.3.2.1 Global transcriptome microarray analysis

In the preliminary array screening, a total of 25,375 gene transcripts were detected. The results showed that a significantly altered expression of 66 gene

transcripts in the METH addiction group which have been exposed to a continuous 15 alternate days METH treatment compared to those of controls, in which 28 transcripts in 13 genes were significantly up-regulated (fold-change ≥ 1.5, $p < 0.05$, FDR ≤ 10%) and 38 transcripts in 8 genes were down-regulated (fold-change ≤ -1.5, $p < 0.05$, FDR ≤ 10%) (Table 4.13). Comparison between the acute METH treatment group without addiction and the control group, showed that there were 21 transcripts in 5 genes significantly up-regulated and 49 transcripts in 12 genes were down-regulated (Table 4.12). However, we observed a significantly altered expression of 30 genes in the METH addiction group which had experienced a continuous 15 alternate days METH treatment compared with the acute METH treatment without addiction group (Figure 4.11). From the 30 genes, twenty-one genes were significantly up-regulated (fold-change ≥ 1.5, $p < 0.05$, FDR ≤ 10%) while 9 genes showed down-regulation (fold-change ≤ -1.5, $p < 0.05$, FDR ≤ 10%). Further stratification shows that none of the genes or gene transcripts has simultaneously significant altered expression within the addiction and non-addiction group.

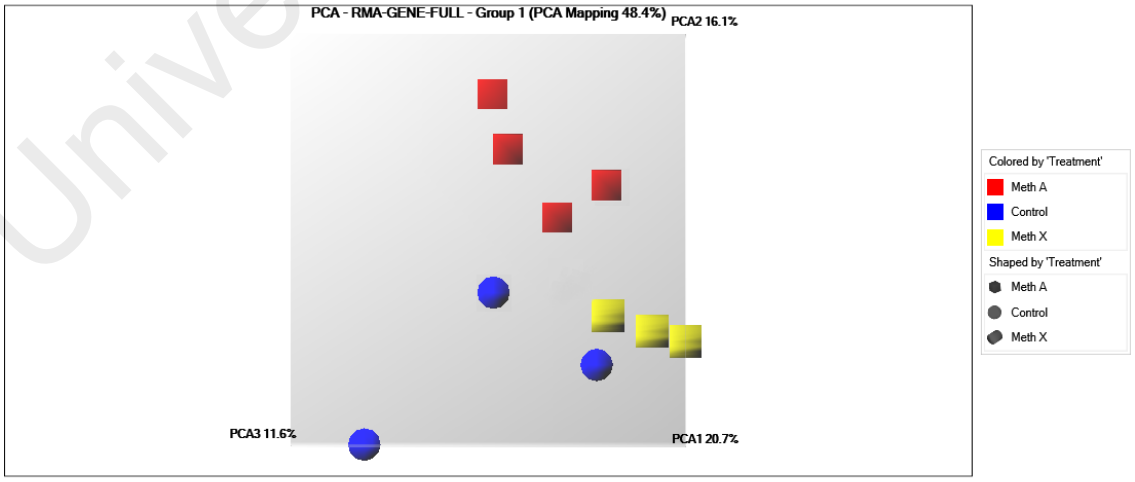


Figure 4.14: Transcriptomic microarray PCA map shows that the samples are clustered in 3 different groups in the hippocampus.

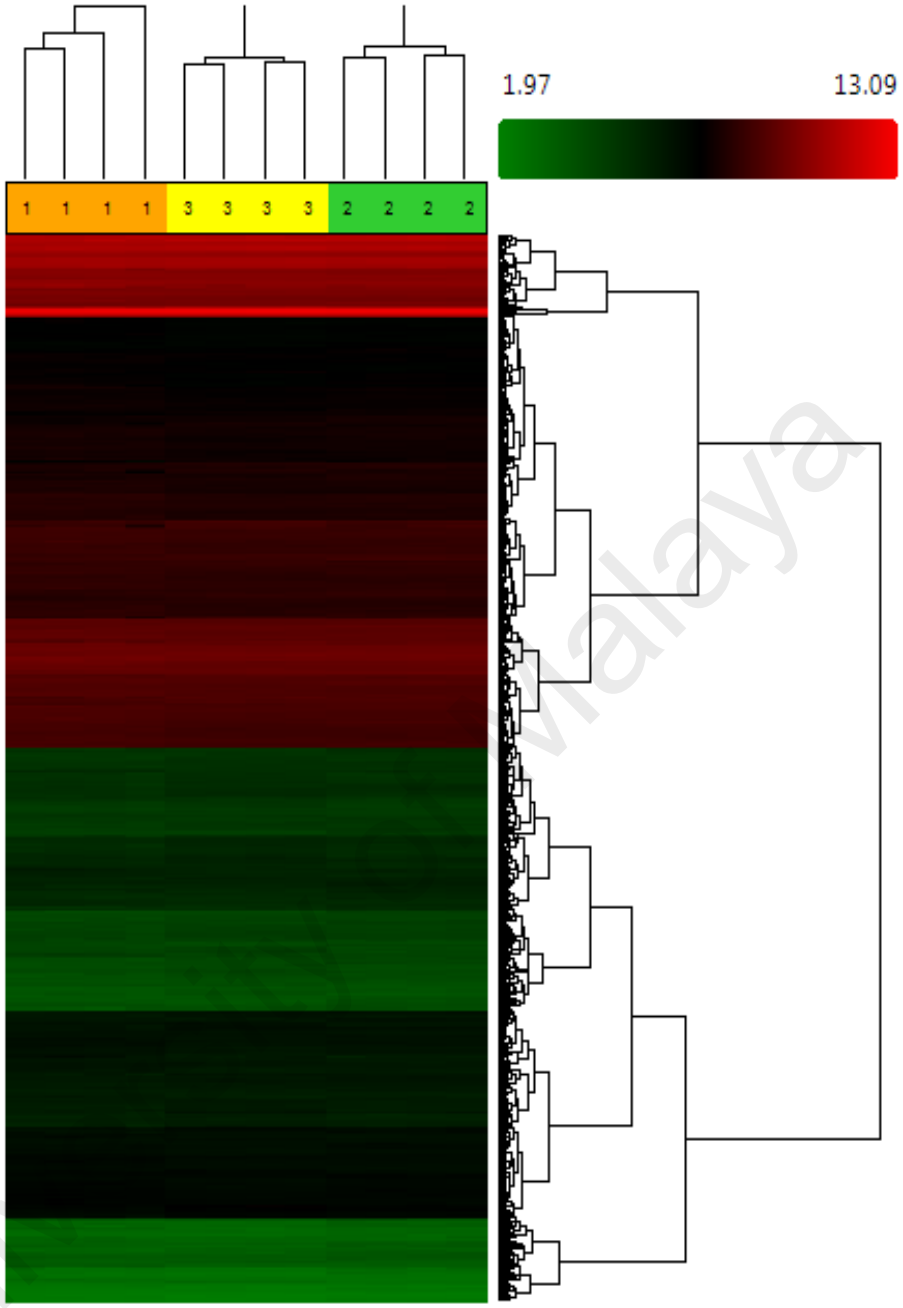


Figure 4.15: Gene transcript expression map shows differentiated genes in the hippocampus.

Table 4.11: The significantly altered gene expression in the hippocampus of the addiction group when compared with the non-addiction group

Gene Symbol	Fold change
Olr235	1.88
LOC102547920	1.82
LOC100360169; LOC691718	1.73
LOC684976	1.70
Mir370; rno-mir-370	1.68
Trib1	1.67
Fos	1.65
Cdk5r2	1.56
Olr1590; Olr1591	1.56
Vom2r6	1.56
LOC691718; LOC100360169	1.55
Olr270	1.51
Olr59	1.50
Olr951	1.50
LOC100362643; LOC100365452	1.50
Grcc10; LOC100911713; Gpx1	1.50
LOC100361644	1.50
RT1-T24-1	1.50
LOC100359616	1.50
LOC102547425	1.50
Olr651	1.50
LOC102553386	-1.50
Pdilt	-1.51
Lyrm5	-1.53
Olr1471	-1.53
ND3	-1.54
LOC684822	-1.56
Olr1458	-1.56
Olr1139; Olr1146; LOC100911988	-1.60
LOC287167	-1.67

Table 4.12: The significantly altered gene expression in the hippocampus of the acute treatment group without addiction as compared with the control group

Gene Symbol	Fold Change	ANOVA p-value
Apeg3	2.02	0.002
Dusp1	1.62	0.014
Junb	1.54	0.024
Vom2r54	1.51	0.013
Olr348	1.5	0.043
LOC102547425	-1.5	0.038
RGD1562381	-1.5	0.008
Mcpt1l2	-1.51	0.033
LOC102551257	-1.51	0.049
LOC684946; Vom2r64; Vom2r63; LOC288574; LOC100912655	-1.52	0.011
Morf4l1	-1.54	0.015
Gzmc; RGD1560650; RGD1563645; LOC100911163	-1.55	0.024
Vom1r50	-1.55	0.008
Vom2r6	-1.61	0.007
Vom1r82	-1.63	0.002
Olr235	-1.78	0.001
RGD1561594	-2.05	0.006

Table 4.13: The significantly altered gene expression in the hippocampus of the continuous treatment group with addiction compared with control group

Gene Symbol	Fold Change	ANOVA p-value
Apeg3	2.09	0.003
LOC100360169; LOC691718	1.82	0.020
Trib1	1.67	0.031
Fos	1.63	0.023
Dusp1	1.62	0.007
Cdk5r2	1.56	0.003
LOC100359616	1.51	0.003
Junb	1.51	0.005
Olr951	1.50	0.010
LOC100362643; LOC100365452	1.50	0.009
Grcc10; LOC100911713; Gpx1	1.50	0.024
LOC100361644	1.50	0.017
RT1-T24-1	1.50	0.021
Olr1471	-1.50	<0.001
LOC287167	-1.50	0.004
ND3	-1.54	0.035
LOC684822	-1.56	0.034
Olr1458	-1.56	0.021
Olr1139; Olr1146; LOC100911988	-1.60	0.029
RGD1560718	-1.60	0.004
Lyrm5	-1.69	0.005

4.3.2.2 Validation with quantitative PCR (qPCR)

For the rat hippocampus tissue, the differential expression of the 5 gene transcripts for 9 samples (3 samples each from METH addicted, acute METH treatment without addicted and control groups, respectively) were further validated by SYBR Green qPCR. The genes were selected for validation according to fold change ≥ ±1.50, low p-value, low FDR, and the availability of the primers sequence. We observed consistent results for all transcripts. In our qPCR findings, TRIB1 shows significantly up-regulation in the continuous treatment group as compared with the controls but no significant difference was seen in the acute treatment group (acute treatment: ΔΔCt = −0.505, 1.42 fold change; continuous treatment: ΔΔCt = −0.75, 1.68 fold change). For the OLR235 gene, it shows significant up-regulation in both of the treatment groups when compared with controls (acute treatment: ΔΔCt = -1.20, 2.29 fold change; continuous treatment: ΔΔCt = -0.325, 1.25 fold change). However, there was no significant difference between continuous treatment and acute treatment in our qPCR finding (ΔΔCt = -0.185, 1.13 fold change), although there was significant down-regulation in our microarray finding. For FOS gene, it shows significant up-regulation in continuous treatment campared to the controls and the acute treatment group (acute treatment: ΔΔCt = −0.01, 1.01 fold change; continuous treatment: ΔΔCt = −0.72, 1.65 fold change). For DUSP1 gene, its expression was significantly up-regulated in both continuous treatment and acute treatment groups, compared with controls (acute treatment: ΔΔCt = -0.79, 1.73 fold change; continuous treatment: ΔΔCt = -0.925, 1.89 fold change). Besides that, expression of LOC287167 shows significant down-regulated in continuous treatment group compared with the controls and the acute treatment group (acute treatment: ΔΔCt = 0.345, 0.78 fold change; continuous treatment: ΔΔCt = 0.86, 0.55 fold change). However, there was significant down-regulation when comparing between the acute treatment group and the controls in qPCR finding (ΔΔCt = 0.515,

0.70 fold change), although no significant difference was found in our microarray finding.

4.4 Global protein profiling analysis

The quality of the protein samples were tested by using SDS-PAGE gel electrophoresis and bioanalyzer. Both of the results indicated that the quality of the protein was good. Thermo Scientific LTQ Orbitrap XL mass spectrometer was applied in the global protein profiling study because this method can provide superior mass accuracy for lower false positive rates, high sensitivity with achievable ultra-high resolution (> 100,000) and dynamic range leading to more protein ID, and its Excellent MS/MS sensitivity, as compared to other methods.

4.4.1 Protein expression in nucleus accumbens

In the preliminary screening, a total of 2447 proteins were identified from control sample, 1893 proteins from acute METH treatment group and 2303 from the METH addiction group which are exposed to a continuous 15 alternate days METH treatment. After filtration according to p-values, there were only 45 proteins which showed significantly altered expression in the METH addiction group as compared to those of controls, after clustering was carried out according to protein families (yellow box in Figure 4.16). A total of 22 proteins were significantly up-regulated (fold-change ≥ 2, $p < 0.05$, FDR $\leq 10\%$) while 23 were down-regulated (fold-change ≤ -2, $p < 0.05$, FDR $\leq 10\%$). In comparing between acute METH treatment group without addiction and the controls, there were 16 proteins that were significantly up-regulated while 2 were down-regulated (blue box in Figure 4.16). Furthermore, the results also showed that all 23 proteins were simultaneously down-regulated in both of the groups when compared with controls (green area in Figure 4.16).

Further stratification among the METH addiction group and non-addiction group revealed that expression of a total of 40 proteins were significantly altered between these two groups. Among the 40 proteins, 25 proteins were up-regulated while 15 were down-regulated (Table 4.14).

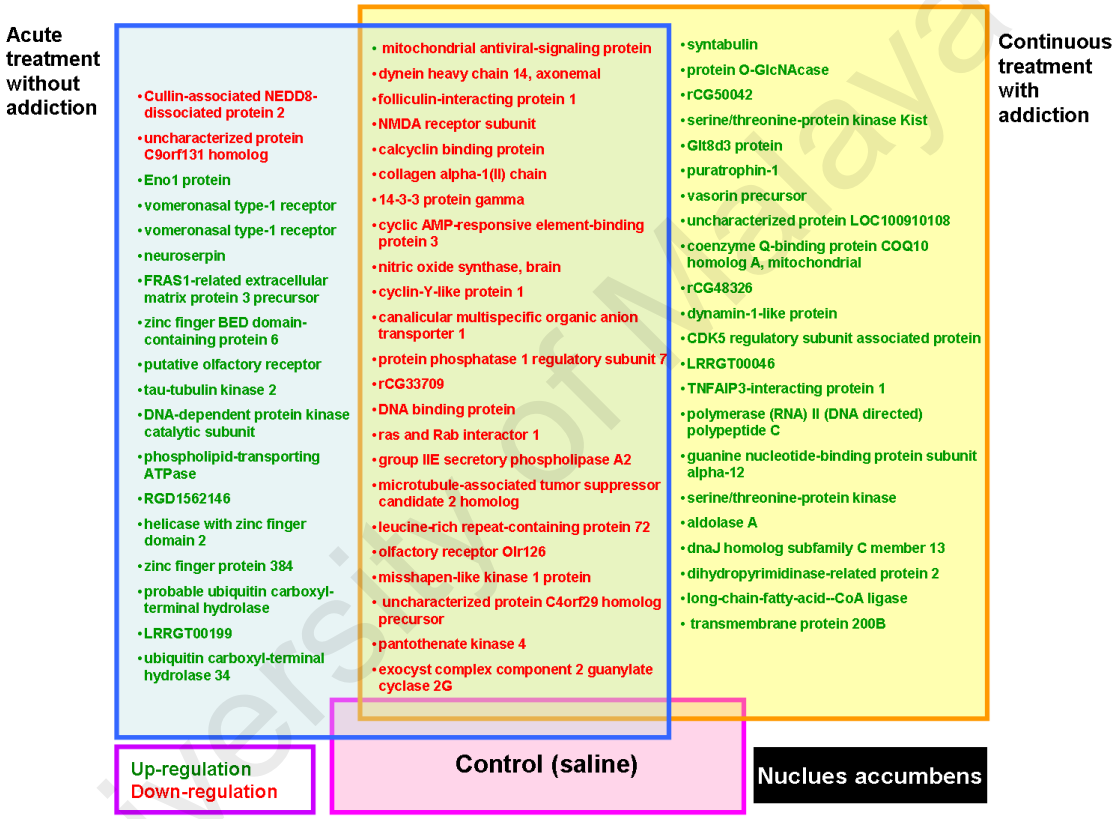


Figure 4.16: The significantly altered expression of proteins in the acute treatment group without addiction and the continuous treatment group with addiction when compared with control group

Table 4.14: The significantly altered expression of proteins in the nucleus accumbens when compared between the addiction group and the non-addiction group

Compounds	Fold change
Transmembrane protein	3.97
Hemoglobin beta chain	3.94
Long chain fatty acid CoA ligase	3.83
hemoglobin epsilon 1	3.78
dihydropyrimidinase-related protein 2	3.74
dnaJ homolog subfamily C member 13	3.69
Aldolase A	3.60
Serine/threonine-protein kinase	3.55
Guanine nucleotide-binding protein subunit alpha	3.25
Polymerase (RNA) II (DNA directed) polypeptide C	3.23
TNFAIP3-interacting protein 1	3.13
LRRGT00046	3.13
CDK5 regulatory subunit associated protein 3	3.10
Dynamin-1-like protein	3.10
rCG48326	3.09
Coenzyme Q-binding protein COQ10 homolog A	3.08
Uncharacterized protein LOC100910108	3.07
Vasorin precursor	3.06
Puratrophin-1	3.06
Glt8d3 protein	3.05
rCG50042	3.02
Protein O-GlcNAcase	3.01
Syntabulin	3.00
Cullin-associated NEDD8-dissociated protein 2	1.85
Uncharacterized protein C9orf131 homolog	1.81
FRAS1-related extracellular matrix protein 3 precursor	-3.00
Putative olfactory receptor	-3.01
Tau-tubulin kinase 2	-3.01
Vomeronasal type-1 receptor	-3.02
Zinc finger protein 384	-3.03
LRRGT00199	-3.03
Helicase with zinc finger domain 2	-3.03
Neuroserpin	-3.07
DNA-dependent protein kinase catalytic subunit	-3.13
Ubiquitin carboxyl-terminal hydrolase	-3.22
RGD1562146	-3.24
Zinc finger BED domain-containing protein 6	-3.27
Phospholipid-transporting ATPase	-3.43
Probable ubiquitin carboxyl-terminal hydrolase	-3.66
Eno1 protein	-3.71

4.4.1.1 Enrichment pathway analysis and target gene prediction

The significant pathway networks, main functions and canonical pathways related with the differentially expressed proteins for each comparison were analyzed by IPA system. From the profiling carried out, among the addiction-associated proteins, pathways for most of the proteins identified are still unknown and following filtering by the IPA system, it was found that 7 proteins were highly involved in neurological and psychological disorder pathway. In this comparison, these proteins are highly associated with 32 canonical pathways which are related to neurological and psychological disorder, e.g.: FGF signaling pathway, Parkinson disease, Wnt signaling pathway, 5HT2 type receptor mediated signaling, CCKR signaling map, Toll receptor signaling pathway and so on (Table 4.15).

Table 4.15: The relationship between the addiction-related proteins and the enriched synaptic signaling pathways in nucleus accumbens which may be associated with METH addiction

Pathways	Proteins
Alpha adrenergic receptor signaling	Epileson 1, Guanine nucleotide-binding protein
EGF receptor signaling	Epileson 1, Serine/threonine-protein kinase
FGF signaling pathway	Epileson 1, Guanine nucleotide-binding protein, Serine/threonine-protein kinase
Hedgehog signaling pathway	Epileson 1, Serine/threonine-protein kinase
Parkinson disease	Epileson 1, Tau-tubulin kinasse 2, Ubiquitin carboxyl-terminal hydrolase, Serine/threonine-protein kinase
Wnt signaling pathway	Epileson 1, Transmembrane protein, Guanine nucleotide-binding protein, Tau-tubulin kinase 2, Phospholipid-transporting ATPase, Serine/threonine-protein kinase
5HT2 type receptor mediated signaling	Epileson 1, Guanine nucleotide-binding protein, Serine/threonine-protein kinase
Oxytocin receptor mediated signaling	Epileson 1, Serine/threonine-protein kinase
CCKR signaling map	Epileson 1, Guanine nucleotide-binding protein, Dynamin-1-like protein, Serine/threonine-protein kinase
Alzheimer disease	Epileson 1, Transmembrane protein, Serine/threonine-protein kinase
Angiogenesis	Transmembrane protein, Serine/threonine-protein kinase
Apoptosis signaling pathway	Transmembrane protein, Serine/threonine-protein kinase
Cadherin signaling pathway	Transmembrane protein, Serine/threonine-protein kinase
Huntington disease	Transmembrane protein, Ubiquitin carboxyl-terminal hydrolase, Serine/threonine-protein kinase
chemokine and cytokine signaling	Transmembrane protein, Serine/threonine-protein kinase
Metabotropic glutamate receptor	Transmembrane protein, Guanine nucleotide-binding protein, Serine/threonine-protein kinase
Muscarinic acetylcholine receptor signaling	Transmembrane protein, Guanine nucleotide-binding protein, Serine/threonine-protein kinase
Nicotinic acetylcholine receptor signaling	Transmembrane protein, Serine/threonine-protein kinase
PDGF signaling pathway	Transmembrane protein, Serine/threonine-protein kinase
Toll receptor signaling pathway	Transmembrane protein, TNFAIP3-interacting protein 1, Serine/threonine-protein kinase
Axon guidance mediated by semaphorins	dihydropyrimidinase-related protein 2, Serine/threonine-protein kinase
Heterotrimeric G-protein signaling pathway	Guanine nucleotide-binding protein, Serine/threonine-protein kinase
PI3 kinase pathway	Guanine nucleotide-binding protein, Serine/threonine-protein kinase
Dopamine receptor mediated signaling	Guanine nucleotide-binding protein, Serine/threonine-protein kinase
Enkephalin release	Guanine nucleotide-binding protein, Serine/threonine-protein kinase
Integrin signalling pathway	Serine/threonine-protein kinase
Interferon-gamma signaling pathway	Serine/threonine-protein kinase
Oxidative stress response	Serine/threonine-protein kinase
VEGF signaling pathway	Serine/threonine-protein kinase
Ras Pathway	Serine/threonine-protein kinase
GABA-B receptor II signaling	Serine/threonine-protein kinase
p38 MAPK pathway	Serine/threonine-protein kinase

4.4.2 Protein expression in hippocampus

In the preliminary array screening, a total of 816 proteins or protein isoforms were detected in the hippocampus sample. A total of 217 proteins were identified from the control group, 280 proteins each from acute METH treatment group and the METH addiction group which is exposed to a continuous 15 alternate days METH treatment. After filtration according to p-values, a total of 28 proteins showed significantly altered expression in the METH addiction group compared to those of controls, after clustering according to protein families (yellow box in Figure 4.17). However, 24 proteins were significantly up-regulated (fold-change ≥ 2, $p < 0.05$, FDR $\leq 10\%$) and 4 were down-regulated (fold-change ≤ -2, $p < 0.05$, FDR $\leq 10\%$). For comparing between acute METH treatment without addiction group and the controls, there were 18 proteins that were significantly up-regulated while 8 were down-regulated (blue box in Figure 4.17). Among 18 proteins, only 8 proteins showed significantly altered expression in the non-addiction group (blue area in Figure 4.17). However, our study did not find any proteins with significantly altered expression only in the METH addiction group; therefore, no proteins are listed within the yellow area of the Figure 4.17.

Further stratification among the METH addiction group and non-addiction group revealed that expression of a total of 42 proteins were significantly altered between these two groups. However, among the 48 proteins, 26 proteins were up-regulated while 16 were down-regulated (Table 4.16).

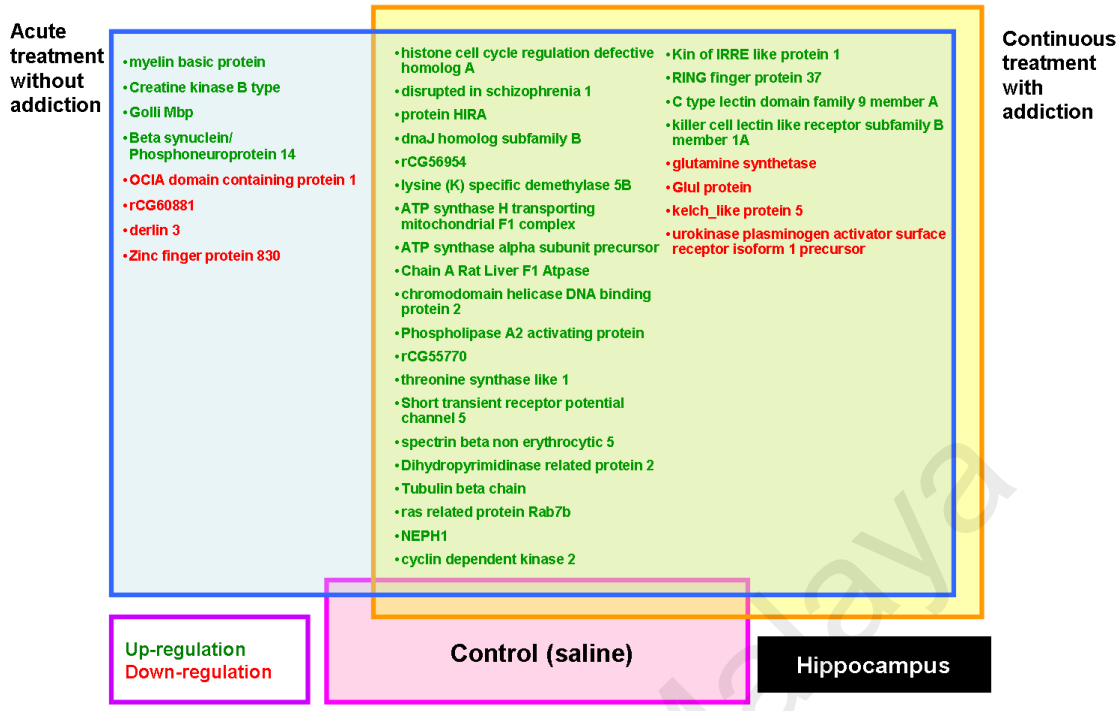


Figure 4.17: The significantly altered expression of proteins in acute treatment group without addiction and the continuous treatment group with addiction when compared with the control group.

Table 4.16: The significantly altered expression of proteins in the hippocampus when comparison is made between the addiction group and the non-addiction group

Compaunds	Fold change
histone cell cycle regulation defective homolog A	9.54
disrupted in schizophrenia 1	9.54
protein HIRA	9.54
dnaJ homolog subfamily B	9.54
rCG60881	8.28
derlin 3	8.28
cell division protein kinase	6.64
rCG56954	6.25
lysine (K) specific demethylase 5B	6.25
Dihydropyrimidinase related protein 2	6.07
Phospholipase A 2 activating protein	5.83
rCG55770	5.83
threonine synthase like 1	5.83
kelch like protein 5	4.91
urokinase plasminogen activator surface receptor	4.91
short transient receptor potential channel 5	4.25
ATP synthase H transporting mitochondrial F1 complex	4.14
ATP synthase alpha subunit precursor	4.14
Chain A Rat Liver F1 Atpase	4.14
spectrin beta non erythrocytic 5	3.91
chromodomain helicase DNA binding protein 2	3.09
OCIA domain containing 1	2.77
Beta synuclein	2.49
glutamine synthetase	2.06
Glul protein	2.00
Zinc finger protein 830	2.00
myelin basic protein	-2.34
Golli Mbp	-2.34
Tubulin beta 2B/4	-3.75
RAB7 like protein	-3.75
tubulin T beta15	-3.75
NEPH1 partial	-4.50
cyclin dependent kinase 2	-4.50
RING finger protein 37	-4.50
C type lectin domain family 9 member A	-4.50
killer cell lectin_like receptor subfamily B	-4.50
Kin of IRRE_like protein 1	-4.89
RING finger protein 37	-4.89
Clathrin light chain B	-4.89
Epsilon 1	-4.89
Chain D Crystal structure of the rat Calcineurin	-4.89
Protein tyrosine phosphatase receptor	-4.89

4.4.2.1 Enrichment pathway analysis and target gene prediction

The significant pathway networks, main functions and canonical pathways related with the differentially expressed proteins for each comparison was analyzed. From the profiling carried out, among the addiction-associated proteins, following filtering by the IPA system, it was found that 20 proteins were highly involved in neurological and psychological disorder pathway. However, the related pathways for other proteins are still unknown. In this comparison, these proteins are highly associated with 25 canonical pathways which are related to neurological and psychological disorders, e.g.: Axon guidance mediated by semaphorins, Wnt signaling pathway, Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway, chemokine and cytokine signaling pathway, CCKR signaling map and so on (Table 4.17).

Table 4.17: The relationship between the addiction-related proteins and the enriched synaptic signaling pathways in hippocampus which may be associated with METH addiction

Pathways	Proteins
Axon guidance mediated by semaphorins	cell division protein kinase, Dihydropyrimidinase related protein 2, Chain D Crystal Structure Of The Rat Calcineurin, protein tyrosine phosphatase receptor, disrupted in schizophrenia 1
p53 pathway	cell division protein kinase, cyclin dependent kinase 2, cell division cycle 2 like 5
Dopamine receptor mediated signaling pathway	cell division protein kinase, cyclin dependent kinase 2
Nicotine pharmacodynamics pathway	cell division protein kinase, cyclin dependent kinase 2, dnaJ homolog subfamily B
Wnt signaling pathway	Chromodomain helicase DNA binding protein, dnaJ homolog subfamily B, Calmodulin, Chain D Crystal Structure Of The Rat Calcineurin, ATP synthase alpha subunit precursor, Epsilon 1, ATP synthase H transporting mitochondrial F1 complex
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	dnaJ homolog subfamily B, Clathrin light chain, ATP synthase alpha subunit precursor, ATP synthase H transporting mitochondrial F1 complex
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	dnaJ homolog subfamily B, Clathrin light chain
Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction	dnaJ homolog subfamily B, Calmodulin, ATP synthase H transporting mitochondrial F1 complex
chemokine and cytokine signaling pathway	dnaJ homolog subfamily B, Chain D Crystal Structure Of The Rat Calcineurin, ATP synthase alpha subunit precursor, ATP synthase H transporting mitochondrial F1 complex
Muscarinic acetylcholine receptor 2 and 4 signaling pathway	dnaJ homolog subfamily B
GABA-B receptor II signaling	dnaJ homolog subfamily B
Angiotensin II-stimulated signaling through G proteins and beta-arrestin	dnaJ homolog subfamily B, ATP synthase alpha subunit precursor, ATP synthase H transporting mitochondrial F1 complex
Parkinson disease	Epsilon 1
Integrin signalling pathway	spectrin beta, protein tyrosine phosphatase receptor
CCKR signaling map	Calmodulin, Chain D Crystal Structure Of The Rat Calcineurin, protein tyrosine phosphatase receptor, Epsilon 1
Gonadotropin-releasing hormone receptor pathway	Chain D Crystal Structure Of The Rat Calcineurin
Cadherin signaling pathway	protein tyrosine phosphatase receptor
FGF signaling pathway	protein tyrosine phosphatase receptor, Epsilon 1
Interferon-gamma signaling pathway	protein tyrosine phosphatase receptor
JAK/STAT signaling pathway	protein tyrosine phosphatase receptor
Glutamine glutamate conversion	Glul protein
Huntington disease	Clathrin light chain
EGF receptor signaling pathway	Epsilon 1
Hedgehog signaling pathway	Epsilon 1
Ionotropic glutamate receptor pathway	Epsilon 1

CHAPTER 5:

DISCUSSION

5.1 Animal behavioral studies

5.1.1 METH addiction development in rat

In our findings, addiction behavior was demonstrated when rats consumed increasing doses over a period of 15 days as outlined in this study. It may therefore be deduced that addiction behavior can occur when the dose of the drug is increased from a low to a high dose. In addition, our study also showed that the addiction phenotype only occurred when a longer period (15 days study period) of METH was used as compared to a single exposure. When comparing between the time spent in pre- and post-CPP, our results indicated that the rats which were exposed to a continuous 15 alternate days treatment spent longer time in the compartment allocated for conditionings, showing a preference for the place preference box with METH injection (Figure 4.1), thereby indicating that the rats were addicted to METH. Furthermore, we found a strong significant difference between the times spent in the black and white compartment, showing a preference for the white color drug-paired compartment of the place preference apparatus. This provides a double confirmation in occurrence of the addiction phenotype in the rats exposed to METH conditioning (Figure 4.2). On the other hand, the results of the study showed that there is no significant difference between the acute METH treatment group and the controls, indicating that a single high dose of METH injection did not induce significant place preference or aversion as compared to the long term METH injection group. This may be because a single dose of METH did not induce obvious tolerance to METH. The saline control rat exhibited no place preference, indicating that there were neither baseline biases for the black vs. white compartment, nor place conditioning effects of saline injection. Our results are in agreement with the findings of Kim and Jang (1997) who also found that METH

induced a place preference, although it is noted that the work was carried out in mice. They demonstrated that treatment with 1-2 mg/kg of METH was sufficient to produce the CPP effect. However, we observed that increasing dose of METH treatment up to 5 mg/kg of METH showed stronger effect of the CPP when compared with controls. Various regimen of METH administration have resulted in the development of different models for METH use. In the neurotoxicity model, high doses of METH were typically used in a short amount of time, which resulted in extensive neurotoxic damage, particularly to the nigrostriatal and mesolimbic systems (Cadet & Krasnova, 2009; Davidson et al., 2001; Machiyama, 1992; Robinson & Becker, 1986). In their study, 8 mg/kg of METH was given twice a day, 4 h apart for the last 4 days to study METH neurotoxicity. Another study however, used a continuous high dose of METH (32 mg/kg/day) in rats for 5 days to study METH neurotoxicity (Armstrong & Noguchi, 2004). In our study, an increasing dose of METH was given to the animal model to prevent the effect of neurotoxicity. It is interesting to note that METH abusers tend to start with low doses of METH, which provides neuroprotective effect to subsequent higher doses (Hsieh, Stein, & Howells, 2014). Recent study suggested that large dose of METH (10 to 15 mg/kg) given to the experimental animals induce METH toxicity (Steven B. Karch, Olaf Drummer, & Karch, 2001). Therefore, METH dose up to 5 mg/kg which was used in our study is not likely to produce METH neurotoxicity in animal model.

5.1.2 METH-induced memory and learning disruption

METH has been known to be a highly addictive stimulant, but besides of addiction, long-term exposure of METH has many negative consequences, including learning and memory impairment. However, only little information about radial-maze learning and memory abilities in drug addicted mice or rat was available. Nevertheless, memory disturbance is a common side effect of drug abuse and suggested that addiction is strongly related to learning and memory (Hyman, Malenka, & Nestler, 2006). Before starting the experiment, optimization of the radial maze was carried out, especially the running time, starving period and the clues around the maze. The upside of the maze is open and in order to navigate themselves in the maze, the animals would have to look upward and find the clues. During optimization steps, it is noticed that some animals used a kinesthetic strategy to complete the maze task by visiting adjacent arms in a clockwise or counter-clockwise pattern. This finding was similar with a previous study (Crusio & Schwegler, 2005). However, these is no direct evident to evaluate whether this method is based on spatial orientation capabilities or not. Therefore, we modified and optimized the radial maze task. The findings for the maze test suggested that rats are probably not using surrounding environment as cues because they seldom seemed to look upwards. To facilitate the use of cues around the maze for spatial orientation, we arranged some objects around the maze to make this task as easy for animals to recognize as possible. Surprisingly, it showed that the rats learned this task easily and the animals seldom exhibit any obvious kinesthetic strategies. To prevent animals from using the undesired cues inside the maze, such as smell, the maze was completely clean with ethanol after each test. Besides that, the maze was rotated 45° at the end of each day and before subsequent trials.

During the actual test, rats were trained to locate three food rewards that were always placed in the same set of three arms. In this study, we decided to use one of the

arm to be the starting point, instead of at the central platform, in order to prevent manipulating biases, because we observed that if the central platform becomes the starting point, the animal is most likely to enter into any arm in front of him, without looking at the clues, when the door of the arms were opened. According to Olton's definition, entries into an arm that is never been baited, is considered as a reference memory (RM) error, whereas repeated entry into an arm that has been visited previously is considered as working memory (WM) errors (Olton & Samuelson, 1976). In our study, we found that it was a strong significant difference for the arms error test and entries error test when compared between the control group and the METH treatment group at the dose of 2, 4 and 5 mg/kg (Table 1), showing that the METH administrated rats at these doses may significantly disturb the reference memory in rats. For the repeated entry test however, our result only show significant difference at 4 and 5 mg/kg, indicating that higher doses of METH are needed to induce working memory disturbance, compared to reference memory. Interestingly, our results also show that the METH group in the three tests has a similar down-trend pattern with the control at the beginning of test. The errors made by the METH group are even lesser than the controls, when less than 2 mg/kg METH was given. This result was consistent with previous study which reported that low dose and short term use of METH may show some positive effects such as improved mood, increased alertness, wakefulness and concentration, increased sociability, and feelings of confidence (Cruickshank & Dyer, 2009) and this may increase the learning and memory ability. Dr Carl Hart, Associate Professor of Psychology at Columbia University revealed that in some tests of cognitive abilities, such as attention, memory and learning, people who use METH at low dose and for short term actually scored better than the control group (Hart et al., 2012). In addition, we also observed that METH group in three of the tests has a same turning point at 2 mg/kg, after which, the METH administrated rats began making more errors.

This dose of METH may be an optimum dose to cause METH-induced memory disturbance in rats. For the latency test, we only found a significant difference at 5 mg/kg and the latency to complete the task was increased at the beginning period. After 2 mg/kg METH treatment, latency for the rats to collect all foods in the maze slowed down and continuosly decreased until 5 mg/kg METH treatment. It shows that higher dose of METH may induce memory disturbance.

Overall, the study suggests that low dose and short term use of METH may improve working and reference memory, while high dose and long term METH use may reduce the ability of learning and memory, either in working or reference memory.

5.2 Molecular analysis

5.2.1 Molecular changes in rat nucleus accumbens following METH administration

With the capability to profile the expression of a large number of genes simultaneously, microarray provides a robust platform for evaluating mRNA and miRNA expression profiles and quantitative the expression levels. This technology is much more efficient than previous methods which are now considered outmoded and time-consuming, and it is becoming among the most popolar research tool for studying gene expression (Calin et al., 2004). In this study, the microarray data indicated that 37 miRNAs were significantly over-expressed only in the addiction group, but not in the acute drug treatment group, so it can be deduced that these miRNAs may be significantly associated with the addiction phenotype (Figure 4.6). For the common miRNAs in both the addiction group and the acute treatment group without addiction, the expression of 13 miRNAs were over-expressed more than 100 percent while 1 miRNAs were down-regulated more than 50 percent when comparing the drug

administrated groups before addiction and after addiction (Table 4.3). Therefore, we speculate that these miRNAs may be strongly associated with METH addiction phenotype because they are only highly expressed in the addiction group, but not in the non-addiction group, while the other common miRNAs may only be associated with METH use. However, the roles of the miRNAs which are expressed only in the acute drug treatment group, but without significant altered expression in the addiction group are still unclear. These may be involved in some physiological or psychological symptoms at the beginning stage of METH use.

In our preliminary results, miRNA profiling in this study indicated that expression of miR-496 was the most significantly altered in the NAc during METH addiction. It showed up-regulation with a 13 fold change when comparing between the METH addiction group and the acute treatment without addiction group. Therefore, this finding suggests that this miRNA may be highly associated with METH addiction phenotype. A recent microarray profiling of the habenular transcripts showed that the expression of miR-496 was differentially altered in mice which were intravenously self-administering nicotine and reported that this miRNA may contribute to nicotine addiction and neuroadaptive changes by nicotine (Lee, Woo, Kim, & Im, 2015). MiRNA studies have been performed on ethanol-treated rats, and changes due to ethanol were observed in both the amygdala and ventral striatum (Ignacio, Mooney, & Middleton, 2014; Lewohl et al., 2011). They found that miR-496 may represent highly robust and persistent indices of ethanol exposure. However, their finding is in contrast with ours with regard to the direction of the changes. We found that this miRNA was up-regulated after METH exposure, while the study above reported down regulation of this miRNA following ethanol exposure. This may due to the different effect of the two drugs used in the experiment. Another study by Lee et al. (2015) reported that miR-496 was significantly up-regulated in mice habenula after intravenously self-administering

nicotine. This finding was consistent with our result that miR-496 was up-regulated after self-administration of the drug, although we used different drugs and different part of the brain in the study. However, addiction-related gene target and canonical neuronal pathways of miR-496 are still unclear.

In contrast, miR-200b-3p was significantly down-regulated with a fold change of 4.7 folds. We found that the expression of this miRNA was significantly decreased in NAc of METH addicted group when compared with non-addicted group and hence it may be associated with METH addiction. This finding is in line with a previous study which reported that miR-200b is down-regulated in the NAc and striatal postsynaptic densities by cocaine administration (Eipper-Mains et al., 2011). However, they did not further stratify the association of miR-200b between cocaine exposure and cocaine addiction in the study. The miR-200b derived from the miR-200 family which included miR-200a, miR-200c, miR-141, and miR-429 play critical role in regulating differentiation and proliferation of neurons (Pandey et al., 2015). According to IPA, miR-200b-3p has 6 gene targets, such as TAOK1, ZC3H6, SSH2, PRTG, USF3 and GNAI3. TAOK1 gene encodes serine/threonine-protein kinase which is involved in the MAPK cascade and has an important role in the drug addiction pathway (Li, Mao, & Wei, 2008). PRTG gene encodes a member of the immunoglobulin superfamily. The encoded transmembrane protein has been associated with the development of various tissues, especially in the process of neurogenesis. It has been suggested that this gene may be associated with attention deficit hyperactivity disorder (ADHD) (Wigg et al., 2008). Beside miR-200b, we also found that PRTG gene is the target for 10 addiction-related miRNAs which are detected in our study, such as miR-26a-5p, miR-181a-5p, miR-194-5p and so on (Figure 5). Furthermore, miR-200b has the potential function to regulate the expression of GNAI3 gene. GNAI3 is a G-protein alpha3 which regulate the activity of polypeptide 3. This protein cooperates with beta-gamma dimmers and a

G-protein signaling 3 activator may be associated with the relapse of heroin-seeking behavior (Yao et al., 2005). Furthermore, G-protein-coupled receptors, for examples 5-hydroxytryptamine-A1 receptor, GABAB receptor and cannabinoid CB1 receptor, are involved in synaptic transmission in neurons through binding to GNAI3 protein (Mannoury la Cour, El Mestikawy, Hanoun, Hamon, & Lanfumey, 2006; Straiker, Borden, & Sullivan, 2002). From the IPA analysis, we found an interaction between GNAI3 and a G-protein, guanine nucleotide-binding protein (GNAT2) (Figure 5.1).

Our finding also shows that expression of miR-194 was significant highly altered in NAc of the METH-addicted rats. It was up-regulated about 5.4 fold when compared with the acute treatment without addiction group. Previous studies which studied miRNA expression in the brains of human alcoholics reported that miR-194 was up-regulated due to alcohol exposure and suggested that this miRNA may be involved in neuroadaptations of alcohol dependence (Nunez & Mayfield, 2012; Yadav et al., 2011). Furthermore, a study from Chen et al. (2013) reported a significantly differential expression of miR-194 in hippocampus when rats acquired cocaine addiction. They found that this miRNA was up-regulated 1.9 fold when compared the cocaine-administrated rats with the saline group. Although a different drug was used in the experiment, their findings is consistent with our result that miR-194 may be strongly associated with drug addiction. From the IPA pathway analysis, we found that DNA (cytosine-5)-methyltransferase 3A (DNMT3A) was one of the gene target of miR-194-5p (Figure 5.1). A previous study demonstrated that DNMT3A expression is regulated in NAc by chronic cocaine administration (LaPlant et al., 2010). NAc specific manipulations block the DNA methylation potentiate cocaine addiction and exert antidepressant-like effects, while NAc specific over-expression of DNMT3A blocks cocaine addiction and is pro-depressant. They found that DNMT3A is induced in a sustained manner after relatively long periods of cocaine withdrawal, and may thereby

play a critical role, not only in the transition to addiction, however also in the maintenance of the addicted circumstance. Besides that, miR-194 also targets glutathione S-transferase beta 2 (GSTT2) which play an important role in the cellular defense against oxidative stress (Figure 5.1). Therefore, GSTs are of interest in the context of association studies of METH abuse (Hashimoto et al., 2005).

In addition, we found that both miR-134-3p and miR34c-5p are significantly over-expressed in the continuous treatment group which is addicted to METH. It shows that the expression of miR-134-3p was increased 2.3 folds, when comparing the expression level in the addiction group with those exposed to METH but without addiction. Athough miR-34c-5p did not show statistically significant difference when compared the METH addiction group with non-addiction group, however, its expression was increased more than 200 percent in addiction group compared with non-addiction group. In our findings, miR-134 and miR-34c-5p have been shown to interact with deacetylase sirtuin-1 (SIRT1) (Figure 5.1), a NAD-dependent protein deacetylase which has emerged as a key regulator of mammalian transcription in response to cellular metabolic status and stress (Jeong et al., 2011) and thus affect learning and memory processes (Gao et al., 2010; Zovoilis et al., 2011). A previous study reported an essential role for SIRT1 in regulating behavioral responses to cocaine and morphine through actions in the NAc and showed that chronic cocaine and morphine administration increases SIRT1 expression in the mouse NAc (Ferguson et al., 2013). Activation of SIRT1 is mediated via post-transcriptional regulation of CREB expression by miR-134 in nucleus accumbens (Gao et al., 2010). This finding is matched with the IPA map in Figure 5.1. SIRT1 normally functions to limit expression of miR-134 via a repressor complex, and unchecked miR-134 expression following SIRT1 deficiency results in the down-regulated expression of CREB and BDNF, thereby impairing synaptic plasticity (Gao et al., 2010). Moreover, many studies also found increase of

CREB activity in NAc after chronic cocaine or amphetamine administration (Shaw-Lutchman, Impey, Storm, & Nestler, 2003), and upon opiate withdrawal (Chartoff, Papadopoulou, Konradi, & Carlezon, 2003; Shaw-Lutchman et al., 2002). In our study, we found that miR-134 was significantly up-regulated during the addiction stage. It may be induced by the over-expression of the CREB protein following METH administration. Besides that, we also noticed that over-expression of miR-134 inhibited the expression of the indirect downstream genes, including brain-derived neurotrophic factor (BDNF) which is strongly linked to the addiction phenotype (Figure 5.1). Moreover, the IPA analysis identified that miR-134 is involved in 16 canonical neuronal pathways, such as glutamate receptor, G beta Gamma, CREB, G-protein coupled receptor, dopamine receptor, GNRH, PKA, MAPK, gap junction and so on, which may be involved in METH addiction.

For miR-34c, it appeared that it regulates its target SIRT1 expression by inhibiting SIRT1 expression via a binding site at the 3' UTR of SIRT1 gene. Inhibition of SIRT1 by miR-34 increase the acetylated p53 level in the cell, and following that increases the expression of p21 and PUMA which are transcriptional targets of p53 that regulate the cell cycle and apoptosis (Yamakuchi, Ferlito, & Lowenstein, 2008). Our profiling analysis shows that SIRT1 gene is one of the targets of miR-34c-5p and it may showing that an increased expression of miR-34c plays an important role to inhibit the over-expression of SIRT1 caused by drug administration (Figure 5.1). In our protein profiling result, two of the proteins, Aldolase A (ALDOA) and Dynamin-1-like protein (DNM1L), were identified in the NAc, indicating their importance in the functional proteome (Figure 5.3). These two proteins are the target gene of miR-34. Recent study revealed an increased expression of brain-type aldolase in rat NAc after chronic cocaine administration. This finding was consistent with their study that ALDOA was significantly over-expressed in NAc after a continuous METH treatment. For DNM1L

protein, a previous proteomic study reported this protein was up-regulated in the cerebral cortex when exposure and dependence to alcohol occurs. Besides that, Iwazaki et al. (2007) reported that DNM1L protein was up-regulated by 2.7 fold in the striatum of the acute METH-treated rats. This finding also consistent with our result that DNM1L protein was significantly over-expressed in the continuous METH treatment with addiction group (Table 4.14).

The expression of two miRNAs from miR-9 family, miR-9-5p and miR-9-3p, were found to be highly altered in rat NAc. MiRNA-9 is an abundant miRNAs in the adult vertebrate brain. This miRNA plays an important role in balancing proliferation in embryonic neural progenitors (Coolen, Katz, & Bally-Cuif, 2013). Brain-enriched miR-9 is highly expressed in the brain. It is a brain-specific miRNAs that has been reported to be associated with neurodegenerative disorders (Marion Coolen & Bally-Cuif, 2015). In our study, we found that miR-9-5p was up-regulated with a fold change of 11.2 in the non-addiction group, while its expression was increased 74.7 fold in the addiction group (Table 4.3). In other words, its expression was more than 500 percent increased in the addiction group when compared with the group without addiction. The highly altered expression of miR-9-5p in the addiction group indicates that this miRNA may play an important role in the addiction pathway. Some studies found that this miRNA is involved in the BDNF pathway, which has been strongly linked to METH addiction (Flanagin, Cook, & de Wit, 2006; Sim, Mohamed, Hatim, Rajagopal, & Habil, 2010). BDNF has been reported to be expressed after acute drug administration, which then causes the subsequent long-lasting increase of DRD3 expression in the NAc, and which may finally induce addiction symptom (Le Foll, Diaz, & Sokoloff, 2005). Interestingly, the up-regulation of miR-9-5p is in contrast with another member of the miR-9 family, miR-9-3p, which demonstrated a down-regulation with 50% reduction in expression in the addiction group when compared with those without addiction (Table 4.3).

Nevertheless, this finding was consistent with a study which reported that miR-9-3p is expressed at a significantly lower level in schizophrenia as compared to controls in rat frontal cortex (Perkins et al., 2007). A research study that had investigated the effects of double deletion of miR-9-3 on brain development in mice indicated that this family of miRNA regulates the proliferation and differentiation of neural progenitor cells in the telencephalon of cerebral cortex. They inhibit the regulator proteins such as homeobox protein Meis2 and the transcription factor Forkhead box protein G1 (FOXG1) which is involved in neurogenesis (Shibata, Kurokawa, Nakao, Ohmura, & Aizawa, 2008; Shibata, Nakao, Kiyonari, Abe, & Aizawa, 2011).

A study by Chandrasekar and Dreyer (2009) had shown that chronic cocaine exposure repressed the expression of miR-124, while inducing the expression of miR-181a in the mesolimbic dopaminergic system. This result was similar with our finding which shows that miR-181a is significantly over-expressed in the addiction group. Previous study by Saba et al. (2012) found that miR-181a is highly expressed in the synaptodendritic compartment of the NAc, suggesting that it might be potentially involved in drug-induced alterations in plasticity and behavior in animal models. They revealed that expression of miR-181a was increased by DA-mediated transmission following cocaine and amphetamines exposure. Furthermore, they also suggested that perturbations in the GluA2 subunit in response to drug-induced expression of miR-181a may cause maladaptive alteration in synaptic transmission and plasticity in brain which are related to drug addiction pathway (Saba et al., 2012). Hence, it is interesting that miR-181a may play an important role in drug-induced remodeling of NAc following drug exposure, thus promoting and regulating the development of addiction. Another miRNA, miR-124, has been reported to play an important role in the differentiation of progenitor cells to mature neurons (Conaco, Otto, Han, & Mandel, 2006; Makeyev, Zhang, Carrasco, & Maniatis, 2007). Previous study reported that miR-124 is over-

expressed in NAc leading to a significant down-regulation of BDNF. This finding was matched with our target prediction map which showed that BDNF is one of the target genes for miR-124 (Figure 5.1). It is well-known that BDNF is highly involved in synaptic plasticity and plays an important role in addiction and memory (Kalivas & Volkow, 2005; Nestler, 2001). The results of our study indicated that miR-124 was over-expressed in both the addicted group and the non-addicted acute treatment group. However, there is a 2.6 times increase in expression following addiction behavior, as compared to the non-addicted acute treatment group. This result is in contrast with the finding by Chandrasekar and Dreyer (2009) which showed down-regulation of miR-124 under chronic cocaine administration. However, a recent study on METH reported that there was a 1.23 fold change of the miR-124 expression in the ventral tegmental area in METH self-administered rats (Bosch, Benton, Macartney-Coxson, & Kivell, 2015). According to previous studies, short- and long-term cocaine administration both cause increases in BDNF levels and are associated with intracellular signaling pathways in the brain reward pathway (Corominas, Roncero, Ribases, Castells, & Casas, 2007; Russo, Mazei-Robison, Ables, & Nestler, 2009). Furthermore, Vargas-Perez et al. (2009) also showed that elevated levels of BDNF in the ventral tegmental area (VTA) of the midbrain increases the likelihood that the rats will become dependent on opiates, such as heroin and morphine. Besides that, our protein profiling also shows that miR-124 was linked to Huntinting protein (HTT) (Figure 5.3). This protein has been suggested to play a role in long-term memory storage (Choi et al., 2014) and it may affect addiction memory in relapse occurrence and maintenance of learned addictive behavior (Boening, 2001).

Our study had also found that expressions of some of the miRNAs are highly significantly altered in the NAc of METH-addicted rats and that these miRNAs may be associated with METH addiction. These miRNAs are miR-299a-5p, miR-127-5p, miR-

411-3p, miR-881a-3p and so on, but their roles in regulating drug addiction have yet to be determined. It would be interesting to further study the roles of these miRNAs in METH addiction pathways in future experimental studies.

Based on our addiction pathway study, the METH addiction related miRNAs were found to be involved in 20 most relevant pathways (Table 4.4). One of the pathways is CREB signaling in neuron. Previous study shows that the nuclear transcription factor CREB is hightly involved in the emergence of addiction, contributing to the alteration in both phenotype and expression in brain regions like NAc which are important for drug-seeking behavior and reward (McPherson & Lawrence, 2007). Long-term potentiation had been associated with addiction-induced neuro-adaptations in glutamatergic transmission and synaptic plasticity (Jones & Bonci, 2005). Another addiction related pathway is MAPK signaling pathway, and studies have reported that this pathway may be involved in the regulation of synaptic plasticity which is strongly linked to memory function and addictive properties (Wang, Fibuch, & Mao, 2007). Interestingly, two other common pathways in our finding, Gonadotropin-releasing hormone (GnRH) signaling and Gap junctions signaling pathways, have been recently identified to be linked to addiction (Li et al., 2008). GnRH signaling pathway had been reported to be involved in the activation of gene expression and the release of gonadotropins, subsequently regulating the stress pathways in the hypothalamus, pituitary gland, and gonadal glands (Tilbrook, Turner, & Clarke, 2002). It has also been suggested to be involved in controlling some emotional behaviors like drug-induced stress during addiction (Li et al., 2008). Gap junctions are inter-cellular membrane channels which is linked to the cytoplasm between adjacent cells (Nakase & Naus, 2004). Gap junctions are also involved in electrical coupling and may also play a role in the recovery of cell function after injury (Nakase & Naus, 2004). Several studies have reported the involvement of gap junctions in neurodegenerative diseases such as

Alzheimer's disease and Parkinson's disease (Nagy, Li, Hertzberg, & Marotta, 1996; Rufer et al., 1996), and epilepsy (Traub et al., 2001). For the gap junctions signaling pathway, a study reported that it can be regulated by the addiction-relevant kinases such as PKA, PKC and ERK in the long-term potentiation pathway (Li et al., 2008). These findings were consistent with a previous study which showed that GnRH signaling and gap junction signaling pathway may play an important role in drug addiction (Li et al., 2008). Our result may also show an additional support to this finding and suggests that they may be new important addiction pathways. Furthermore, although some of the pathways identified in this study, such as G Beta Gamma Signaling, GABA Receptor Signaling, CXCR4 Signaling and G-Protein Coupled Receptor Signaling, had not been directly linked to addiction, however, because many of the addiction-related miRNAs are also involved in these pathways, we speculate that these pathways may also play a role in drug addiction. Therefore, it would be interesting to study the roles of these pathways in the future study.

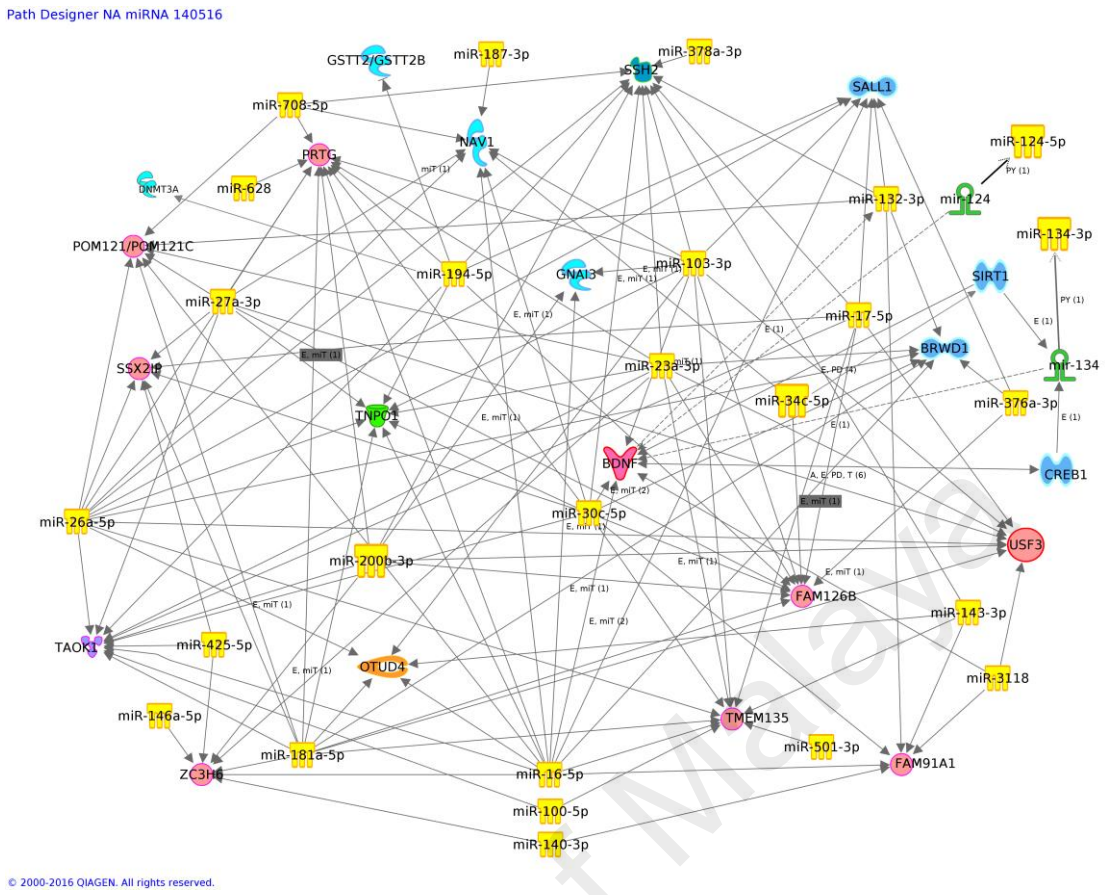


Figure 5.1: Ingenuity Pathway Analysis (IPA) canonical pathway analysis network for miRNAs which are significantly differentiated in expression between METH addiction and those without addiction.
The relationship between the METH addiction-related miRNAs with their gene targets, such as transcription regulators, SIRT1 and CREB, growth factor BDNF and enzymes like GSTT2 and GNAI3.

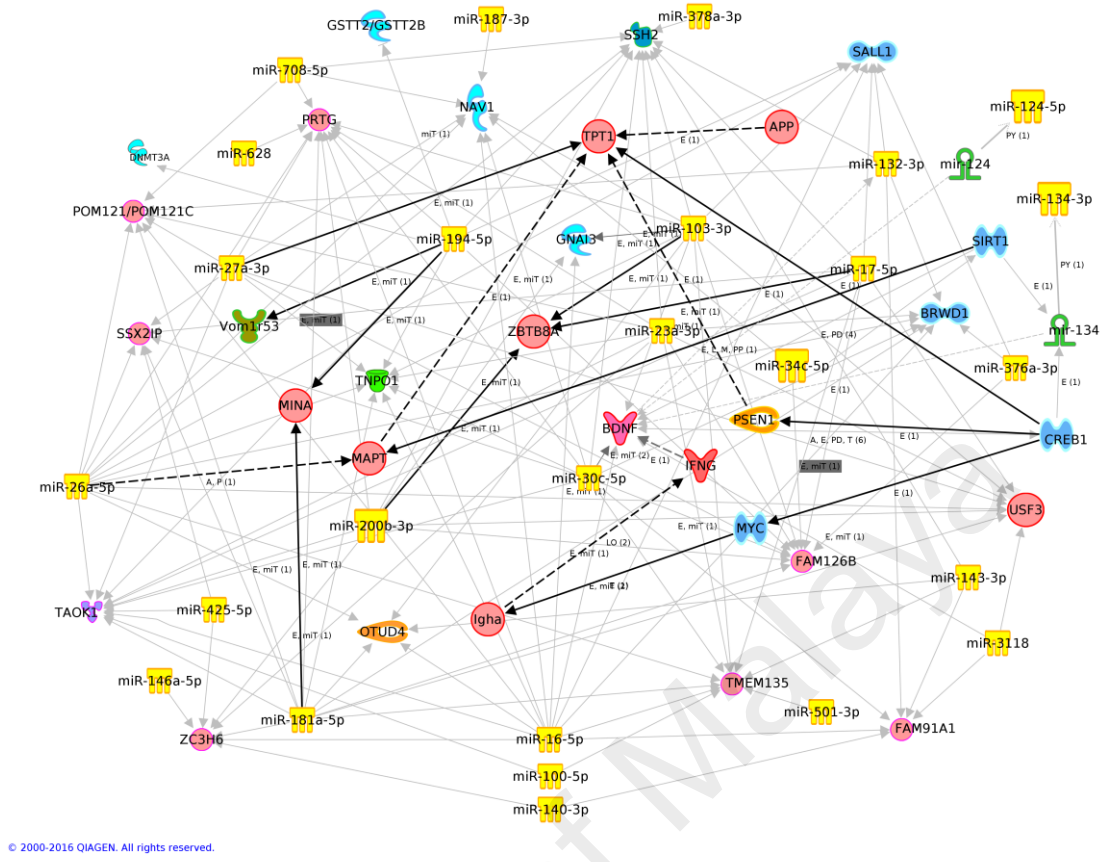


Figure 5.2: Ingenuity Pathway Analysis (IPA) canonical pathway analysis network for miRNAs – gene interactions in which are significantly differentiated in expression between METH addiction and those without addiction.
The relationship between the METH addiction-related miRNAs with their gene targets and METH addiction-related genes, such as TPT1 gene and so on.

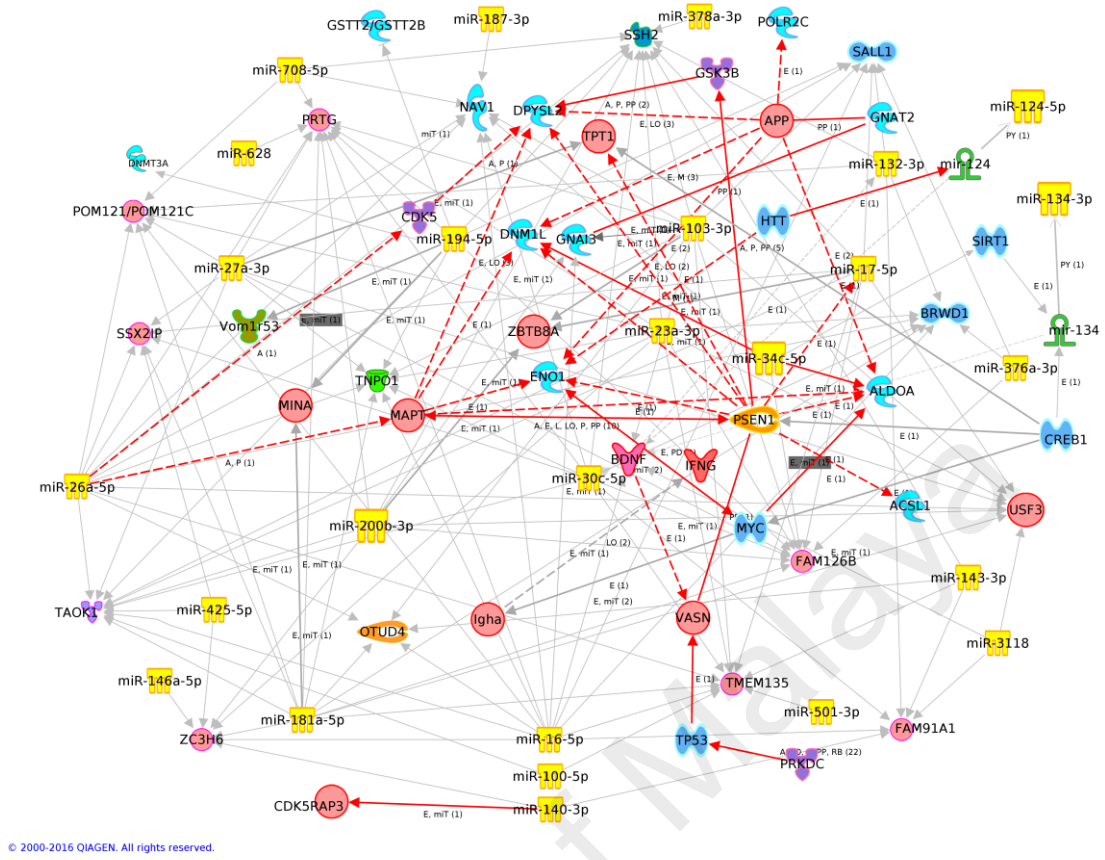


Figure 5.3: Ingenuity Pathway Analysis (IPA) canonical pathway analysis network for miRNAs – gene – protein interactions in which are significantly differentiated in expression between METH addiction and those without addiction.
The relationship between the METH addiction-related proteins with the METH addiction-related miRNAs and genes which are identified from miRNA and gene profiles.

5.2.2 Molecular changes in rat hippocampus

In this study, a pair-wise significance analysis of the microarray data indicated significant differential expression of 190 miRNAs in hippocampus between the METH treatment in the addiction group and the controls. Amongst these, 109 miRNAs are highly expressed only in the addiction group, but not in the acute drug treatment group, so it can be deduced that these miRNAs may be significantly associated with the addiction phenotype (Figure 4.10). For the common miRNAs in both the addiction group and the acute treatment group without addiction, 11 miRNAs were over-expressed more than 50% while 4 miRNAs was down-regulated around 40-50% when comparing the drug administrated animal before addiction and after addiction. Therefore, these miRNAs may be also strongly associated with METH addiction phenotype because they are only highly expressed in addiction group, but not in the non-addiction group, while the other common miRNAs may only be associated with METH use. However, the roles of the miRNAs which are expressed only in the acute drug treatment group, but without significantly altered expression in the addiction group are still unclear. These may be involved in some physiological or psychological symptoms at the beginning stage of METH use.

In the preliminary result, we found that miR-448-3p is the highest over-expressed miRNA in the nucleus accumbens when compared between METH addiction and non-addiction groups. It may show that this miRNA may strongly associate with METH addiction phenotype. However, the relationship of this miRNA with neurological and psychological disorders and related pathways are still unknown. A previous study reported that miR-448 in rat hippocampus was significant associated with morphine exposure. They found that this miRNA in stressed neonatal mice was down-regulated after morphine treatment when compared to the controls. This contrasts with our finding that this miRNA was highly up-regulated in rat hippocampus after

METH exposure. It may due to different drug given and different comparative parameter applied in the study. Nevertheless, both of us found that this miRNA was significantly associated with drug use and addiction.

The total number of miRNA gene target in hippocampus which are related to METH addiction are much lesser than in nucleus accumbens, which indicated that 1016 gene targets of the addiction related miRNAs in nucleus accumbens, while only 191 gene targets in hippocampus. Besides the nucleus accumbens, we also found that miR-9a-5p is also significant highly expressed in the hippocampus. MiR-9a-5p showed up-regulation with a fold change of 4.16 in the METH addiction group when compared with non-addiction group. MiR-9 is highly expressed in the brain, especially in the hippocampus, and it had been suggested to involve in regulating neuronal differentiation (Delaloy et al., 2010). The highly altered expression of miR-9-5p in the addiction group indicates that this miRNA may play an important role in METH addiction, either in nucleus accumbens or hippocampus. A previous study reported that the expression of this miRNA was up-regulated by alcohol exposure in adult mammalian brain and suggested that miR-9 dependent mechanism contributes to alcohol tolerance (Pietrzykowski et al., 2008). They showed that alcohol causes a rapid increase in miR-9 expression in striatum and down-regulated the expression of DRD2 receptor which is one of the miR-9 regulated target (Figure 5.3). DRD2 receptor was reported to be strongly associated with METH use and addiction (Barr et al., 2006). Besides that, we also noticed that one of the gene targets of miR-9a is FBX033. A recently published study in GWAS suggested the involvement of FBX033 in attention-deficit/hyperactivity disorder (ADHD) and frontal gray matter volume (Sanchez-Mora et al., 2015). Since attention is one of the symptoms on METH use, we suggest that it may be an interesting candidate gene to be studied in future.

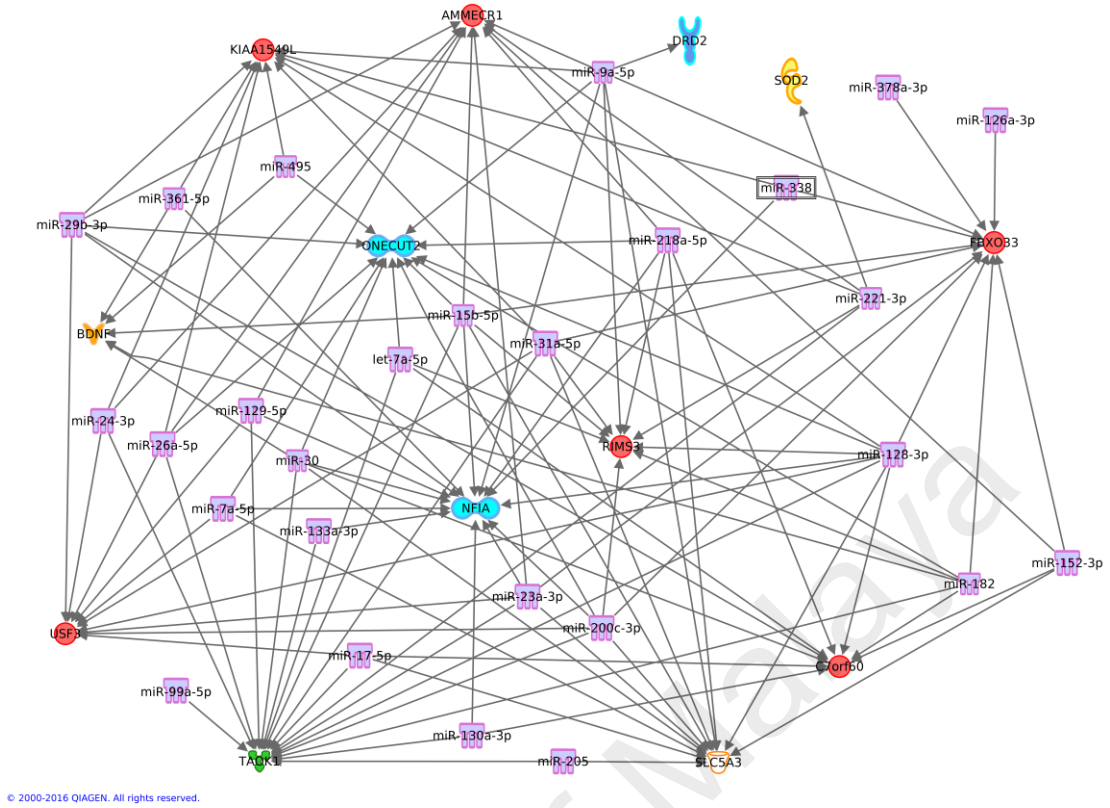


Figure 5.4: Ingenuity Pathway Analysis (IPA) canonical pathway analysis network for miRNAs which are significantly differentiated in expression between METH addiction and those without addiction.
The relationship between the METH addiction-related miRNAs with their gene targets and some addiction-related receptors, such as serotonin and DA receptors, growth factor BDNF and enzymes like SOD2 and Tao kinase.

In our finding of the transcriptome profile, we found that the expression of the FOS gene was significantly altered in the hippocampus when comparing the METH addiction group with non-addiction group. Therefore, we suggest that this gene may be associated with METH addiction phenotype. The IPA analysis revealed that FOS expression in hippocampus was directly regulated by 3 miRNAs, miR-29b-3p, miR-7a-5p and miR-221-3p (Figure 5.3), which had shown significant association with METH addiction (Figure 4.10). FOS have been implicated in regulation of cell proliferation, differentiation, and survival (Tulchinsky, 2000). Previous studies reported that cocaine, METH (Graybiel, Moratalla, & Robertson, 1990), heroin (Curran, Akil, & Watson,

1996) and other psychoactive drugs (Nichols & Sanders-Bush, 2002; Singewald, Salchner, & Sharp, 2003) increase the expression of *c-fos* in the mesocortical and mesolimbic reward pathways. This finding was consistent with our result that the expression of the FOS gene was significantly up-regulated by 1.6 fold, when compared between METH addiction group and non-addiction group (Figure 4.9). However, this gene only showed significantly altered expression in hippocampus, not in NAc. Besides, this gene only showed significantly altered expression when addiction occurred and it did not show any significantly differential expression in acute METH treatment group when compared to the controls. Again, this finding shows that FOS gene may be only associated with METH addiction phenotype, but not in acute METH use. Furthermore, our IPA analysis data also showed that the interaction between FOS and DRD2 receptor with may be involved in METH addiction (Figure 5.4). A study suggested that *c-Fos* may be implicated in regulation of gene expression in DA receptor-expressed neuron following cocaine exposure (Zhang et al., 2005). They also reported that a *c-Fos* in the dopamine-receptor-bearing neurons plays an important role in the memory of the reinforcing effects of cocaine. Singewald et al. (2003) found that *c-fos* can be suppressed by a complex, named ΔFosB's AP-1, which can lead to a long-term induction of ΔFosB. Therefore, the *c-fos* promoter is found to be useful in the addiction study, especially in the context-induced relapse to drug-seeking and other behaviors related with chronic drug exposure.

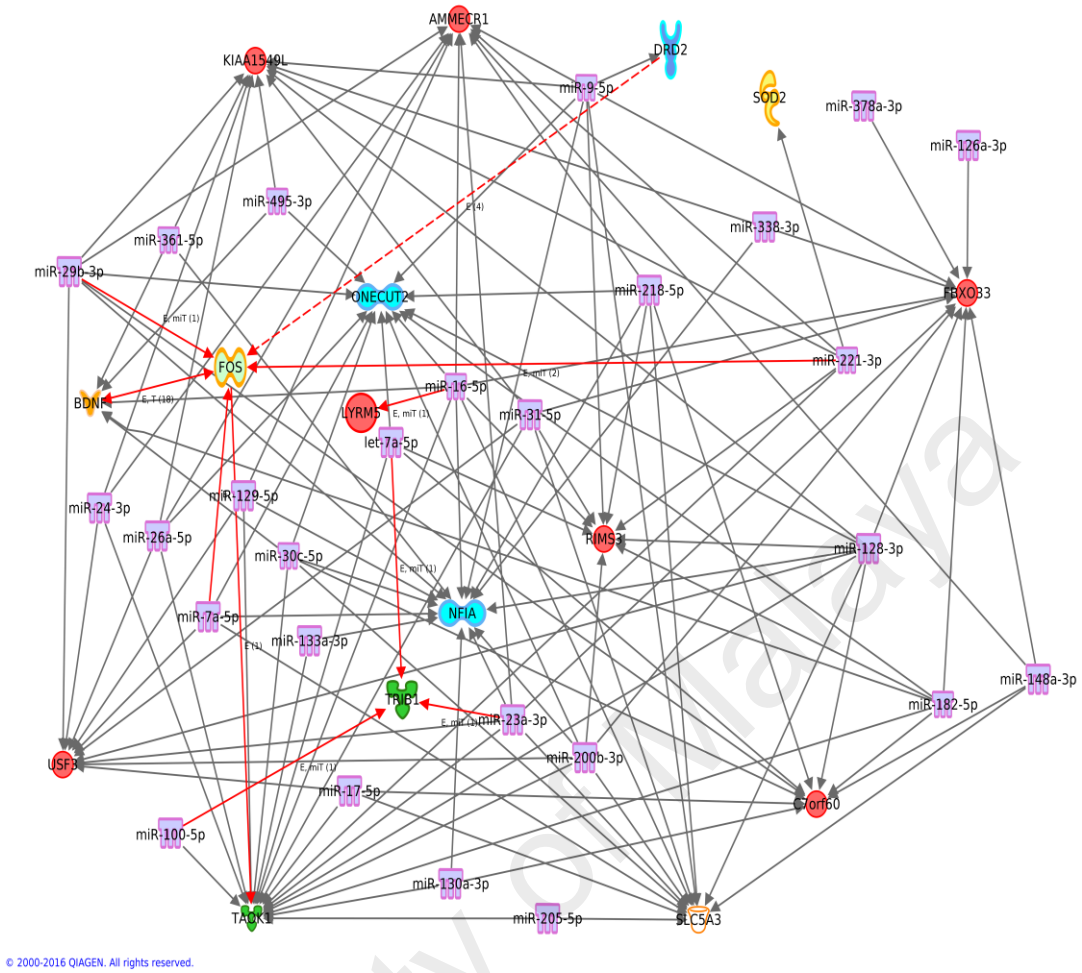


Figure 5.5: Ingenuity Pathway Analysis (IPA) canonical pathway analysis network for miRNAs – gene interactions in which are significantly differentiated in expression between METH addiction and those without addiction.
The relationship between the METH addiction-related miRNAs with their gene targets and METH addiction-related genes, such as FOS and TRIB1 gene.

Our finding from IPA analysis also revealed that 16 miRNAs regulating same down-stream gene target, TAOK1 (Figure 5.3). TAOK1 are serine-threonine kinases and also a member of the mitogen-activated protein kinase (MAPK) family. TAOK1 plays a prominent role in numerous neuronal functions, such as control of cell growth, cell differentiation, neuronal survival, and synaptic plasticity (Girault, Valjent, Caboche, & Herve, 2007; Hetman & Gozdz, 2004; Thomas & Huganir, 2004). This kinase is higly enrished in the brain, especially in the mesolimbic dopaminergic system.

It is highly expressed in the amygdala and prefrontal cortex and moderately expressed in the ventral tegmental area (VTA), hippocampus, NAc, and midbrain (Lein et al., 2007). A study suggested that a functional conservation of TAO kinase in mediating ethanol's biological action and identify this kinase as a putative candidate gene for ethanol use disorder in human (Kapfhamer et al., 2013). Besides that, our finding also showed that TAOK1 was directly interacted with FOS gene which had shown significant association with METH addiction (Figure 5.4). These findings demonstrate that FOS is a target for several protein kinases including TAO kinase, involved in signal transduction and suggest that phosphorylation could regulate the transcriptional properties of FOS. Two of the miRNAs, miR-133a and miR-26b, had been shown a direct interaction with TAOK1 (Figure 5.4). A recent study indicated that dynamic changes in miRNA expression in rat hippocampus following repeated cocaine exposure and reported that miR-133 and miR-26b were involved in brain disorders and drug abuse (Chen et al., 2013).

In general, substance abuse and other psychiatric diseases like schizophrenia may share in molecular pathology. In our protein profiling study, we identified the relationship between 2 identified miRNAs (miR-24 and miR-128), Disrupted in Schizophrenia 1 (DISC1) and DRD2 receptor. Although MiR-24 and miR-128 did not show any significant difference between the METH addiction group and the non-addiction group, we noticed that these were only significantly up-regulated in the addiction group when compared with controls, while there were no significant altered expressions in the non-addiction group (Figure 4.10). This may suggests that these two miRNAs may also be associated with METH addiction phenotype. MiR-24 was reported to be up-regulated in the rat prefrontal cortex when comparison was made between the METH self-administration group with the controls (Du et al., 2016), while miR-128 was associated with fear-extinction memory formation (Lin et al., 2011). Their

findings were consistent with our results that both of the miRNAs were over-expressed in the METH addiction group when compared with controls. The study demonstrated that knockdown of miR-128 can prevent the occurrence of fear-extinction memory, suggesting that this miRNA may play an important role in extinction-induced memory (Lin et al., 2011). IPA analysis from our study shows that these 2 miRNAs directly interacted with Disrupted in Schizophrenia 1 (DISC1) gene which may be linked to the addiction pathways (Figure 5.5). DISC1 is a gene which is disrupted by a translocation, thereby segregating the major psychiatric disorders, including schizophrenia, recurrent major depression and bipolar affective disorder (Millar et al., 2001; Millar et al., 2000; St Clair et al., 1990). Many subsequent studies demonstrated that DISC1 is not only implicated in schizophrenia and mood disorders, but also in autism spectrum disorder, Asperger syndrome, attention deficit and hyperactivity disorder (ADHD) (Callicott et al., 2005; Hennah et al., 2003; Kilpinen et al., 2008). DISC1 also has been shown to be involved in neurodevelopment mechanism like neuronal cell proliferation, migration, differentiation, and regulation of DISC1 gene in rats, subsequently leads to behavioral alteration (Hayashi-Takagi et al., 2010; Wang et al., 2011). A study demonstrated the role of DISC1 as a psychiatric risk factor in cocaine self-administration in which there is significantly increased expression of DISC1 in the NAc (Gancarz et al., 2016). This study provides the first mechanistic evidence of a critical role of DISC1 in drug-induced behavioral neuroadaptations and sheds more light at the shared molecular pathology of drug abuse and other major psychiatric disorders. Further genetic interaction studies showed that DISC1 regulates axon guidance through activation of RAC-PAK signaling pathways. This was consistent with our finding that DISC1 was involved in axonal guidance signaling pathway (Table 4.17). We also found that 16 addicted-related miRNAs were over-expressed in the hippocampus in which 13 miRNAs are involved in

axon guidance signaling pathway, while 3 miRNAs are involved in PAK signaling pathway (Table 4.7).

Further IPA analysis revealed that miR-128 together with another 4 miRNAs (miR-29b, miR-31, miR-148 and miR-17) showed an interaction with dihydropyrimidinase related protein 2 (DPYSL2) (Figure 5.5). DPYSL2 showed significant up-regulation by 9.7 fold when comparing between METH addiction group and non-addiction group (Table 4.16). DPYSL2 is widely expressed in neuronal tissues and has been reported to be involved in several psychiatric disorders, especially Alzheimer's disease and schizophrenia (Taylor & Wang, 2014). Taylor and Wang (2014) reported that DPYSL2 gene polymorphisms may be associated with alcohol dependence in Caucasian population. These finding suggests that disruption of DPYSL2 may be associated with susceptibility to alcohol addiction. Studies of protein expression profiles in the striatum and frontal cortex of METH-administrated rats demonstrated that DPYSL2 can be related to underlying mechanisms of METH-induced behavioral sensitization, protein degradation, redox regulation, energy metabolism, cellular growth, cytoskeletal modifications and altered synaptic function (Faure, Hattingh, Stein, & Daniels, 2009; Iwazaki et al., 2007). Therefore, we suggest that the involvement of the 4 detected miRNAs with DPYSL2 has high possibility to be associated with METH use and addiction.

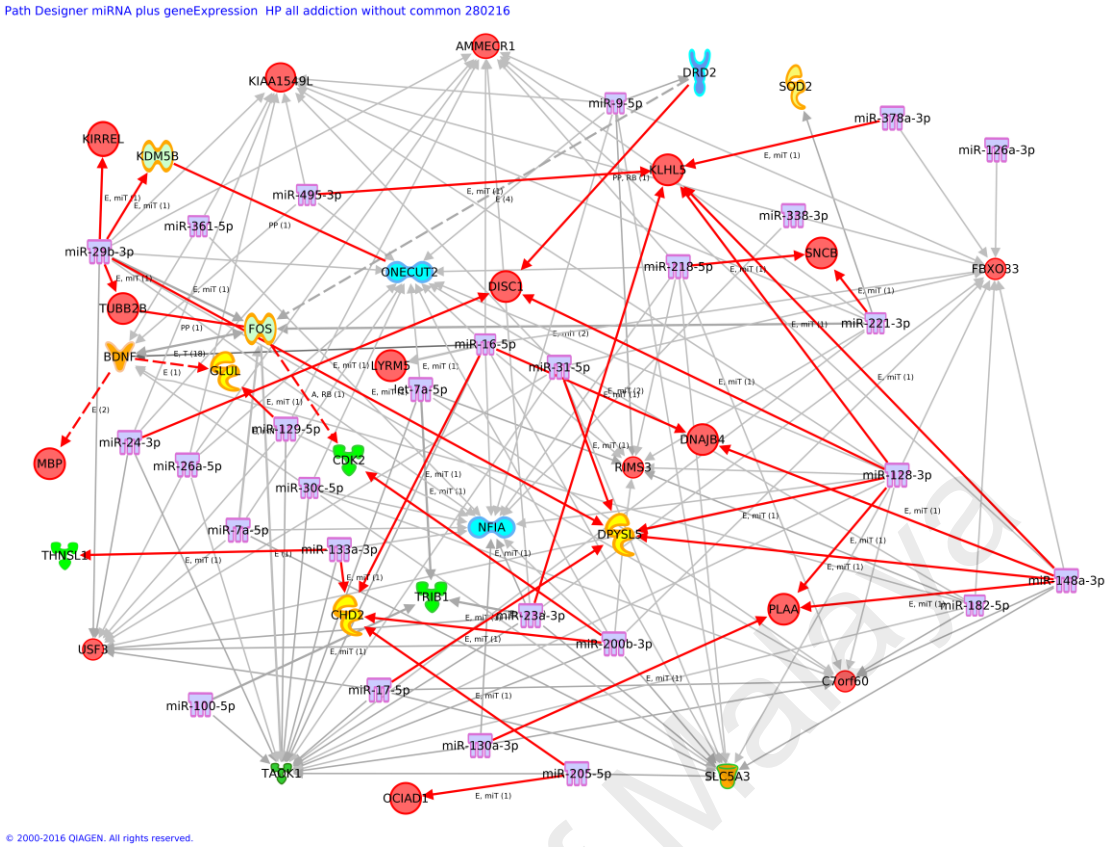


Figure 5.6: Ingenuity Pathway Analysis (IPA) canonical pathway analysis network for miRNAs – gene – protein interactions in which are significantly differentiated in expression between METH addiction and those without addiction.
The relationship between the METH addiction-related proteins with the METH addiction-related miRNAs and genes which are identified from miRNA and gene profiles.

5.3 Interaction of the molecular expression in nucleus accumbens and hippocampus

In general, we found that some miRNAs expressions were significantly altered in both of the brain tissues, nucleus accumbens and hippocampus. These are miR-200b-3p, miR-30c-5p, miR-26a-5p, miR-16-5p and miR-100-5p, in which their expressions were highly altered following METH addiction. This indicated that some interaction occur between these miRNAs in NAc and hippocampus in the regulation of drug addiction. Gene targets for METH addiction-related miRNAs such as BDNF and TAOK1 play important roles in METH addction in both of the brain tissues. Besides that, we found some common signaling pathways in both NAc and hippocampus which interacted with the addiction-related miRNAs, such as CREB, G Beta Gamma, GABA receptor, Glutamate receptor, GNRH, G-protein coupled receptor, PDGF, synaptic long term depression and protein Kinase A signaling pathways. Furthermore, protein profile from NAc and hippocampus showing DPYSL2 protein were significantly over-expressed following METH addiction. While protein pathways analysis showed that there are some common pathways in NAc and hippocampus which may be involved in METH addiction, namely axon guidance mediated by semaphorins, dopamine receptor mediated signaling pathway, Wnt signaling pathway, and CCKR signaling map. These findings show that these pathways may be related to the mechanism of the drug addiction, and it would be interesting to investigate this further in the future.

5.4 Limitation of the study

There are some limitations to the current study. A broad observation relevant to many studies that utilize any animal model system to assess neurobehavioural or cognitive disorders is that, it cannot be ignored that one is trying to recapitulate a complex human phenotype in an animal model that, by its nature, will not be able to fully model all aspects of that disorder as it presents in human patients. In this sense, the validity of the model is reliant on its ability to effectively map the etiology, behavior, and biological mechanisms of human psychopathology.

Secondly, this is a molecular profiling study which was not able to provide the information regarding the effect of the miRNAs, mRNAs and protein on various metabolism pathways. It is therefore recommended that functional studies be carried out to determine how they affect the metabolism pathways and to determine the mechanisms of addiction.

In our transcritptome study, we found only small to moderately significant changes in gene expression resulting in weak p-values and small fold changes between the different conditions. This is not surprising as psychiatric disorders are associated with subtle changes in neuronal function which translates into slightly altered expression of genes involved in neurotransmitter production, secretion and reception, synaptic function and cellular signal processing.

In general, the sample size of the study is relatively small for it to be able to reflect the METH addiction phenotype completely, the reason mainly being imposed by cost constraints and the need by animal ethics to reduce numbers as far as possible. However, further studies using larger sample size may be necessary to confirm these results.

Furthermore, neuropsychiatric disorders are multigenic by nature and include the interaction of multiple genes and proteins that are expressed spatially on a small scale and are in different stages of the psychological disorder. Therefore, our findings may not completely represent the addiction phenotype. Nevertheless, our study compared the molecular profiling between METH use without addiction and those with addiction, thereby eliminating biases caused by effects of drug use. However, it is not possible to eliminate the effect of some phychological symptoms, such as panic, pain and so on.

5.5 Future studies

In spite of the above study limitations, we had been able to identify new candidate miRNAs, genes and proteins which may be highly associated with METH addiction, and detect significant expression differences in two of the brain tissues. In addition, several recommendations for future studies in addiction research can be made based on this thesis. As discussed above, the currently available microarray technology is plagued by a variety of issues including the multiple testing problems, the vast amount of probes lacking biological significant annotation and a probe design which reflects an outdated state of knowledge concerning the rodent genome. It is therefore worthwhile to try a different approach in order to test smaller, previously selected sets of genes. This could be done using techniques such as SYBR Green or TaqMan PCR arrays or RNA in situ hybridisation assays which all allow for the parallel testing of up to several hundred genes (Spurgeon et al., 2008).

Furthermore, the brain consists of multiple components that every component of the brain performs their specific functions. However, drugs of abuse can change the brain structure and leads to neuroadaptation that induce compulsive drug use or

addiction. Therefore, besides nucleus accumbens and hippocampus, many other regions of the brain areas may be affected by drug abuse, such as prefrontal cortex (Du et al., 2016), striatum (Chen & Chen, 2005) and ventral tegmental area (Bosch et al., 2015). These parts of the brain should be studied in future.

Moreover, using cell type-specific samples for gene expression measurements would significantly increase the biological conclusiveness of the studies. Therefore, laser capture microdissection might be an interesting method to achieve this goal in the future study (McCullumsmith & Meador-Woodruff, 2011).

For the functional part of the study, we plan to study in future, the functional aspects of the identified miRNAs, genes or proteins which showed highly significant association with METH addiction from our current study, either in-vivo or in-vitro, to identify the role of the addiction-related miRNAs, genes or proteins in the regulation of the addiction pathways.

CHAPTER 6:

CONCLUSION

In the first part of the study, which is the development of addiction behaviour in an animal model, we observed that methamphetamine (METH) is indeed a highly addictive drug and that a 15 alternate day treatment with increasing doses of METH up to 5 mg/kg showed a much stronger effect of the CPP when compared to the control group, indicating that the addiction phenotype had developed in the experimental rats following METH exposure.

In the second part of the study, the effects of the METH exposure on learning and memory disturbance were analyzed, and the results indicated that low dose and short term use of METH may improve working and reference memory, while high dose and long term use of METH may reduce the ability of learning and memory, be it the working memory or reference memory. Interestingly, our finding demonstrated that the animals which were given 4 and 5 mg/kg of METH showed a highly significant difference in working or reference memory as compared with the control group. As the doses were increasing, a turning point occurred at 2 mg/kg of METH whereby the number of the errors in the radial maze test started to increase. This finding indicated that the METH-induced memory and learning disturbance may occur at 2 mg/kg onwards and two of the higher doses, 4 and 5 mg/kg may strongly decrease memory and learning ability.

For the miRNA profiling study, we observed many changes in miRNA expression in our microarray analyses of METH addiction and found a strong relationship between addiction biology and the genes that were differentially expressed. The expression of miRNAs which are highly altered in METH addiction such as miR-496-3p, miR-194-5p, mir-200b-5p, miR-181a-5p and miR-134-3p in the nucleus

accumbens, and miR-448-3p, miR-374-5p, miR-329-5p and miR-7a-5p in the hippocampus, may be strongly associated with the METH addiction phenotype. The study highlights some interesting relationships between these miRNAs and METH addiction which would warrant further study. Our findings implicate a role for miRNAs in METH use and addiction, and have led us to hypothesize that there is an alteration of miRNAs expression in the miRNA biogenesis process in the drug addiction pathways. Our finding strongly supports the occurrence of brain-specific miRNAs that could bind to the mRNAs of their target genes to perform gene-silencing processes which may lead to the addiction phenotype.

Next, our findings on transcriptomics study showed that FOS and TRIB1genes showed significant over-expression in the METH addiction group when compared to non-addiction group, while in the protein profiling study, DPYSL2, ALDOA and DISC1 proteins were significantly over-expressed in the METH addiction group. These findings indicated that these proteins may be highly associated with METH addiction.

We believe that the findings in the study have contributed to our understanding of the regulatory network associated to METH use and addiction. This information can be utilized in the effort to look for new targets for the future management of METH abuse. Our findings also showed that neuronal molecular expressions in the brain remain an important field of discovery in psychiatric research. Its scientific outcome as well as the proposed follow-up studies should contribute to the body of knowledge of the molecular aspects of METH addiction and to the development of efficient treatment options for METH addiction.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

A) Research publications

1. Sim, M. S., A. Hatim, G. P. Reynolds & Z. Mohamed (2013) Association of a functional FAAH polymorphism with methamphetamine-induced symptoms and dependence in a Malaysian population. *Pharmacogenomics,* 14**,** 505-14. **(Tier 1; Impact factor = 3.97) (ISI-Cited Publication)**

   Article accepted for publication: 1-Februry-2013

   Citation: 7

2. Maw Shin Sim, Ahmad Hatim, Shiau Hui Diong, Zahurin Mohamed (2014) Genetic polymorphism in DTNBP1 gene is associated with methamphetamine-induced panic disorder. *Journal of Addiction Medicine*, 8(6):431-7. **(Tier 3; Impact factor = 1.756) (ISI-Cited Publication)**

   Article accepted for publication: December-2014

3. Shiau Hui Diong, Nor Shuhadah Mohd Yusoff, Maw Shin Sim, Raja Elina Raja Aziddin, Zamri Chik, Rusdi Abdul Rashid, Norliza Chemi, Zahurin Mohamed (2014). Quantitation of methadone and its metabolite in patients under maintenance treatment. *Journal of Analytical Toxicology*, 38(9):660-6.

   (Tier 2; Impact factor = 2.017) (ISI-Cited Publication)

   Article accepted for publication: 8-August-2014

   Citation: 2

4. Maw Shin Sim, Tomoko Soga, Vijayapandi Pandy, Yuan Seng Wu, Ishwar S. Parhar, Zahurin Mohamed. Global microRNA expression profiling of methamphetamine exposure and addiction in the rat nucleus accumbens. (Submitted to Metabolic Brain Disease Journal on 24th Sept 2016)

B) Conference publications

1. M.S.Sim, I.S.Parhar, T.Soga, P.Vijayapandi and Z.Mohamed (2015) MicroRNA Expression Profiling of Methamphetamine Dependence in Rat Nucleus Accumbens Tissue. *Public Health Genomics*,18(suppl 1):1-51. Publisher: Karger. **(Tier 2; IF = 2.208) (ISI Publication)**

2. M.S.Sim, I.S.Parhar, T.Soga, P.Vijayapandi and Z.Mohamed (2015) MicroRNA Expression Profiling of Methamphetamine Dependence in Rat Hippocampal Tissue. *European Journal of Human Genetics*;23(suppl 1):183 Publisher: Nature. **(Tier 1; IF = 4.349) (ISI Publication)**

LIST OF CONFERENCE PROCEEDINGS

1. **Golden Helix Symposia 2015**

   Level: International

   Date: 11th – 13th March 2015

   Venue: Connexion@Nexus, Bangsar South, Kuala Lumpur, Malaysia

   Organizer: The Golden Helix Foundation, London & University of Malaya, Kuala Lumpur

   (Apendix A)

2. **European Human Genetics Conference 2015**

   Level: International

   Date: 6th – 9th June 2015

   Venue: SECC, Glasgow, Scotland, United Kingdom

   Organizer: European Society of Human Genetics (ESHG)

   (Apendix B)

LIST OF AWARDS

1. **Best Poster Award**

   Prize: **2nd**

   Conference: Golden Helix Symposia 2015

   Level: International

   Date: 11th – 13th March 2015

   Title poster: MicroRNA Expression Profiling of Methamphetamine Dependence in Rat Nucleus Accumbens Tissue

   (Apendix C)

APPENDIX A

GOLDEN HELIX SYMPOSIA 2015

P014: MicroRNA Expression Profiling of Methamphetamine Dependence in Rat Nucleus Accumbens Tissue

M. S. Sim [a], I. S. Parhar [b], T. Soga [b], P. Vijayapandi [a], Z. Mohamed [a]

[a]Department of Pharmacology, Faculty of Medicine, University of Malaya, [b]Brain Research Institute, School of Medicine and Health Sciences, Monash University, Sunway, Malaysia.

Objective: Metamphetamine is a highly addictive psychostimulant that induces behavioral changes, the nucleus accumbens being the part of the brain that plays the important role in these changes, especially in drug addiction. However, little is known about the underlying mechanisms of methamphetamine effects on global miRNA expression. The objective of this study was to determine the global miRNA profiling of the methamphetamine dependence from the rat nucleus accumbens tissue and to identify the miRNAs which are associated with methamphetamine use and dependence.

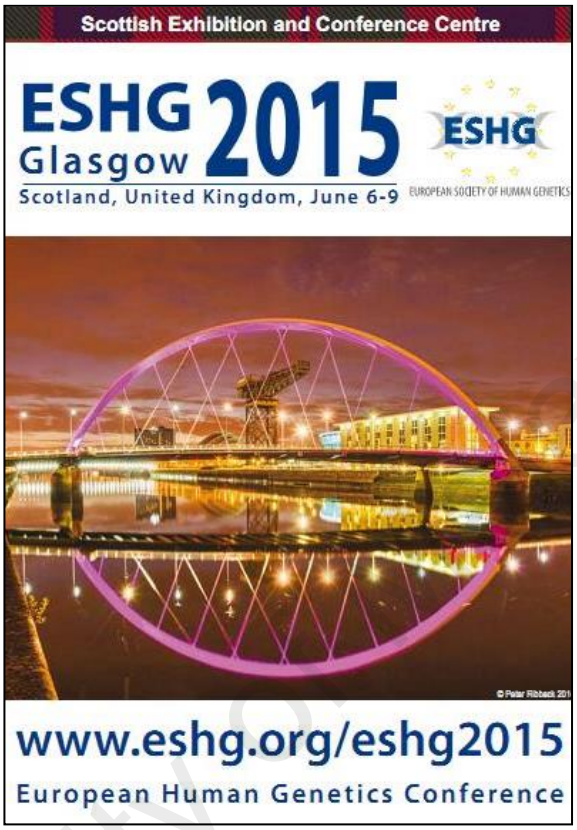
Material and Methods: The study comprised of 18 male rats which were divided into 3 groups: 15 days continuous methamphetamine treatment(0.25,0.5,1,2,3,4,5mg/kg), single dose acute methamphetamine treatment (5 mg/kg), and a control group. Addiction behavior was determined using Conditioned Place Preference task. The analysis of the miRNA profiling was performed using Affymetric microarray GeneChip® System.

Results: For behavior test, we found that the addiction behavior only occur with continuous treatment of methamphetamine, but not in acute treatment. Differential profiling of miRNAs indicated that 25 miRNAs were significantly up-regulated and 1 down-regulated when the acute treatment was given; 55 miRNAs were up-regulated and 15 down-regulated in the continuous methamphetamine treatment group. Comparing between acute treatment without addiction and the continuous treatment with addiction, 20 miRNAs were up-regulated with 3 down-regulated for addiction phenotype. The miRNAs were selected when there are more than 2 times fold changes, ANOVA test with $p<0.05$ and FDR test with $p>0.05$.

Conclusion: Our results suggest that dynamic changes occur in the expression of miRNAs, which may be associated with the methamphetamine use and dependence.

APPENDIX B

EUROPEAN SOCIETY OF HUMAN GENETICS CONFERENCE 2015



PM09.078

MicroRNA expression profiling of methamphetamine dependence in rat hippocampal tissue

M. Sim[1], *I. S. Parhar*[2], *T. Soga*[2], *P. Vijayapandi*[1], *Z. Mohamed*[1];
[1]*Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia,* [2]*Brain Research Institute, School of Medicine and Health Sciences, Monash University, Sunway, Malaysia.*

Metamphetamine is a highly addictive psychostimulant that induces behavioral changes, the hippocampus being the part of the brain that plays the important role in these changes, especially in drug addiction. However, little is known about the underlying mechanisms of methamphetamine effects on global miRNA expression. The objective of this study was to determine the global miRNA profiling of the methamphetamine dependence from the rat hippocampal tissue and to identify the miRNAs which are associated with methamphetamine use and dependence. The study comprised of 18 male rats which were divided into 3 groups: continuous methamphetamine treatment(0.25,0.5,1,2,3,4,5mg/kg), single dose acute methamphetamine treatment (5 mg/kg), and a control group. Addiction behavior was determined using Conditioned Place Preference task. The analysis of the miRNA profiling was performed using Affymetric microarray GeneChip® System. For behavior test, we found that the addiction behavior only occur with continuous treatment of methamphetamine, but not in acute treatment. Differential profiling of miRNAs indicated that 30 miRNAs were significantly up-regulated and 1 down-regulated when the acute treatment was given; 40 miRNAs were up-regulated and 1 down-regulated in the continuous methamphetamine treatment group. Comparing between acute treatment without addiction and the continuous treatment with addiction, 29 miRNAs were up-regulated with 1 down-regulated for addiction phenotype. The miRNAs were selected when there are more than 2 times fold changes, ANOVA test with $p<0.05$ and FDR test with $p>0.05$. Conclusion, our results suggest that dynamic changes occur in the expression of miRNAs, which may be associated with the methamphetamine use and dependence.

*Research University Grant RG443/12HTM from University of Malaya

APPENDIX C

BEST POSTER AWARD