SHOTGUN PROTEOMICS ANALYSIS OF PROTEINS RESPONDING TO DRUG ADDICTION IN RAT CEREBELLUM

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ABSTRACT

Drug addiction in Thailand is becoming an increasing problem. A trend in the use of methamphetamine (METH) and dextromethorphan (DXM) addictive drugs is found mostly in the teenagers. There are many brain regions that might be relevant to addiction, including the cerebellum. Addictive drugs have affected on the alteration of neurotransmitter systems and other related proteins underlying the mechanism of drug addiction in multiple brain regions such as prefrontal cortex, hippocampus, striatum and amygdala. However, the molecular mechanisms underlying the processes of METH and DXM addiction in the cerebellum are still not fully understood. Therefore, the aim of this study was to examine proteins expression profiles in the cerebellum of METH and DXM addiction. Male Sprague-Dawley rats were used in this study and divided into two addiction models, including METH and DXM. In METH addiction model, animals in control group received saline for 15 days. Animal in METH group received METH 0.1-4 mg/kg for 14 days and on day 15, animals received METH 6 mg/kg. In DXM addiction model, animals were divided into 6 groups; control, DXM-induced addiction, drug withdrawal (DW), DXM-GABA standard (DG), DXM-PGBR (DR) and DXM-diazepam drug (DD). Animals in control groups received saline for 15 days and distilled water for 60 days. Animals in DXM group received DXM 30 mg/kg for 15 days. Following DXM administration, animals in DW, DG, DR and DD groups received distilled water, GABA standard 0.8 mg/kg, PGBR 5 g/kg and diazepam drug 10 mg/kg, respectively for 15, 30 and 60 days. The alteration of differential expressed

protein profiles were evaluated by label-free shotgun proteomics. An expression level of some protein was validated by using western blotting technique.

The proteomics results showed the alteration of differential protein expression profiles in rat cerebellum of drug addiction, drug withdrawal and addiction treatments (PGBR, GABA standard and diazepam drug). These proteins were associated with biological functions, including cell-cell signaling (calbindin, excitatory amino acid transporter 1, protein shisa-6 and neuronal acetylcholine receptor subunit alpha-10) and signal transduction (plexin-B3, epidermal growth factor receptor, serine/threonine-protein kinase SIK1 and NUAK family SNF1-like kinase 2) functions. In addition, we confirm the role of calbindin, a calcium binding protein, in drug addiction. A significant increase in the protein expression levels of calbindin was observed in METH and DXM groups comparing with control. The expression of calbindin protein was significantly attenuated in all treatment groups such as DG, DR and DD groups when compared with DXM group. Nevertheless, no significant difference in DW group was observed when compared to DXM-administered rats.

These findings suggest that cell-cell signaling and signal transduction proteins, including calbindin play important roles in neuropathology underlying drug addiction, drug withdrawal and addiction treatments.

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ABBREVIATIONS

µg	=	microgram
µl/min	=	microliter per minute
µm	=	micrometer
ACN	=	acetonitrile
ANOVA	=	analysis of variance
APS	=	ammonium persulfate
BCA	=	bicinchoninic acid
BSA	=	bovine serum albumin
CaBPs	=	calcium binding proteins
°C	=	degree Celsius
DTT	=	dithiothreitol
DXM	=	dextromethorphan
eg	=	exempli gratia (for the sake of example)
FA	=	formic acid
g	=	gram
GABA	=	gamma aminobutyric acid
GAD	=	glutamic acid decarboxylase
GO	=	gene ontology
H	=	hour
IAA	=	iodoacetamide
kDa	=	kilodalton
LC-MS/MS	=	Liquid chromatography-tandem mass spectrometry
mA	=	milliAmpere
METH	=	methamphetamine
MeV	=	*Multi Experiment Viewer*
mg	=	milligram
mg/kg	=	milligram per kilogram
mg/ml	=	milligram per milliliter
min	=	minute

ABBREVIATIONS (CONT.)

mm	=	millimeter
mM	=	milliMolar
m/z	=	mass-to-charge ratio
NaCl	=	sodium chloride
NH_4HCO_3	=	ammonium bicarbonate
nm	=	nanometer
PANTHER	=	Protein Analysis Through Evolutionary Relationships
PGBR	=	pre-germinated brown rice
rpm	=	round per minute
SDS	=	sodium deoxycholate
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	=	standard error of the mean
TEMED	=	Tetramethylethylenediamine
TBS	=	tris buffer saline
TBST	=	tris buffer saline Tween-20
TMB	=	3, 3', 5, 5' - tetramethylbenzidine
Tris-HCl	=	tris-hydrochloride
V	=	voltage
vs	=	versus
VTA	=	ventral tegmental area

CHAPTER I

INTRODUCTION

Rational and significance of the study

Over the last few years, drug addiction is a major problem affecting the development of country, including economic, social, and public health. It is found that the problem of drug addiction has more severe and widespread in the workplace, community and educational institutions. The incidence of addictive drugs such as cannabis, opiates, cocaine, amphetamine, or methamphetamine is recently increasing throughout the world. Use of these drugs may lead to addiction and develop to psychotic disorders. Methamphetamine, an amphetamine derivative, is a psychostimulant drug with potent effects on the central nervous system (Srisurapanont et al., 2003). The mental disorders resulted from methamphetamine exposure are similar to the symptoms of schizophrenic patients, including hallucinations, delusions, and cognitive impairment (Srisurapanont et al., 2011). Moreover, a previous study in animal models found that methamphetamine administration can induce the behavioral changes such as hyperlocomotion, sensitization, and stereotypes (Segal, Kuczenski, O'Neil, Melega, & Cho, 2003). These behavioral changes may regard as animal models of human stimulant-induced addiction and psychotic disorders (Iwazaki, McGregor, & Matsumoto, 2006). In addition, behavioral changes have been described by complex interactions of methamphetamine addiction and neurotransmitter systems. Methamphetamine administration has been reported to affect dopaminergic neurotransmission by disrupting dopamine transporter functions, consequently extracellular dopamine levels (Howell, & Kimmel, 2008). The glutamatergic system has also been found to be disrupted after methamphetamine exposure including an increase in extracellular glutamate levels (Mark, Quinton, Russek, & Yamamoto, 2007). Moreover, previous studies have shown that methamphetamine administration changes GABAergic functions. Accordingly, the alteration of multiple neurotransmitter systems is complicated in drug addiction induced by methamphetamine exposure. Recently, it is not only psychostimulant drugs but the over-the-counter medicine has

also been reported to become addicted substances among youths in Thailand. One of over-the counter drugs which has been addicted is dextromethorphan. Dextromethorphan has been widely used as an antitussive drug for over 50 years. Furthermore, dextromethorphan is a more potent antagonist of the glutamate receptor *N*-methyl-D-aspartic acid (NMDA) which is responsible for psychotropic effects (Mayhew, 2007). At high doses, it can also produce sensation such as euphoria, distorted perception and dissociative anesthesia that are seen with other NMDA antagonists such as phencyclidine and ketamine (Banken, & Foster, 2008). An animal study showed an increase in the levels of tyrosine hydroxylase, enzyme of dopamine synthesis, after dextromethorphan administration (Zhang, Jahng, & Kim, 2001).

As it has been reported for long, addiction involves alteration in several brain regions that are associated with functions such as memory, motivation and executive control. Previous studies indicated abnormalities of several brain areas such as frontal cortex, hippocampus and amygdala underlying the processes of addiction (Belujon, & Grace, 2011; Wearne et al., 2015). Apart from these regions, an increasing amount of data suggests that the cerebellum is also involved in addicted behaviors (Anderson et al., 2006). It has been reported to relate with drug-induced homeostatic adaptations which are tolerance and physical dependence after repeated stimulation of addictive drug (Edwards, & Rizk, 1981). Both tolerance and physical dependence are considered to be primary symptoms of addiction. Numerous imaging studies in cocaine and alcoholic addictions have shown cerebellar activations during exposure to drug-associated cues (Anderson et al., 2006; Moulton, Elman, Becerra, Goldstein, & Borsook, 2014). In addition, a cocaine-sensitized regimen is reported to increase the binding of an NMDA antagonist in the cerebellum (Bhargava, & Kumar, 1999). Furthermore, a dopaminergic direct ventral tegmental area (VTA)-cerebellar projection has been demonstrated (Ikai, Takada, & Mizuno, 1994; Ikai, Takada, Shinonaga, & Mizuno, 1992) and found the expression of dopamine transporters (DAT) (Melchitzky, & Lewis, 2000). In addition, a previous study has reported that GABA receptors are expressed in granule cells of the cerebellum (Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000) and the deficits of GABAergic system have been found after drug administration (Zhang et al., 2006). Several studies have pointed out to the role of GABAergic neurotransmission in drug addiction by disrupting gamma-

aminobutyric acid (GABA) receptors, GABA transporters and calcium binding proteins (neuronal marker) (Kumari, Lakhan, Kalita, Misra, & Mittal, 2010; Veerasakul, Thanoi, Reynolds, & Nudmamud-Thanoi, 2016). GABAergic system is a major inhibitory neurotransmitter in the central nervous system, which is a target of drug, including antidepressants, anticonvulsants, and anxiolytics (anti-anxiety). Nevertheless, the conventional treatment with these drugs might produce several side effects such as insomnia, dizziness and confusion. Therefore, an alternative treatment has been studied for reducing the side effects from the conventional treatment. As it has been mentioned above, GABA is a neurotransmitter that may be involved in drug addiction and may be a target of drug treatments. A variety of foods contains GABA such as fish, tomato, chocolate, and whole grains. GABA has been reported to have and high amounts in pre-germinated brown rice (PGBR) (Moongngarm, & Saetung, 2010). GABA supplements are used to treat stress and anxiety. PGBR has also been investigated for antidepressant activities in mice (Mamiya et al., 2007). Thus, the natural GABA was used in this study for investigating the potential protective effect after drug addiction.

Recently, the proteomics has been employed to evaluate the differential expression of proteins and functional outputs of a biological system in drug addiction such as oxidative stress responses, protein modification and degradation, synaptic functions and neurotransmission (Wang, Wu, Zhao, & Li, 2016). Proteomics analysis revealed the effects of METH sensitized rats including synaptic regulation, protein phosphatase signaling, mitochondrial functions and alterations to the inhibitory GABAergic network in the prefrontal cortex (Wearne et al., 2015). Moreover, the proteome study in rat cortex indicated the alteration of protein after acute METH exposure (Kobeissy et al., 2008). This study has identified the involvement of cell-cell signaling and signal transduction proteins in animal models of drug addiction, drug withdrawal, and addiction treatment of the rat cerebellum. Moreover, the molecular mechanisms characterized of these proteins, relevant to drug addiction, should have utility in the therapeutic drug target including alternative treatments.

Purpose of the study

A general experiment objective

This study was to investigate the protein expression profiles in rat cerebellum after exposure to addictive drugs and addiction treatments.

Specific objectives

1. To investigate changes of differential protein expression profiles in animal models of drug addiction.

2. To investigate changes of differential protein expression profiles in rat cerebellum after exposure to drug withdrawal.

3. To examine the comparative effects of GABA-enriched pre-germinated brown rice, GABA standard and diazepam drug treatments in an animal model of drug addiction.

4. To confirm the expression of identified protein levels in rat cerebellum after exposure to drug addiction, drug withdrawal and addiction treatments.

Hypotheses

1. The effects of drug addiction may lead to changes in protein expression profiles of rat cerebellum.

2. Whether or not, GABA-enriched pre-germinated brown rice treatment can recover the protein expression from the effect of drug addiction

3. GABA-enriched pre-germinated brown rice treatment may be a protective effect better than other treatments including GABA standard and diazepam after exposure to drug addiction.

Scope of the study

The present study used Sprague-dawley rats to investiagte the alteration of protein expression profiles in the cerebellum of drug addiction models induced by methamphetamine and dextromethorphan using proteomics technique. In addition, changes of protein expression profiles in rat cerebellum were also observed in withdrawal and treatments (GABA standard, pre-germinated brown rice and diazepam drug) periods of DXM addiction model. The selected protein was then validated the proteomics results using the western blotting technique.

Keywords

Drug addiction, Methamphetamine, Dextromethorphan, Cerebellum, Proteomics

Anticipated outcomes

1. To provide an understanding about the alteration of differential protein expression profiles in rat cerebellum of methamphetamine and dextromethorphan addictions.

2. To provide an information about the possible role of the cerebellum in methamphetamine and dextromethorphan addiction.

3. To support information about the proteomics data in the cerebellum of methamphetamine and dextromethorphan addictions.

4. To support information about the neuroprotective effect of pre-germinated brown for addiction treatment in the future.

Conceptual framework

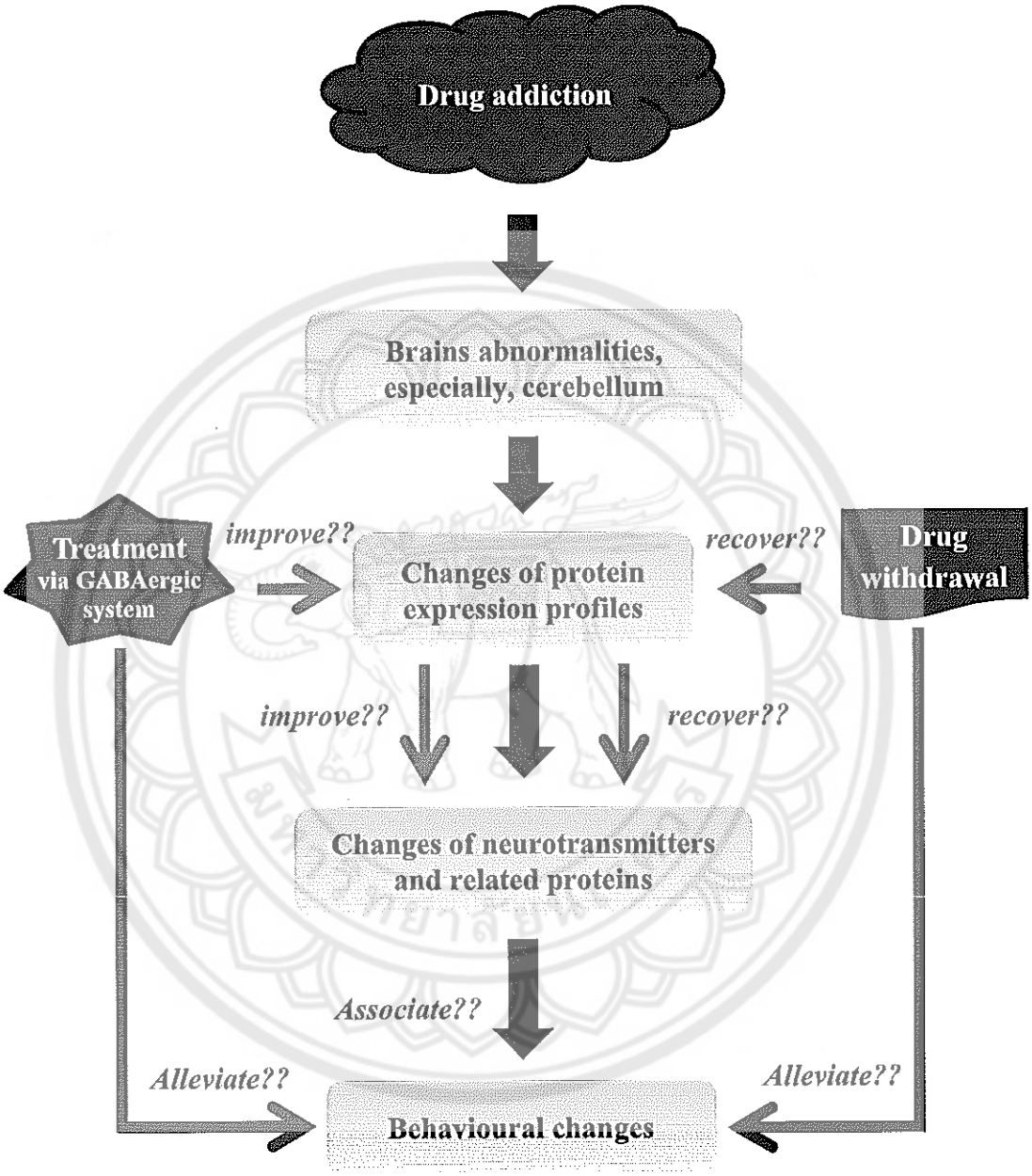


Figure 1 Conceptual framework of this study

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Drug addiction

Drug addiction or substance use disorder is a complex neuro-behavioral disorder. The abuse of addictive drug can induce to alter functions of the brain leading to several symptoms of addiction and drug-related disorders. The distinctive features of addictive behaviors are craving (a strong desire for drug taking), tolerance (the reducing effect of a drug following repeated administration at a certain dose), and withdrawal symptoms (occur when they stop suddenly the drug or cut down extremely dosage, eg. dysphoria, anxiety, depression) (Koob, & Volkow, 2010). In patients, clinician can describe an addictive person according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM), fifth edition as shown in Table 1 (American Psychiatric Association, 2013). The effect of addictive drugs can also contribute to psychosis, deficits in attention and memory and motor-dysfunction (Chang, Alicata, Ernst, & Volkow, 2007). Currently, addictive drugs are found in a variety types such as cannabis, opioids, cocaine, amphetamine-type stimulants and antitussive drugs, including dextromethorphan. It has been estimated that about 5.2% of the world population aged 15 to 64 years used an addictive drug at least once in the previous years (United Nations Office on Drugs and Crime, 2015). Addictive drug is a favored throughout the world in the adolescent users. The rates of adolescents seeking addictive drug, particularly methamphetamine increased in Asia (Ren, Ma, & Hashimoto, 2016). In addition, dextromethorphan have become the most commonly abused among Thai adolescents and young adults (Chomchai, & Manaboriboon, 2012). The repetition of addictive drug has led to addiction which is harmful consequences and changes in the brain. As such, to further understand the molecular marker in the brain that involve in addiction will help to understand the pathogenesis of addiction.

Table 1 The DSM-V criteria for substance use disorder

Criteria	Symptoms
1	Taking the substance in larger amounts or for longer than you're meant to.
2	Wanting to cut down or stop using the substance but not managing to.
3	Spending a lot of time getting, using, or recovering from use of the substance.
4	Cravings and urges to use the substance.
5	Not managing to do what you should at work, home, or school because of substance use.
6	Continuing to use, even when it causes problems in relationships.
7	Giving up important social, occupational, or recreational activities because of substance use.
8	Using substances again and again, even when it puts you in danger.
9	Continuing to use, even when you know you have a physical or psychological problem that could have been caused or made worse by the substance.
10	Needing more of the substance to get the effect you want (tolerance).
11	Development of withdrawal symptoms, which can be relieved by taking more of the substance.
	Disorder severity: Presence of two to three symptoms: mild Presence of four to five symptoms: moderate Presence of six or more symptoms: severe

Source: American Psychiatric Association, 2013

Neurocircuitry of drug addiction

The brain circuitry underlying addiction is complex that describes as a simplification by the cycle with three stages, including binge/intoxication, withdrawal/ negative effect and preoccupation/anticipation. In the binge/intoxication stage, a part

of the brain activated the reward system. Several addictive drugs, a primary neurobiological mechanism that drives drug-related disorder is increased activation of the dopamine reward pathway. It originates dopaminergic neuron projections from ventral tegmental area to other reward-related regions including the nucleus accumbens and prefrontal cortex (figure 2). Many studies have shown the elevation of extracellular dopamine concentration that is activated by addictive substances such as cocaine and methamphetamine (Creed et al., 2016; Lin, Sambo, & Khoshbouei, 2016). In addition, cocaine evoked synaptic plasticity glutamate excitatory inputs onto dopaminergic neuron at ventral tegmental area that contributes to rewarding seeking behavior (Chen et al., 2008).

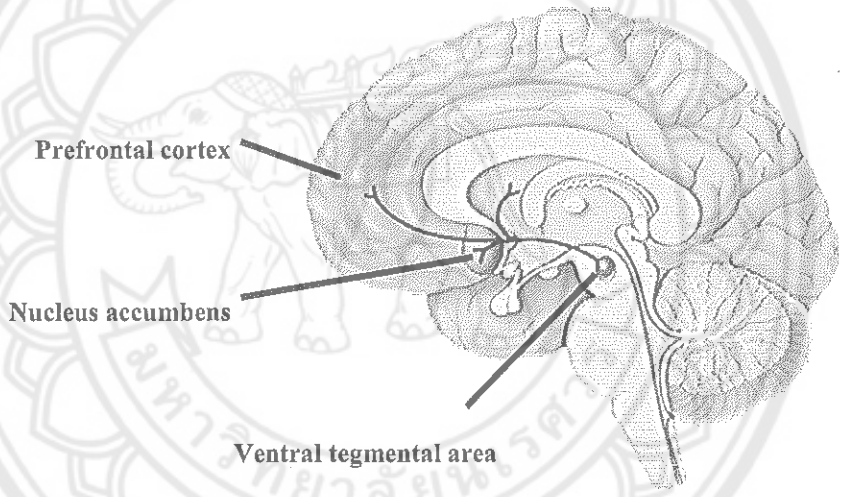


Figure 2 Illustration of dopamine projections from ventral tegmental area to nucleus accumbens and prefrontal cortex by promoting reward circuit system

Source: https://i1.wp.com/knowingneurons.com/wp-content/uploads/2012/10/reward_750.jpg?fit=750%2C414&ssl=1

During drug abstinence, chronic users can trigger in withdrawal symptoms. This stage of addiction may involve in a diminishing function of the brain reward system and activation of stress neurotransmitters, included corticotropin-releasing factor, norepinephrine and dynorphin. Imaging studies in the brains of cocaine, alcohol

and methamphetamine addicted subject have shown a reduction of dopamine receptor when compared with normal subject as shown in Figure 3 (Goldstein, & Volkow, 2002). A previous research has shown an impairment of endocannabinoid signaling within amygdala contributes to increased stress response and negative emotional states in prolonged drug exposure (Parsons, & Hurd, 2015).

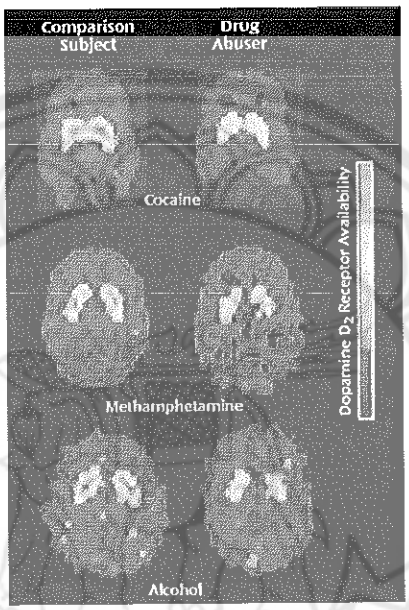


Figure 3 Lower dopamine receptor during withdrawal in the brain of cocaine, alcohol and methamphetamine than in normal comparison subjects

Source: Goldstein, & Volkow, 2002

The reduction of reward effect may contribute compulsive augmentation of substance use that attempt to regain euphoria/pleasurable feelings (Koob, & Le Moal, 2001). The preoccupation/anticipation stage, also called craving stage, is the stage of the addiction that a person may relapse to drug taking behavior again after a period of abstinence. Craving is a motivational state associated with learning response to drug experience and loss of control to drug taking. A previous study found that the addicted subjects are liable to return to compulsive drug taking and consuming (Langleben et al., 2008). This circuit involves the prefrontal cortex and its connections to two

other structures, the amygdala and the nucleus accumbens. An increasing of glutamate projection from the prefrontal cortex to nucleus accumbens drives substance use habits associated with craving (Kalivas, 2009; Kalivas, & O'Brien, 2008).

Methamphetamine

Methamphetamine (METH) is also known as methylamphetamine or desoxyephedrine. It is called in different names that depend on country and characteristic such as meth, ice or YABA in Thailand. Methamphetamine in Thailand is available mainly as crystal, or ICE, and pills in which pure methamphetamine has been intermix with other substances and made into pill form. Methamphetamine can be ingested, smoked, snorted or injected (Mandyam et al., 2007, 2008; Kuczenski et al., 2007). Following methamphetamine administration, it is well absorbed into the bloodstream and metabolized by the liver and excreted by urine (Meyer, & Guenzer, 2005; Kumihashi et al., 2007). The chemical structure of methamphetamine is similar to amphetamine (Figure 4). Methamphetamine has similar effect to amphetamine type of psychostimulant drugs but it has a half-life longer than amphetamine depending on the drug, dosage and routes of the drug (Mendelson et al., 2006). Therefore, methamphetamine has a longer lasting effect and can induce psychological changes to abusers over amphetamine.

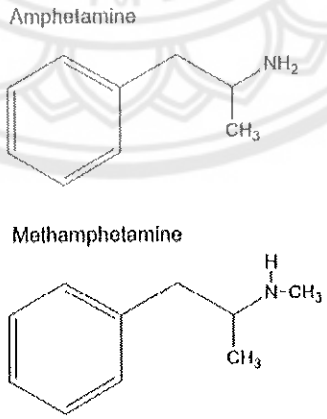


Figure 4 Chemical structures of amphetamine and methamphetamine

Source: Kish, 2008

Effects of Methamphetamine

Methamphetamine is a psychostimulant drug by inducing the harmful effect to drug users. A previous study has shown the effect of methamphetamine that induced psychotic symptoms in patients including hallucination and delusion. At the same time, the withdrawal symptoms such as dysphoria, anxiety and depression has been investigated in methamphetamine user. In animal studies, the administration of methamphetamine has displayed the dysfunctional behaviors by increasing of locomotor activity (Fukushima et al., 2007; Hayashizaki et al., 2013). The addicted-behaviors in human or animal are thought to be mediated by alteration of the neurotransmission system in circuitry of drug addiction. The disturbances of neurotransmitters have shown in the several changes including dopamine, glutamate and GABA (Guilarte, Nihei, McGlothan, & Howard, 2003; Howell, & Kimmel, 2008). The dopamine level is up-regulated in mice prefrontal cortex after methamphetamine exposure resulting in hyperactivity (Tanaka et al., 2017). In related study, the high level of dopamine has been found in rat striatum resulting from the down-regulation of dopamine transporter following methamphetamine administration (German, Hanson, & Fleckenstein, 2012). Methamphetamine-induced behavioral alteration may regulate by cyclic adenosine 3',5'-monophosphase (cAMP) levels in the brain by stimulating adenylate cyclase activity. It has been reported that the methamphetamine treatment reduced dopamine-adenylate cyclase activity and cAMP levels in the striatum (Barnett, Segal, & Kuczenski, 1987; Iyo et al., 1995). Furthermore, the lacking of phosphodieasterase (PDE), intracellular second messenger cyclic nucleotide hydrolyzing enzyme, has been implicated in hyperactivity and impaired spatial learning in response to methamphetamine (Ehrman et al., 2006; Reed, Repaske, Snyder, Greengard, & Vorhees, 2002). Abnormalities of glutamatergic system have also been reported in the multiple brain areas after exposure to methamphetamine. An elevation of glutamate has been found in several brain regions such as striatum, hippocampus, ventral tegmental area and nucleus accumbens (Mark et al., 2007). The reduction of excitatory amino acid transporter 3 (EAAT3), glutamate transporter, expressed in rat brain after methamphetamine exposure leading to imbalance extracellular glutamate concentration (Kerdsan, Thanoi, & Nudmamud-Thanoi, 2012). Interestingly, the inhibitory GABAergic neurotransmission system, is an important

role in the control of functional normality other neurotransmission systems, have been shown in several previous studies for drug addiction processes, in particular synaptic connectivity. The GABA-A and GABA-B receptors were up-regulated in the prefrontal cortex of rat sensitized to methamphetamine (Wearne, Parker, Franklin, Goodchild, & Cornish, 2016). These changes results from a consequence of the reduction of GABA concentrations after methamphetamine exposure (Bu et al., 2013).

Dextromethorphan

Dextromethorphan (DXM) has been widely used as an antitussive or cough suppressant drug. Dextromethorphan is available in many over-the-counter cough and cold preparations worldwide (Hendrickson, & Cloutier, 2007). Non-medical use of dextromethorphan is prevalent among teenagers. The American Poison Control System reported a 300% increase in dextromethorphan cases in teenagers 13-19 years of age in 2000-2003. In Thailand, the most common using dextromethorphan is in a group situation where the drug is distributed among friends and several pills are mixed in soft drinks such as coke or orange juice. The chemical structure of dextromethorphan is similar to codeine, an opioid analgesic which is often abused due to euphoric side effect (Figure 5). Dextromethorphan is a more potent antagonist of the voltage-dependent calcium channels or glutamate receptor *N*-methyl-D-aspartic acid (NMDA) that similarly related with psychotropic effects of ketamine and phencyclidine (Mayhew, 2007).

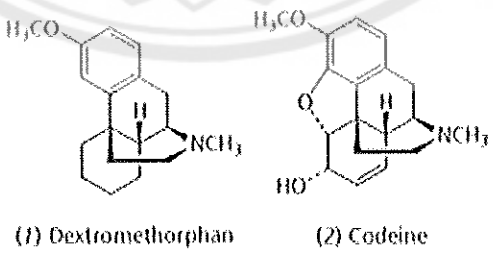


Figure 5 Chemical structures of dextromethorphan and codeine

Source: https://www.chemistryworld.com/news/cold-comfort/3001045.article

Effects of Dextromethorphan

The pharmacodynamic effects of dextromethorphan may relate to addictive properties and toxicity. Taken together, the human and non-human data suggest that dextromethorphan is psychoactive and might contribute to the behavioral effects experienced by recreational dextromethorphan users. The effects of dextromethorphan can produce the symptoms similar to psychotic disorders including, auditory hallucination and paranoid (Lachover, 2007). Moreover, dysphoria and depression are presented in the withdrawal period of dextromethorphan abuse (Wolfe, & Caravati, 1995). In human subject, high doses of dextromethorphan resulted in acute cognitive impairments (Carter et al., 2013). At very high doses of dextromethorphan, it can also produce sensation such as euphoria, distorted perception, and dissociative anesthesia that are seen with other NMDA antagonists such as phencyclidine and ketamine (Banken & Foster, 2008; Romanelli & Smith, 2009). The animal models have demonstrated adverse effects of dextromethorphan. For instance, an up-regulation of tyrosine hydroxylase gene (enzyme of dopamine synthesis) found in the midbrain dopamine neurons that may lead to the rewarding effect (Zhang et al., 2001). A previous study presented that dextromethorphan impaired the hippocampal long-term potential and learning (Krug, Matthies, Wagner, & Brodemann, 1993). In another study, the dose-dependent of dextromethorphan in rat were found in the memory impairment, spatial memory using the Morris water maze (Bane, Rojas, Indermaur, Bennett, & Avery, 1996). These cognitive impairments appeared to become permanent that the rat showed water maze impairment 18 months following dextromethorphan exposure during postnatal days 28-37 (Zhang et al., 2006). Inhibition of the phosphorylation of cAMP response-element-binding protein (p-CREB) by the NMDA receptor antagonist has been reported to induce memory impairment (Pittenger et al., 2002). The inhibition of CAMKII (calcium calmodulin-dependent kinase II) -CREB signaling pathway in hippocampal and prefrontal cortex results from the dextromethorphan exposure that leads to impairment of memory similar to morphine-like effect (Ghasemzadeh, & Rezayof, 2016). Moreover, dextromethorphan is suggested to be dissociative hallucinogen that can act as a nonselective serotonin reuptake inhibitor (Schwartz, Pizon, & Brooks, 2008). Vollenweider and Kometer (2010) proposed that the hallucinogen increases glutamate

levels in the prefrontal cortex via serotonin receptor localized on pyramidal cells in the cortex. The changes in glutamate neurotransmission in the brain may be responsible for cognitive and perceptual effects of hallucinogens.

Brain regions and drug addiction

There have been many studies indicating abnormalities of multiple brain regions in drug addiction. Mechanisms of drug addiction have been suggested to involve many brain regions such as frontal cortex, limbic system and basal ganglia. Some of these brain regions such as prefrontal cortex, hippocampus and amygdala have been associated with functions such as memory, motivation, and executive control. However, there are other brain regions such as prefrontal cortex, hippocampus, and amygdala that have also been reported to be involved in symptoms of drug addiction (Belujon, & Grace, 2011; Wearne et al., 2015). The cerebellum has been reported in related to drug-induced homeostatic adaptations (tolerance and physical dependence) (Edwards, & Rizk, 1981). Both tolerance and physical dependence are considered to be primary symptoms of addiction. Moreover, there has been investigated molecular and structural plasticity in the cerebellum of sensitized mice (Vazquez-Sanroman, Carbo-Gas, et al., 2015; Vazquez-Sanroman, Leto, et al., 2015). These studies have led to the hypothesis that the cerebellum dysfunction may involve in mechanism of drug addiction and relevant to addictive behaviors. Therefore, this section points out to the specific brain region particularly cerebellum in drug addiction models.

Abnormalities in cerebellum related with drug addiction

Cerebellum is a part of the brain that plays a critical role in motor coordination, including postural control, eye movements and motor learning. Previous research investigations of addictive drug have implicated the cerebellum in addiction (Miquel, Toledo, Garcia, Coria-Avila, & Manzo, 2009; Strick, Dum, & Fiez, 2009). Much evidence revealed the involvement of the cerebellum in emotional memory, planning, and decision making (Moers-Hornikx et al., 2009; Rosenbloom, Schmahmann, & Price, 2012; Schutter, & van Honk, 2006). Furthermore, a previous study has been shown the cerebellum promoting the cocaine-addicted behaviors

(Anderson et al., 2006). Human neuroimaging study in cocaine addicts and alcoholics users has shown the activation of cerebellum during additive process (Moulton et al., 2014). The dopaminergic projection from ventral tegmental area has been investigated innervation to the cerebellar cortex may be exert co-function of the cerebrum and cerebellum by promoting the execution, co-ordination of voluntary movement, or cognitive function (Ikai et al., 1994; Ikai et al., 1992). The recent study has supported the function of cerebellum implicated in cognitive functions (Locke et al., 2018). In addition, studies have observed the cerebellar abnormalities in subjects with obsessive- compulsive disorder and attention deficit hyperactivity disorder (Seidman et al., 2011; Zarei et al., 2011). Bostan, & Strick (2010) identified the cerebellum that drive a role in reward by sharing interconnection with dopaminergic system in the basal ganglia. In opioid-addicted patients, it has been reported the implication of opioid addiction and the functional changes among the cerebellum, nucleus accumbens and amygdala connectivity leading to dysregulation of reward and motivational functions (Upadhyay et al., 2010).

As mentioned above, the cerebellum is possibly a key modulator that influences an addiction. Such studies have shown neuroimaging of the cerebellum in the addicted subjects that associated with addiction/ addictive disorder. Addictive drugs may lead to disturbances in key signaling systems associated with addiction. There are evidence to support dysfunction of multiple neurotransmitter signaling systems in cerebellum including gamma-aminobutyric acid (GABA)ergic and glutamatergic systems (Fatemi, Folsom, Kneeland, & Liesch, 2011; Fatemi, Folsom, Rooney, & Thuras, 2013).

Effects of drug addiction on neurotransmitter systems

Much evidence indicated that the mechanisms underlying drug addiction are complex cascade of neurotransmitter systems. Changes in dopamine levels in the brain have been found after exposure to addictive drugs. In addition to dopamine, other neurotransmitters are affected by addictive drugs, including glutamatergic and gamma-aminobutyric acid (GABA)ergic systems.

Effects on dopaminergic neurotransmitter system

Dopamine is the neurotransmitter that has been implicated in the process of drug addiction. The abnormality functions of dopaminergic transmission has been observed in animal exposed to addictive drug, particularly disruption of dopamine transporter, consequently increasing extracellular dopamine concentrations in limbic regions including nucleus accumbens (Howell, & Kimmel, 2008). Animals behaviorally sensitized to amphetamine, cocaine or nicotine show enhanced dopamine release in nucleus accumbens in response to addictive drug exposure (Kalivas, & Duffy, 1990; Robinson, Jurson, Bennett, & Bentgen, 1988; Shoaib, Benwell, Akbar, Stolerman, & Balfour, 1994). Dopamine neuron excitability is enhanced in the ventral tegmental area (dopamine projection to nucleus accumbens) in animals with a history of repeated exposure to amphetamine, cocaine or ethanol. Nicotine increases dopamine levels by directly stimulating ventral tegmental area dopamine neurons through nicotinic receptor (Maskos et al., 2005). In addition to changes in neurotransmitter release, addictive drugs have been observed a disruption of dopaminergic markers including dopamine synthesizing enzymes, dopamine transporters (Segal et al., 2003) and dopamine receptors (Riddle, Fleckenstein, & Hanson, 2006).

Effects on glutamatergic neurotransmitter system

Much evidence suggested that glutamate excitatory neurotransmitter can be modulated by chronic drug use at a variety of levels. The glutamate neurotransmitter system has been found to be disrupted after methamphetamine exposure resulting in an increase in extracellular glutamate levels (Mark et al., 2007). Previous reports indicated that dopamine neurons in ventral tegmental area were responsive to glutamate and enhanced dopamine release in the nucleus accumbens during drug addictive process (Tzschentke, & Schmidt, 2003). An elevation of glutamate levels in the ventral tegmental area has also revealed in cocaine sensitized animals (Kalivas, & Duffy, 1998). The dopamine releasing effect of glutamate in the nucleus accumbens have been found mediated by AMPA and NMDA glutamate receptor (Youngren, Daly, & Moghaddam, 1993). It has also been observed an upregulation of NMDA receptor protein expression in frontal cortex after repeated methamphetamine exposure (Kerdsan, Thanoi, & Nudmamud-Thanoi, 2009). Injection of the NMDA antagonist

into nucleus accumbens impaired learning in the acquisition to obtain food reward (Kelley, Smith-Roe, & Holahan, 1997). Disrupted glutamate excitatory amino acid transporter (EAAT) function is linked to a variety of excitotoxicity-relate disease, including drug addictive disorder. Downregulation of EAAT2 protein expressions have been shown in the brains after repeated cocaine (Reissner et al., 2015) and nicotine (Gipson et al., 2013) to rats. Changes in EAAT3 transporter have also been observed by decreasing protein expression in prefrontal cortex following methamphetamine (Kerdsan et al., 2012) and opiates (Wu et al., 2013) exposure to rats.

Effects on GABAergic neurotransmitter system

GABAergic system is an important neurotransmitter system which is involved in the regulation of other neurotransmitter such as dopamine, glutamate and serotonin. GABA or gamma-aminobutyric acid is the major inhibitory neurotransmitter in the central nervous system. Previous studies investigated that GABAergic system related to drug addiction (Moeller, London, & Northoff, 2016; Veerasakul, Watiktinkorn, Thanoi, Reynolds, & Nudmamud-Thanoi, 2017). In particular, Steffensen et al (2009) reported that the activation of GABAergic neurotransmission is connected with mesolimbic dopamine neurotransmission during rewarding processes. The reduction of GAD67 protein, an enzyme of GABA synthesis was found in rat nucleus accumbens after methamphetamine administration (Zhang et al., 2006). In the human studies, the reduction of GAD67 protein and mRNA were observed in several brain areas including prefrontal cortex, hippocampus and cerebellum in postmortem brain of schizophrenic patients, suggesting the high content of GABA among addictive users (Akbarian, & Huang, 2006). Moreover, the concentrations of GABA were also reduced in the cerebrospinal fluid of schizophrenic patients (Orhan et al., 2018). GABA-A receptor deficits were reported to be associated with cognitive deficits, schizophrenia, and substance abuse (Jacob, Moss, & Jurd, 2008). Down-regulation of GABA transporter 1 (GAT1) protein and mRNA expressions in the prefrontal cortex has been reported in postmortem of schizophrenic patient (Lewis, & Moghaddam, 2006). Furthermore, the impairment of GABAergic neurons have been found in the methamphetamine psychosis (Hsieh, Stein, & Howells, 2014). Likewise the reduction of subgroups of GABAergic neurons included

parvalbumin, calretinin and calbindin, have shown in rat frontal cortex and hippocampus following methamphetamine exposure to addiction (Veerasakul et al., 2016). These subgroups of inhibitory neurons are defined as the calcium binding proteins (CaBPs) that regulates by intracellular calcium level (calcium homeostasis) and participates in the cellular functions by promoting calcium ion transporters across cell membranes or calcium ion modulated sensors. These proteins interact with calcium by promoting many neurobiological processes including the development of addictive process similar to the psychosis. Dysfunction of selective subgroups of GABAergic neurons have revealed in the frontal cortex and hippocampus of schizophrenic patients (Nakazawa et al., 2012; Zhang, & Reynolds, 2002).

Treatment of addiction

A variety of addiction treatments is mostly increase the level of GABA in the brain. GABAergic medications including gabapentin, tiagabine, lamotrigine, topiramate and pregabalin have been mentioned as possible treatments in various types of addictive drugs including alcohol, stimulants and opioids. Their ability increase the action of GABA contributing to treat seizures, sleep disorders, anxiety or mood disorders. Moreover, the benzodiazepines (BDZ) class including diazepam which act on the central nervous system by enhancing GABA activity via binding to BDZ site on the GABA-A receptor, facilitating chloride ion influx (Figure 7). Their action enhances GABA's inhibitory effects resulting in an overall decrease in neuron activity. Diazepam is mainly used to treat anxiety, sleep disorder, seizure, and symptoms of alcohol withdrawal. Adverse effects of diazepam include amnesia, ataxia, depression, confusion and sedation. Long-term use of diazepam can cause tolerance, physical dependence, substance use disorder, and withdrawal symptoms. Diazepam can also impair short-term memory and learning of new information. In addition, a previous study observed the reward behaviour in rats after diazepam exposure (Thiebot, Soubrie, & Simon, 1985).

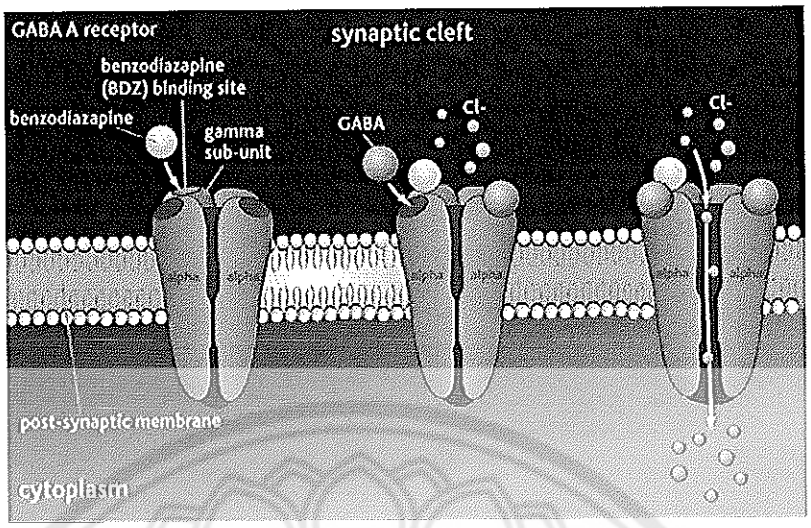


Figure 6 Schematic mechanism of diazepam drug

Source: https://innovareacademics.in/journals/index.php/ijap/article/download/27975/15716/137589

Therefore, the alternative treatments have been studied as a potential protective effect for reducing the side effects. GABA supplement is used widely treatment for stress, anxiety, nervousness, and depression. A previous study has been reported that GABA can decrease blood pressure and relieve depressive score (Kayahara, Tsukahara, & Tatai, 2001). GABA is found in chicken, fish, eggs, dairy products, beans, and pre-germinated brown rice (PGBR). During germination has higher nutritional components such as magnesium, potassium, zinc, dietary fiber and amino acids and GABA (Moongngarm, & Saetung, 2010). In human, improved depression, anger-hostility and fatigue scores were observed in the maternal PGBR diet group, resulting in depletion of total mood disturbance (Sakamoto et al., 2007). The physiological effects of PGBR administration in animals has been reported to anti-diabetic, hypocholesterolemic and anti-cancer (Cho, & Lim, 2016). A previous study found a protective effect of PGBR on amyloid β-peptide induced neurotoxicity of a neuroblastoma cell line (Soi-ampornkul, 2013). The preventative effect of PGBR was found in β-amyloid induced learning and memory deficits in mice (Mamiya et al., 2004). PGBR can also improve learning and memory deficits induced by lead in rats

(Zhang et al., 2010). Furthermore, PGBR has a protective effect as an improvement of depressive behaviours in mice (Mamiya et al., 2007).

This study used the PGBR with strain of *Oryza sativa var. glutisa* which is known as purple glutinous rice. The purple pigmented rice bran contains high amounts of anthocyanin. The PGBR purple glutinous rice has effectively reduced the depressive activity in rat (Thaweethee, Unpublished doctoral dissertation) and reduced addictive behaviour in rats (Veerasakul, Unpublished doctoral dissertation). Therefore, the understanding of molecular mechanisms of PGBR purple glutinous rice can be developed therapeutic strategies for drug addiction.

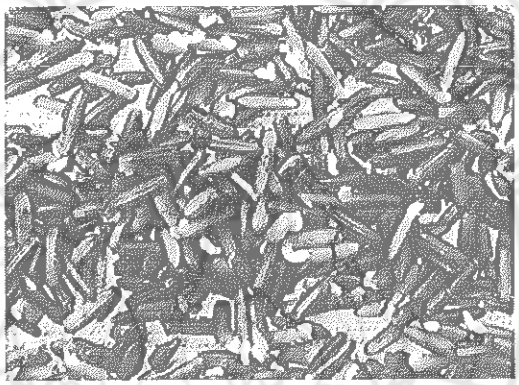


Figure 1 Pre-germinated purple glutinous rice

Proteomics

Proteomics technique allows the investigation of protein profiling and protein identification in the perturbation of states, such as disease states or drug effects. The application of proteomics techniques to neuroscientific research is starting to provide an understanding of nervous system structure and function. A proteomic approach serves to drug-induced changes in a specific proteome. In addition, applying proteomics becomes better understand their mechanism of action. Interestingly, the advantage of proteomics techniques examine in the process of drug addiction. There are several advantages to using Liquid chromatography tandem-mass spectrometry (LC-MS/MS) technique for protein identification. This method provided a high level of certainly in protein identification. Mass spectrometry-based proteomics has been applied to amphetamine, cocaine and methamphetamine administration which

identified a large of proteins that persist to abstinence (Lull et al., 2009; Brenan et al., 2010; Charntikov et al., 2015). Using proteomic techniques, changes in energy metabolism, oxidative stress, protein modification and degradation have been shown in the rat following methamphetamine administration (Iwazaki et al., 2006; Faure et al., 2009). Previous studies using neurotoxic effect doses of methamphetamine showed differential expression of protein involved in oxidative stress, mitochondrial dysfunction, cell cytoskeletal and apoptosis (Li et al., 2008; Faure et al., 2009; Kobeissy et al., 2010). Proteomics analysis revealed the effects of methamphetamine sensitized rats including synaptic regulation, protein phosphatase signaling, mitochondrial function and alterations to the inhibitory GABAergic network in the prefrontal cortex (Wearne et al., 2015). Moreover, the proteome study in the prefrontal cortex has indicated alteration of protein after methamphetamine administration that is relevant to neurotoxicity (Kobeissy et al., 2008; Faure et al., 2009).

CHAPTER III

RESEARCH METHODOLOGY

This study comprises of four parts as follows: (1) animal models of drug addiction, (2) tissue preparations for proteomics analysis and western blotting, (3) proteomics analysis of protein expression profiles and (4) western blot analysis of protein expression changes. Protocols for experimental study were approved by the Animals Research Committee of Naresuan University, Thailand.

Animal models of drug addiction

Animal models of drug addiction were divided into two models as follows:

Animal model of METH addiction

Animals

Male Sprague-Dawley rats weighing 200-250 g were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. Animals were housed in cages and maintained at 24±1°C under a 12-h light/dark cycle with free access to food and water. All animal procedures were carried out in compliance with Mahidol University Code of Practice the National Institutes of Health Guidelines for treatment of laboratory animals. Animals were divided into two groups; control (n = 10) and escalating dose METH binge (ED-Binge, n = 10) groups.

Methamphetamine administration

D-METH hydrochloride (Alletch, palatine & DC, IL, USA) was used for the experiment with permission from the Thai Ministry of Public Health. METH was dissolved in saline and administered intraperitoneal (i.p.) injections. Animals were intraperitoneally injected (i.p.) with saline or METH as previously described (Segal et al., 2003; Veerasakul et al., 2016). Animals were exposed to ED phase consisting of three injections per day at 3-h intervals of saline or gradually increasing doses of METH at the initial dose 0.1 mg/kg up to 4.0 mg/kg for 14 days. On the day following the ED pretreatment, animals were exposed to binge treatment consisting of four successive injections at 2-h intervals of 6.0 mg/kg METH, while groups of control

animals were received equivalent injections of saline. All animals were sacrificed by cervical dislocation. The cerebellar tissues were collected and were stored at -80°C until used.

Table 2 Schedule of methamphetamine administration

Day	Time		
	07.30	10.30	13.30
	METH dose (mg/kg)		
Day 1	0.1	0.2	0.3
Day 2	0.4	0.5	0.6
Day 3	0.7	0.8	0.9
Day 4	1.0	1.1	1.2
Day 5	1.3	1.4	1.5
Day 6	1.6	1.7	1.8
Day 7	1.9	2.0	2.1
Day 8	2.2	2.3	2.4
Day 9	2.5	2.6	2.7
Day 10	2.8	2.9	3.0
Day 11	3.1	3.2	3.3
Day 12	3.4	3.5	3.6
Day 13	3.7	3.8	3.9
Day 14	4.0	4.0	4.0
Day 15	(4 x 6.0 mg/kg; 2 h intervals)		

Source: Segal et al., 2003; Veerasakul et al., 2016

Animal model of dextromethorphan addiction

Animals

Male Sprague-Dawley rats weighing 200-250 g were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand.

The animals were housed in cages and maintained at 24±1°C under a 12-h light/dark cycle with free access to food and water. All animal procedures were carried out in compliance with Mahidol University Code of Practice the National Institutes of Health Guidelines for treatment of laboratory animals.

Dextromethorphan and substances administration

Dextromethorphan hydrobromide was purchased from Sigma-Aldrich® Lot#090M1298V. Diazepam (psychotropics drug) was obtained from Naresuan Hospital. Pre-germinated brown rice (PGBR) powder was supplied by the Laboratory of Faculty of Agriculture Natural Resources and Environment, Naresuan University. Briefly, PGBR with strain of *Oryza sativa var. glutinosa*, which obtain from KhekNoi, KhaoKho, Phetchabun (Thailand) was soaked for 24 hours until germinated. Subsequently, PGBR was dried and produced to powder. The substances in PGBR were inspected by the Central Laboratory (Thailand) Co., Ltd. as shown in Table 3. Synthetic GABA was purchased from Sigma Chemical Company, St. Louis, USA. Amount of GABA standard were equalled with the GABA content found in PGBR.

Table 3 The substances in pre-germinated brown rice

Substances	Volumes (mg/ 100g)
GABA	16.51
Gamma oryzanol	29.61
Alpha tocopherol (Vitamin E)	0.91
Pyridoxine (Vitamin B6)	0.11
Thiamine (Vitamin B1)	0.05

The animals were divided into six groups (Figure 8); control (C), dextromethorphan (D), withdrawal (DW), pre-germinated brown rice (DR), GABA standard (DG) and diazepam (DD). The animals of each group of C, D, DW, DR, DG and DD have seven animals. Control group (C): animals were received saline i.p. injection for 15 days and were received distilled water by oral administration via gavage for 60 days. Animals in DXM, DW, DR, DG and DD groups: animals were received DXM for 15 days (30 mg/kg, i.p.), DXM animals were then sacrificed.

Animals in the DW group were divided into three subgroups; DW15, DW30 and DW60 and were withdrawn for 15, 30 and 60 days, respectively. Animals in the DR group were divided into three subgroups; DR15, DR30 and DR60 and were received 5 g/kg PGBR by oral administration via gavage for 15, 30 and 60 days, respectively. Animals in DG group were divided into three subgroups; DG15, DG30 and DG60 and were received 0.8 mg/kg GABA standard by oral administration via gavage for 15, 30 and 60 days, respectively. Animals in DD group were divided into three subgroups; DD15, DD30 and DD60 and were received 10 mg/kg diazepam standard by oral administration via gavage for 15, 30 and 60 days, respectively. All animals were sacrificed by cervical dislocation. The cerebellar tissues were collected and were stored at -80°C until further assayed.

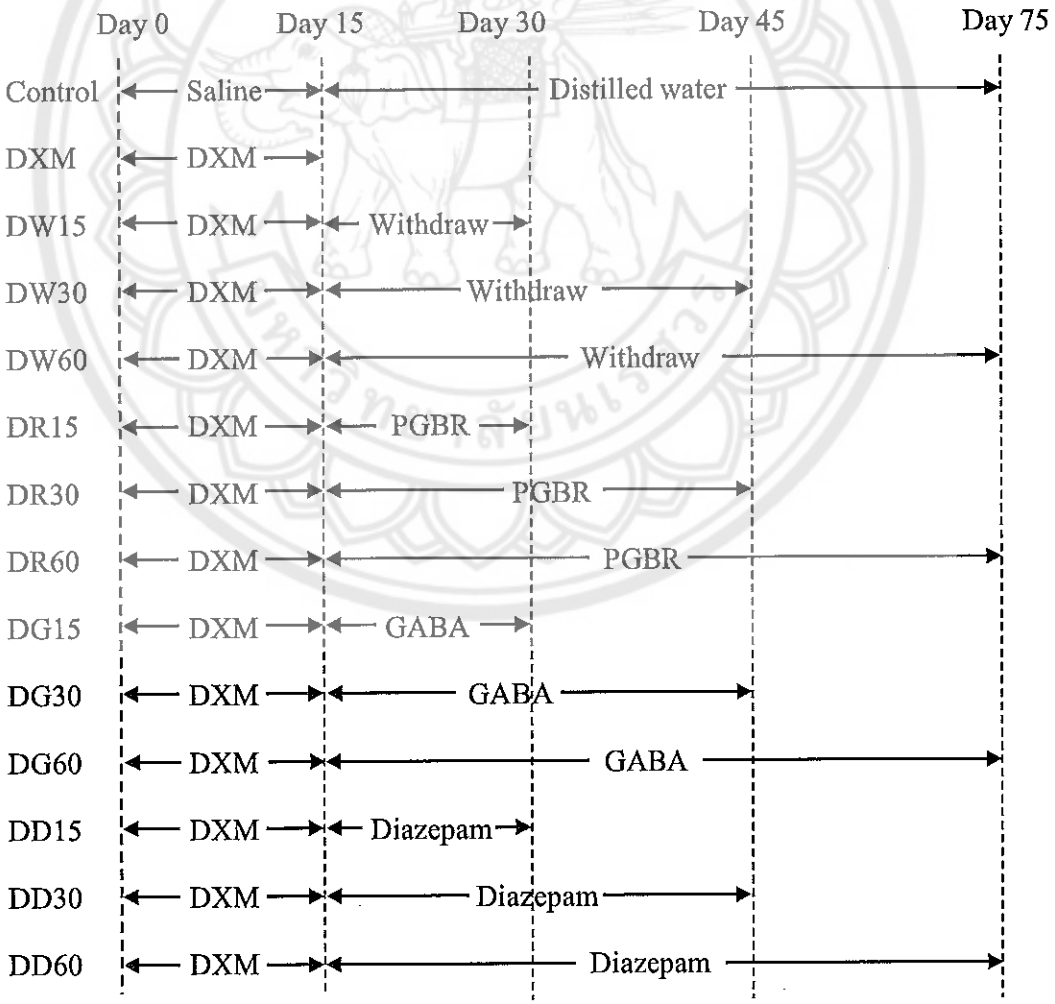


Figure 8 The schematic diagram of DXM and substances administration

Tissue preparations for proteomics analysis and western blotting

Protein extraction

Cerebellar tissues were homogenized in homogenate buffer (5 mM Tris-HCl and 20 mM NaCl, pH 8.0). Tissue homogenates were then centrifuged at 14,000 rpm for 10 min at 4°C. The pellets were collected and homogenized again in lysis buffer (50 mM Tris-HCl, 0.15 M NaCl, 0.1% SDS, and 0.25% sodium deoxycholate, and 1% protease inhibitor cocktail (P8340, Sigma-Aldrich)). Cerebellar lysates were kept on ice for 1 h to complete tissue lysis.

Protein concentration

Protein concentration was determined using the BCA (bicinchoninic acid) protein assay (Instruments of BCA Protein Assay Kit, Thermo Scientific, Pierce, Rockford, IL., USA) with bovine serum albumin (BSA) as the protein standard. BSA protein concentrations for a standard curve are between 0.25 mg/ml and 1.0 mg/ml. The homogenate sample was diluted in lysis buffer and measurements were performed in triplicate. BCA working reagent containing a combination of 50 part of reagent A and 1 part of reagent B was added to the sample and protein standard. The solution mixture was mixed and incubated by a block heater (Labnet) at 37°C for 30 min. The absorbance was read at a wavelength of 562 nm by a microplate reader (Labsystem iEMS Reader MF). The samples of unknown concentration were determined in comparison with a standard curve of known protein concentrations.

Proteomics analysis of protein expression profiles

In-solution trypsin digestion

Protein samples of each group were pooled (n = 3) for LC-MS/MS analysis. A total of 5 μg of lysate samples was reduced by the addition of 10 mM dithiothreitol in 10 mM ammonium bicarbonate (NH_4HCO_3) and incubation at 56°C for 1 h, and then alkylated by the addition of 30 mM iodoacetamide in 10 mM NH_4HCO_3 and incubation at room temperature in the dark for 1 h. To perform in-solution digestion, the proteins were digested with trypsin (Promega, Madison, WI, USA) overnight at 37°C. The protein-to-enzyme ratio was 20:1, and protein digestion was stopped by adding formic acid at a final concentration of 0.1%. The tryptic peptides were purified with C18 Tips (Merck Millipore, Darmstadt, Germany) as follows: C18 Tips were

wetted with 70% acetonitrile (ACN)/0.1% FA, equilibrated with 0.1% FA, bind up the sample, and washed with 5% ACN/0.1% FA. The purified peptides were then eluted with 70% ACN/0.1% FA and transferred to a new low binding microcentrifuge tube. Samples were dried and resuspended in 0.1% FA. The peptide sample was transferred to the vial for injecting or keeping at -20°C prior to further mass spectrometric analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The digested peptide solutions were analyzed with Impact II UHR-TOF MS System (Bruker Daltonics Ltd., Germany) coupled to a nanoLC system: UltiMate 3000 LC System (Thermo Fisher Scientific, USA). Peptides were separated on a nanocolumn (PepSwift monolithic column 100 µm i.d. x 50 mm). Eluent A was 0.1% formic acid and eluent B was 80% acetonitrile in water containing 0.1% formic acid. Peptide separation was achieved with a linear gradient from 10% to 45% B for 8.5 min at a flow rate of 1 µl/min, including a regeneration step at 90% B and an equilibration step at 1% B, one run took 20 min. Peptide mass spectra were acquired in a data-dependent AutoMS mode by selecting most abundant precursor ions in the 3-second cycle for fragmentation. The mass range of the MS scan was set to extend from 150 to 2200 *m/z*. The MS/MS data were submitted for a database search using the Mascot software (Matrix Science, London, UK) (Perkins, Pappin, Creasy, & Cottrell, 1999). The data were searched against the NCBI database for protein identification. Database interrogation were; taxonomy (*Rattus*); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (±0.6 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages.

Protein quantitation and identification

For protein quantitation, DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare) was used (Johansson et al., 2006; Thorsell, Portelius, Blennow, & Westman-Brinkmalm, 2007). Acquired LC-MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ions signal intensities in MS mode. The analyzed MS/MS data from DeCyderMS were submitted for a database search using the Mascot software (Matrix Science, London, UK) (Perkins et al., 1999).

The data were searched against the NCBI database for protein identification. Database interrogation were; taxonomy (*rattus*); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (±0.6 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages. The maximum value of each group was used to determine the presence or absence of each identified protein.

Differentially expressed proteins were statistically analyzed by Multi Experiment Viewer (MeV) software (Howe E., 2010). The identified proteins alterations in animal models of METH and DXM addiction were statistically analyzed by MeV software. Significant proteins changes between control and drug-treated groups were determined by t-test at p-value level of 0.05 and a threshold cut-off of 1.3-fold change. Protein function family classification, including biological process, molecular function, protein class and cellular component were performed based on Gene Ontology (GO) terms using Protein Analysis Through Evolutionary Relationships (PANTHER) classification system which available at http://www.pantherdb.org (Mi et al., 2017).

Western blot analysis of protein expression changes

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Calbindin differentially-expressed protein was chosen for validation of the quantitative LC-MS/MS data using Western blotting. After protein extraction (described above), the homogenate sample 20 ug was dissolved in lysis buffer and 2X Laemmli Sample Buffer. The samples were boiled at 100°C for 5 min to completely denature the protein. The samples were centrifuged at 3,000 rpm for 3 min, and the supernatant were then loaded on 4% stacking gel and 12.5% separating gel using constant 90 volts. The BLUelf Prestained SDS-PAGE ladder (GeneDirex) was loaded in parallel with the sample to visualize the molecular weight during electrophoresis. After electrophoresis, the proteins on gel were transferred onto 0.45µm polyvinylidene fluoride (PVDF) membranes (GE Healthcare Life Sciences Corp., Piscataway, NJ, USA) using semi-dry transfer apparatus (Amershams Biosciences Corp., Piscataway) at 120 mA and 25 V for 90 min. Following the transfer, the gel was stained with

Coomassie Blue staining solution and was destained with destaining solution until transparent background, while the membrane was stained with Ponceau S solution (Sigma-Aldrich Co. LLC., St. Louis, Mo, USA) to confirm that proteins were completely transferred to a membrane. The membrane was washed with distilled water and allowed to dry at room temperature. The dried membranes were then kept at 4C until immunodetection.

Protein blotting for anti-calbindin and anti-β-actin antibody

The membrane was soaked by adding methanol and washed three times in TBST (Tris-buffered saline, 0.1% Tween-20). Following washing, the proteins on membranes were blocked with 5% skimmed milk in TBST for rabbit polyclonal anti-calbindin for 2 h at room temperature (RT) and 5% BSA in TBST for mouse monoclonal anti-β-actin for 2 h at RT. The membrane was then incubated with primary for anti-calbindin (dilution 1:4,000, C2724, Sigma-Aldrich Co. LLC., St. Louis, Mo, USA) for 3 h at RT and anti-β-actin (dilution 1:1,000, ab8226, abcam, UK) for 4 h at RT and overnight for 16 h at 4°C. After washed with TBST every 5 min for three times, the membrane was incubated with goat-anti-rabbit (1:400 dilution, BA-1000, Vector Laboratories Inc., Burlingame, CA, USA) for 1 h at RT and goat-anti-mouse (1:200 dilution, BA-9200, Vector Laboratories Inc., Burlingame, CA, USA) for 2 h at RT. The membrane was washed three times in TBST and incubated with avidin-biotinylated horseradish complex (ABC) kit (dilution 1:100, PK4000, Vector Laboratories Inc., Burlingame, CA, USA) for 1 h at RT. The membrane was washed in TBST and TBS for three times and two times, respectively. Eventually, the proteins on the membrane of interest were visualized by peroxidase substrate, 3, 3', 5, 5' - tetramethylbenzidine (TMB) (Promega Corporation, Madison, WI, USA) and stopped reaction with distilled water for 5 min. The detected membranes were scanned and integrated optical density was measured by ImageJ software (base on NIH). All specific bands were measured with the same dimension to obtain their integrated optical density (Nudmamud-Thanoi, & Reynolds, 2004). Expressions of calbindin were normalized against that of β-actin.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was done using SPSS 17.0 (SPSS Inc., USA) by one-way analysis of variance (ANOVA) followed by Dunnett test, and differences between groups were using independent samples t-test. $P < 0.05$ was considered statistically significant for all data analyzed.

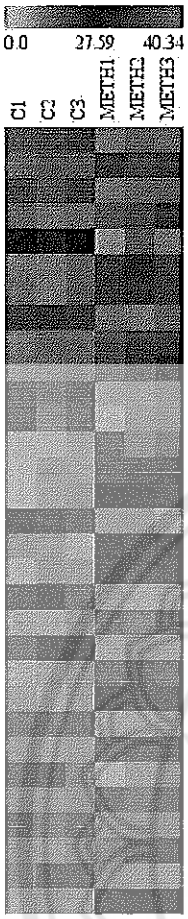
CHAPTER IV

RESULTS

Protein expression profiles in rat cerebellum after addictive drugs exposure

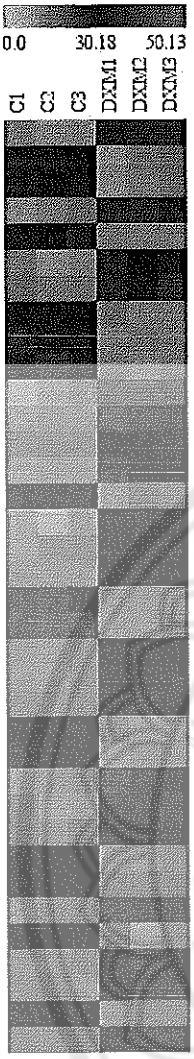
The LC-MS/MS analysis revealed a total of 1,273 differentially expressed proteins between control and METH groups (see appendix Figure 27), of which 31 proteins were differentially expressed at a significance level of $p<0.01$. A total of 1,758 proteins were differentially expressed between control and DXM groups (see appendix Figure 28). The expression pattern profiles of 31 and 36 differentially changed proteins in METH and DXM compared to control was shown in Figure 9 and Figure 10, respectively. These proteins in each model were classified by the PANTHER software and displayed as a pie chart (Figure 11, 12). According to the biological process, the results showed that categories of the biological process found to be predominantly in the cellular process of METH (32.60%) and DXM (33.30%) groups as shown in Figure 11A and Figure 12A, respectively. Their child categories of the cellular process linked cell communication are divided into 2 groups including cell-cell signaling (28.60%; METH, 33.30%; DXM) and signal transduction (71.40%; METH, 66.70%; DXM). Moreover, the most of proteins were mapped to protein classes consisted of enzyme modulator (18.80%; METH, 15.80%; DXM), transferase (18.80%; METH, 15.80%; DXM), calcium-binding protein (12.50%; METH, 10.50%; DXM) and transporter (12.50%; METH, 15.80%; DXM) as shown in Figure 11B and Figure 12B.

This study has focused on the cell-cell signaling and signal transduction categories. A value of protein expression as fold changes with statistically significant difference between control and addictive drugs treated-rats were derived from cell-cell signaling and signal transduction categories and presented in Table 4 (METH-treated rats) and Table 5 (DXM-treated rats).



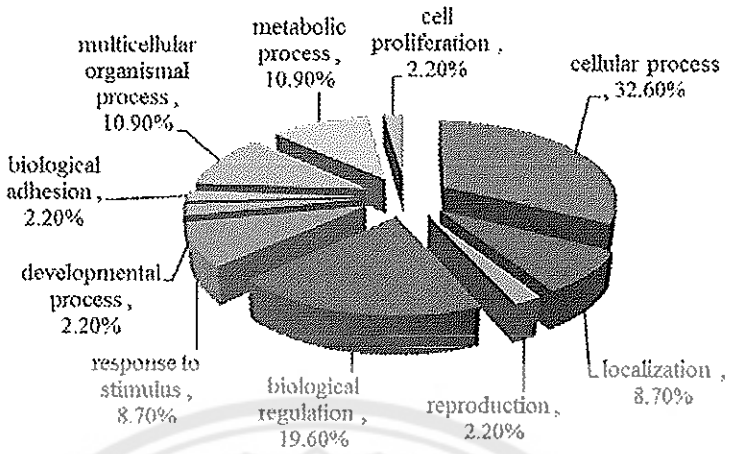
sodium/potassium-transporting ATPase subunit gamma isoform X2
usherin precursor
NUAK family SNF1-like kinase 2
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 4-like
neuronal acetylcholine receptor subunit alpha-10 precursor
heat shock cognate 71 kDa protein
78 kDa glucose-regulated protein precursor
alcohol dehydrogenase class-3
prostaglandin E2 receptor EP1 subtype
G protein-coupled receptor kinase 4
"1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase eta-2 isoform b"
syntaxin binding protein 3A
calbindin
serine/threonine-protein kinase SIK1
synaptotagmin-7 gamma isoform
"malate dehydrogenase, mitochondrial precursor"
epidermal growth factor receptor isoform 1 precursor
Protein shisa-6 homolog
coiled-coil domain-containing protein 38 isoform X4
calcium/calmodulin-dependent protein kinase type 1B
"cAMP-specific 3',5'-cyclic phosphodiesterase 4D"
E3 ubiquitin-protein ligase Rnf220
plexin-B3 precursor
mitogen-activated protein kinase kinase kinase kinase 1
"stress-70 protein, mitochondrial"
phosphatidylethanolamine-binding protein 1
paralemmin-3
dihydropyrimidinase-related protein 2
heat shock 70 kDa protein 12B isoform X2
beta-2-glycoprotein 1 precursor
excitatory amino acid transporter 1

Figure 9 Heatmap of significantly identified proteins after METH exposure

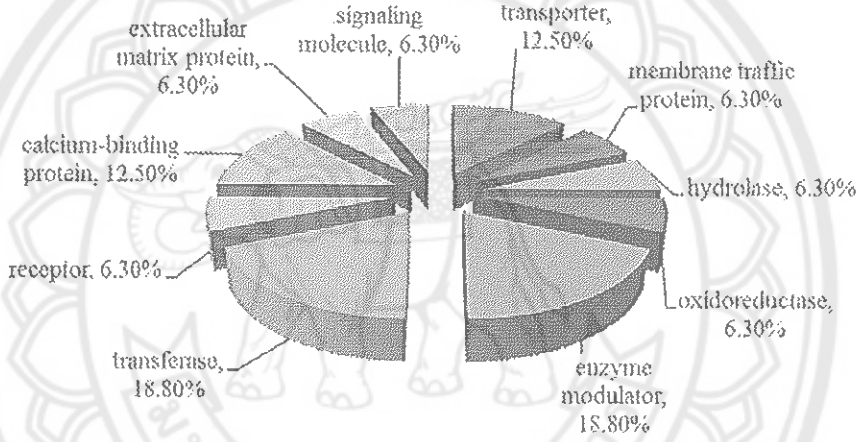


calcium/calmodulin-dependent protein kinase type 1B
fructose-bisphosphate aldolase C
neuronal acetylcholine receptor subunit alpha-10 precursor
usherin precursor
"1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase eta-2 isoform b "
"citrate synthase, mitochondrial precursor"
leucine carboxyl methyltransferase 1 isoform X1
"aspartate aminotransferase, mitochondrial "
WD repeat and SOCS box-containing protein 1 isoform X1
excitatory amino acid transporter 1
calbindin
synaptotagmin-7 gamma isoform
E3 ubiquitin-protein ligase listerin
plexin-B3 precursor
coiled-coil domain-containing protein 133 isoform X5
epidermal growth factor receptor isoform 1 precursor
Protein shisa-6 homolog
heat shock 70 kDa protein 1-like
Creatine kinase B-type
sodium/glucose cotransporter 1
78 kDa glucose-regulated protein precursor
G-protein coupled receptor 98 isoform X2
"NADH dehydrogenase (ubiquinone) complex I, assembly factor 6"
"malate dehydrogenase, mitochondrial precursor "
"cAMP-specific 3',5'-cyclic phosphodiesterase 4D"
metabotropic glutamate receptor 5-like
"NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial "
heat shock cognate 71 kDa protein
phosphatidylethanolamine-binding protein 1
beta-2-glycoprotein 1 precursor
triosephosphate isomerase
NUAK family SNF1-like kinase 2
clathrin heavy chain 1
prostaglandin E2 receptor EP1 subtype
syntaxin binding protein 3A
60S ribosomal protein L31-like

Figure 10 Heatmap of significantly identified proteins after DXM exposure

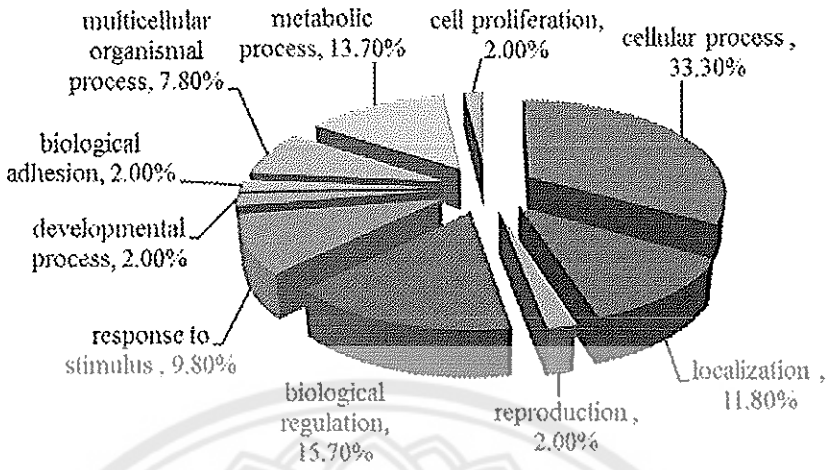


(A) Biological process

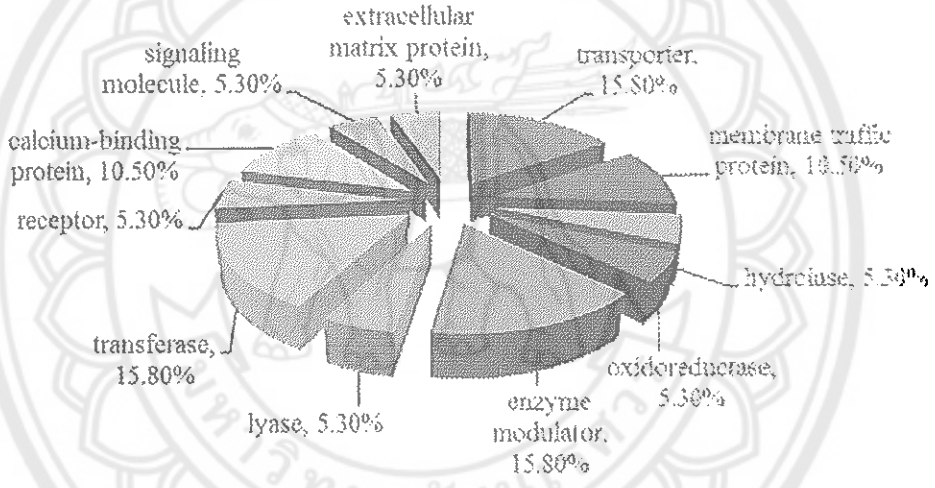


(B) Protein class

Figure 11 Protein classified in rat cerebellum after METH administration



(A) Biological process



(B) Protein class

Figure 12 Protein classified in rat cerebellum after DXM administration

Table 4 List of significantly identified protein after METH exposure

Protein name	Accession number	Fold change (log 2)	P-value
Cell-cell signaling			
calbindin	gi|6753242	2.11	0.005
excitatory amino acid transporter 1	gi|24233554	1.67	0.003
protein shisa-6	gi|123794581	1.64	0.002
neuronal acetylcholine receptor subunit alpha-10	gi|12018340	-1.51	0.006
Signal transduction			
plexin-B3	gi|209529650	1.63	0.004
G protein-coupled receptor kinase 4	gi|341940776	1.60	0.008
epidermal growth factor receptor	gi|46560582	1.61	0.002
serine/threonine-protein kinase SIK1	gi|148747268	1.83	0.005
NUAK family SNF1-like kinase 2	gi|110815882	-1.57	0.005
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase eta-2	gi|164565357	-1.72	0.004
phosphatidylethanolamine-binding protein 1	gi|84794552	-1.78	0.004
Others			
usherin	gi|145699097	1.37	0.001
heat shock cognate 71 kDa protein	gi|31981690	1.65	0.001
heat shock 70 kDa protein	gi|1147366048	1.63	0.008
synaptotagmin-7	gi|160948571	1.56	0.003
calcium/calmodulin-dependent protein kinase	gi|884859030	1.80	0.005
E3 ubiquitin-protein ligase Rnf220	gi|81911415	1.76	0.004
stress-70 protein, mitochondrial	gi|913506278	1.68	0.001
prostaglandin E2 receptor EP1 subtype	gi|507579036	1.70	0.003
78 kDa glucose-regulated protein	gi|254540168	1.64	0.002
NADH dehydrogenase [ubiquinone] 1 alpha	gi|861479060	1.67	0.004
paralemmin-3	gi|913517846	1.62	0.003
malate dehydrogenase	gi|31982186	-1.66	0.007
coiled-coil domain-containing protein 38	gi|1046835069	-1.78	0.001

Table 4 (Con.)

Protein name	Accession number	Fold change (log 2)	P-value
alcohol dehydrogenase class-3	gi|880945936	-1.63	0.002
cAMP-specific 3',5'-cyclic phosphodiesterase 4D	gi|57015263	-1.61	0.007
mitogen-activated protein kinase 1	gi|1104887319	-1.81	0.004
syntaxin binding protein 3A	gi|148670038	-1.80	0.005
beta-2-glycoprotein 1	gi|160358825	-1.79	0.002
dihydropyrimidinase-related protein 2	gi|731192474	-1.68	0.002
sodium/potassium-transporting ATPase	gi|984107881	-1.65	0.003

Table 5 List of significantly identified protein after DXM exposure

Protein name	Accession number	Fold change (log 2)	P-value
Cell-cell signaling			
excitatory amino acid transporter 1	gi|24233554	-1.86	0.003
calbindin	gi|6753242	2.58	0.002
protein shisa-6	gi|123794581	1.45	0.001
neuronal acetylcholine receptor subunit alpha-10	gi|12018340	-1.60	0.005
Signal transduction			
plexin-B3	gi|209529650	1.83	0.004
epidermal growth factor receptor	gi|46560582	1.87	0.006
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase eta-2	gi|164565357	-1.71	0.005
NUAK family SNF1-like kinase 2	gi|110815882	-1.55	0.006
phosphatidylethanolamine-binding protein 1	gi|84794552	-1.67	0.005
Others			
heat shock 70 kDa protein 1-like	gi|124339838	1.48	0.002
heat shock cognate 71 kDa protein	gi|31981690	1.53	0.002
synaptotagmin-7	gi|160948571	1.98	0.002
clathrin heavy chain 1	gi|51491845	1.72	0.003
usherin	gi|145699097	1.73	0.004
metabotropic glutamate receptor 5-like	gi|1147391889	1.77	0.005

Table 5 (Cont.)

Protein name	Accession number	Fold change (log 2)	P-value
E3 ubiquitin-protein ligase listerin	gi|198041681	1.47	0.001
78 kDa glucose-regulated protein	gi|254540168	1.55	0.003
G-protein coupled receptor 98	gi|1046874075	1.52	0.001
NADH dehydrogenase (ubiquinone) complex I	gi|395406791	1.50	0.003
NADH dehydrogenase (ubiquinone) iron-sulfur protein 3	gi|157817227	1.82	0.002
triosephosphate isomerase	gi|226958349	1.69	0.001
citrate synthase	gi|13385942	1.83	0.001
leucine carboxyl methyltransferase 1	gi|564330837	1.66	0.002
calcium/calmodulin-dependent protein kinase	gi|884859030	1.84	0.001
prostaglandin E2 receptor	gi|507579036	1.65	0.002
60S ribosomal protein L31-like	gi|672049066	1.56	0.002
fructose-bisphosphate aldolase C	gi|6978489	-1.59	0.007
malate dehydrogenase	gi|31982186	-1.58	0.008
coiled-coil domain-containing protein 138	gi|1046877484	-1.51	0.007
cAMP-specific 3',5'-cyclic phosphodiesterase 4D	gi|57015263	-1.76	0.003
syntaxin binding protein 3A	gi|148670038	-1.53	0.003
beta-2-glycoprotein 1	gi|160358825	-1.40	0.003
aspartate aminotransferase	gi|6754036	-1.59	0.004
WD repeat and SOCS box-containing protein 1	gi|1046849453	-1.78	0.002
creatine kinase B-type	gi|122065316	-1.64	0.005
sodium/glucose cotransporter 1	gi|261824023	-1.67	0.002

Protein expression profiles in rat cerebellum after drug withdrawal

The LC-MS/MS analysis revealed a total of 1,274 differentially expressed proteins among DXM, DW15, DW30 and DW60 groups (see appendix Figure 29), of which 27 proteins were differentially expressed at a significance level of $p<0.01$. The expression pattern profile of 27 differentially changed proteins was shown in Figure 13. These proteins in each model were classified by the PANTHER software and displayed as a pie chart (Figure 14). The differentially identified proteins were almost mapped in cellular process sub-categories of biological process (31.80%) as shown in Figure 14A. Cell communication (child categories of cellular process) category was divided into 2 groups including cell-cell signaling (30.00%) and signal transduction (70.00%). Furthermore, the most of proteins were mapped to protein classes consisted of enzyme modulator (14.30%), membrane traffic protein (14.30%), transferase (21.40%) and transporter (21.40%) (Figure 14B).

These identified proteins were derived from cell-cell signaling and signal transduction categories. A value of protein expression as fold changes with statistically significant difference between DXM and drug withdrawal groups was presented in Table 6.

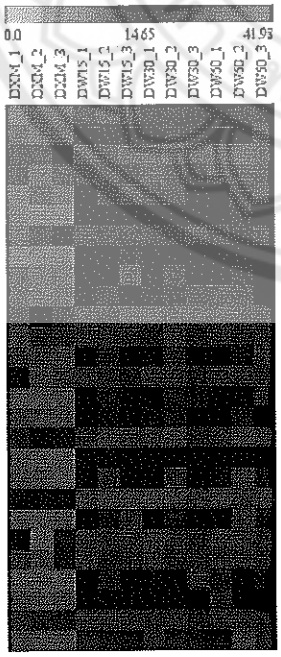


Figure 13 Heatmap of significantly identified proteins after drug withdrawal

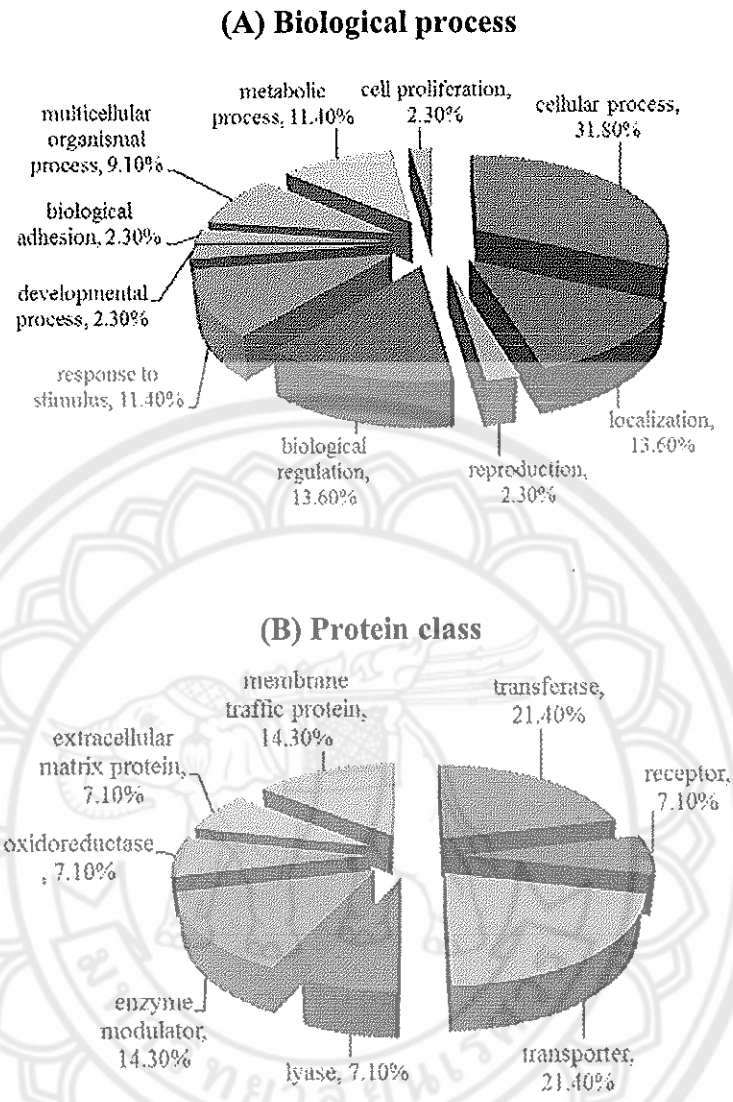


Figure 14 Protein classified in rat cerebellum after drug withdrawal

Table 6 List of significantly identified protein after drug withdrawal

Protein name	Accession number	Fold change (log 2)			P-value		
		DW15_1	DW15_2	DW15_3	DW15_1	DW15_2	DW15_3
Cell-cell signaling							
excitatory amino acid transporter 1	gi|24233554	1.44	1.47	1.582	0.006	0.005	0.003
neuronal acetylcholine receptor subunit alpha-10	gi|12018340	1.36	1.38	1.373	0.009	0.003	0.005
protein shisa-6	gi|123794581	-1.40	-1.56	-1.598	0.005	0.002	0.003
Signaling transduction							
plexin-B3	gi|209529650	-1.73	-1.59	-1.608	0.002	0.003	0.002
epidermal growth factor receptor	gi|46560582	-1.42	-1.41	-1.371	0.005	0.005	0.004
NUAK family SNF1-like kinase 2	gi|110815882	1.41	1.45	1.514	0.004	0.008	0.009
phosphatidylethanolamine-binding protein 1	gi|84794552	1.47	1.55	1.566	0.004	0.003	0.005
Others							
heat shock cognate 71 kDa protein	gi|31981690	-1.50	-1.59	-1.589	0.001	0.001	0.001
heat shock 70 kDa protein 1-like	gi|124339838	-1.35	-1.43	-1.439	0.002	0.003	0.002
synaptotagmin-7	gi|160948571	-1.50	-1.44	-1.497	0.003	0.003	0.004
NADH dehydrogenase (ubiquinone) complex I	gi|395406791	-1.38	-1.39	-1.485	0.001	0.002	0.003
usherin	gi|145699097	-1.57	-1.43	-1.660	0.002	0.003	0.003
clathrin heavy chain 1	gi|51491845	-1.57	-1.52	-1.523	0.002	0.002	0.002
E3 ubiquitin-protein ligase listerin	gi|198041681	-1.37	-1.43	-1.416	0.004	0.001	0.003
78 kDa glucose-regulated protein	gi|254540168	-1.38	-1.49	-1.605	0.001	0.001	0.004

Table 1 (Cont.)

Protein name	Accession number	Fold change (log 2)			P-value		
		DW15_1	DW15_2	DW15_3	DW15_1	DW15_2	DW15_3
G-protein coupled receptor 98	gi|1046874075	-1.34	-1.46	-1.494	0.003	0.002	0.008
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	gi|157817227	-1.43	-1.69	-1.686	0.002	0.002	0.001
malate dehydrogenase	gi|31982186	1.42	1.50	1.527	0.002	0.005	0.002
coiled-coil domain-containing protein 138	gi|1046877484	1.43	1.46	1.434	0.006	0.003	0.001
cAMP-specific 3',5'-cyclic phosphodiesterase 4D	gi|57015263	1.61	1.60	1.660	0.002	0.003	0.002
syntaxin binding protein 3A	gi|148670038	1.34	1.35	1.446	0.002	0.006	0.001
beta-2-glycoprotein 1	gi|160358825	1.32	1.36	1.370	0.002	0.002	0.001
sodium/glucose cotransporter 1	gi|261824023	1.34	1.42	1.460	0.003	0.007	0.009
creatine kinase B-type	gi|122065316	1.34	1.32	1.384	0.001	0.002	0.004
calcium/calmodulin-dependent protein kinase type 1B	gi|884859030	-1.52	-1.56	-1.721	0.002	0.002	0.002
citrate synthase	gi|13385942	-1.53	-1.59	-1.598	0.005	0.006	0.004
60S ribosomal protein L31-like	gi|672049066	-1.46	-1.46	-1.494	0.001	0.002	0.005

Protein expression profiles in rat cerebellum after PGBR, GABA standard and diazepam treatments

The LC-MS/MS analysis revealed a total of 1,440, 1,474 and 1,323 differentially expressed proteins among DXM, DXM-treated with PGBR, GABA and Diazepam (see appendix Figure 30-32), of which 31, 34 and 30 proteins were differentially expressed at a significance level of $p<0.01$ in treatment for 15, 30 and 60 days, respectively. The expression pattern profiles of differentially changed proteins were shown in Figure 15, 16, 17. These proteins in each model were classified by the PANTHER software and displayed as a pie chart (Figure 18, 19, 20). The significantly identified proteins during treatment for 15, 30 and 60 days were almost expressed 32.60%, 34.10% and 34.10% in cellular process sub-categories of the biological process function, respectively (Figure 18A, 19A, 20A). High percentages of proteins in cell communication sup-group of the cellular process were involved in cell-cell signaling (36.40%;treatments 15 days, 33.30%;treatment 30 days, 33.30%;treatments 60 days) and signal transduction (63.60%%;treatments 15 days, 66.70%;treatment 30 days, 63.60%; treatments 60 days). In addition, proteins in categories of the protein class were found to be the predominant child categories in transferase, transporter, membrane traffic protein and enzyme modulator of treatments for 15, 30 and 60 days as shown in Figure 18B, 19B and 20B, respectively.

The identified proteins alterations in an animal model of DXM addiction after PGBR, GABA standard and diazepam treatments were derived from cell-cell signaling and signal transduction categories. A value of protein expression as fold changes with statistically significant difference between addictive drugs- and different treatments periods exposed-rats were presented in Table 7-9.

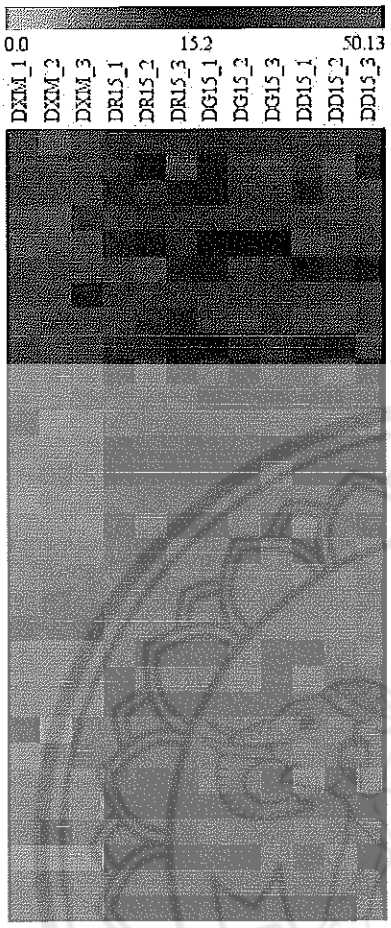


Figure 15 Heatmap of significantly identified proteins after treatment for 15 days

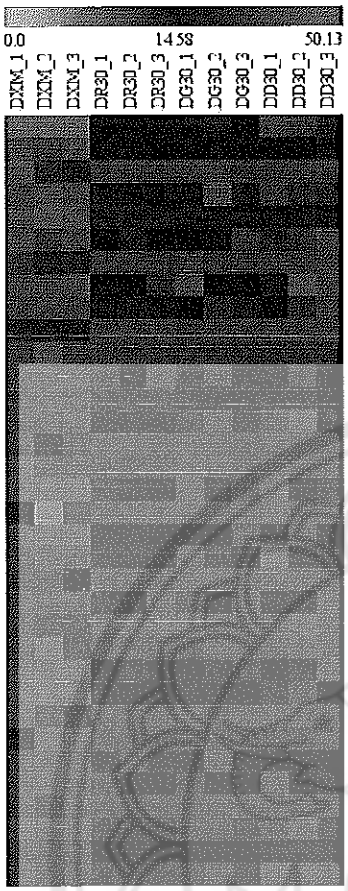


Figure 16 Heatmap of significantly identified proteins after treatment for 30 days

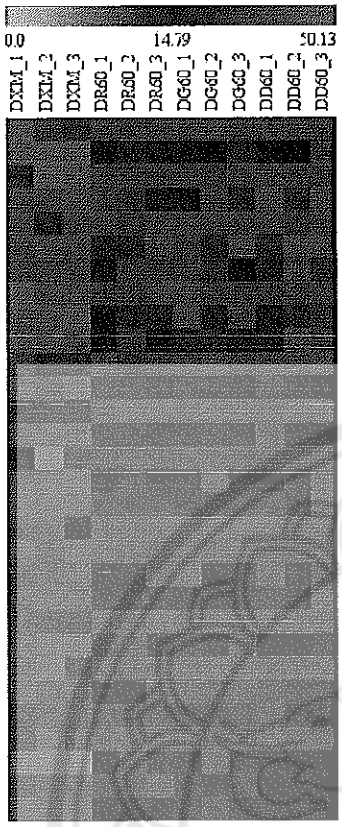


Figure 17 Heatmap of significantly identified proteins after treatment for 60 days

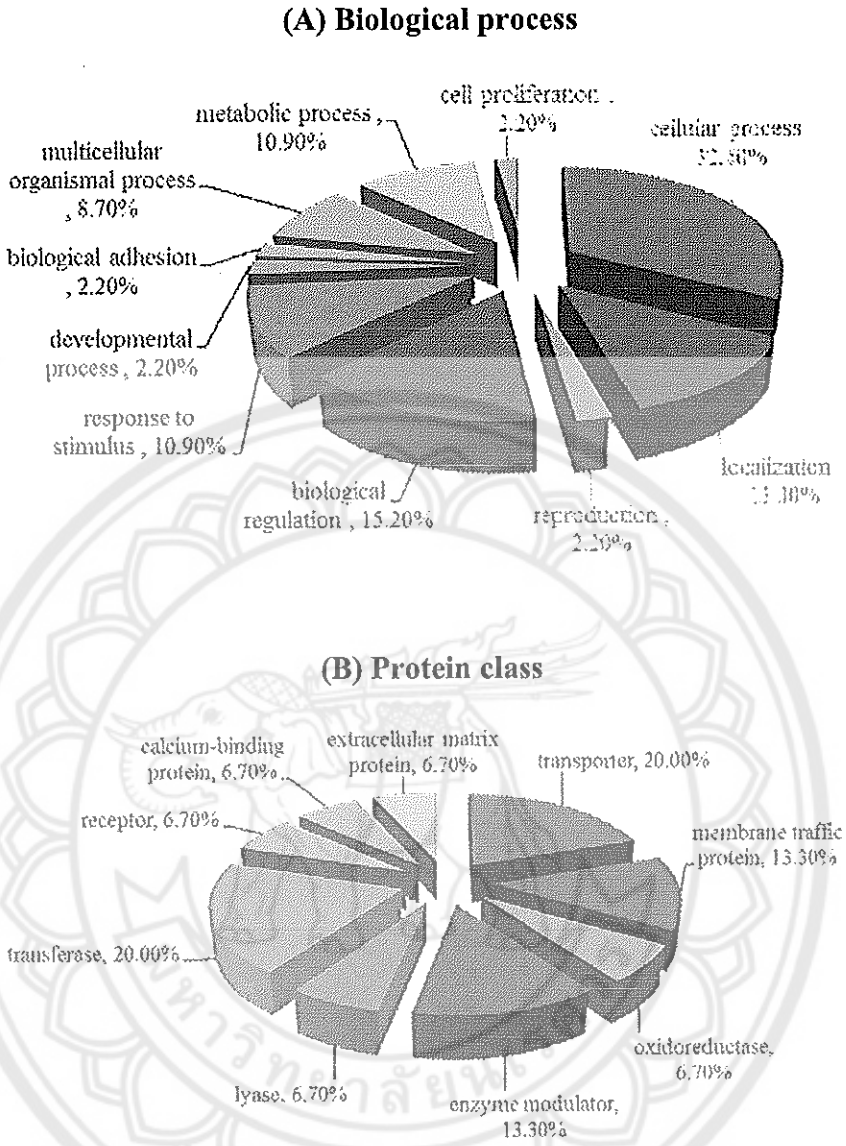


Figure 18 Protein classified in rat cerebellum after PGBR, GABA standard and diazepam treatment for 15 days

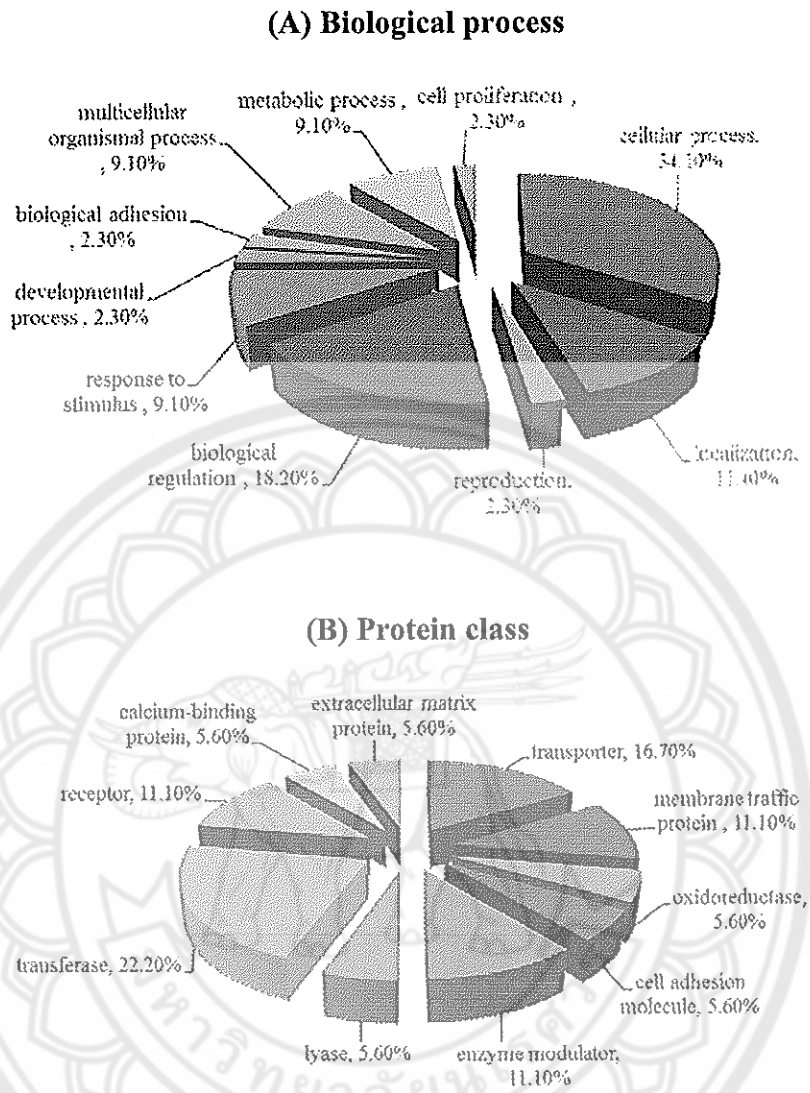


Figure 19 Protein classified in rat cerebellum after treatment for 30 days

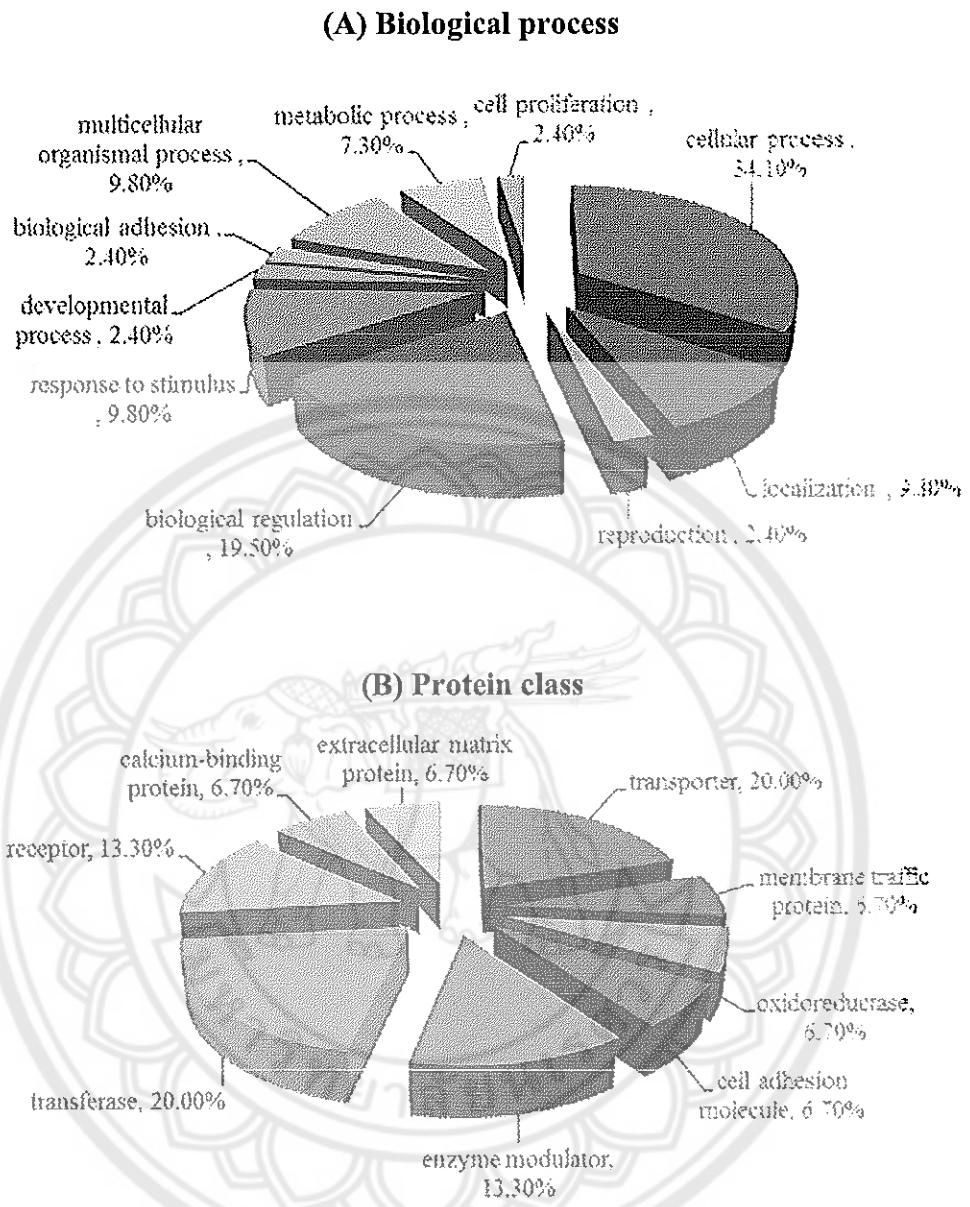


Figure 20 Protein classified in rat cerebellum after treatment for 60 days

Table 7 List of significantly identified protein after different treatments for 15 days

Protein name	Accession number	Fold change (log 2)			P-value		
		DR15_1	DG15_2	DD15_3	DR15_1	DG15_2	DD15_3
Cell-cell signaling							
excitatory amino acid transporter 1	gi|24233554	1.58	1.60	1.72	0.001	0.002	0.001
neuronal acetylcholine receptor subunit alpha-10	gi|12018340	1.46	1.49	1.59	0.006	0.007	0.004
protein shisa-6	gi|123794581	-1.43	-1.43	-1.43	0.002	0.001	0.001
calbindin	gi|6753242	-1.59	-1.87	-1.95	0.002	0.001	0.003
Signal transduction							
plexin-B3	gi|209529650	-1.59	-1.69	-1.75	0.005	0.003	0.003
epidermal growth factor receptor	gi|46560582	-1.41	-1.44	-1.48	0.002	0.004	0.002
NUAK family SNF1-like kinase 2	gi|110815882	1.49	1.54	1.58	0.005	0.009	0.009
phosphatidylethanolamine-binding protein 1	gi|84794552	1.55	1.61	1.69	0.001	0.003	0.003
Others							
heat shock 70 kDa protein 1-like	gi|124339838	-1.50	-1.29	-1.38	0.001	0.002	0.002
heat shock cognate 71 kDa protein	gi|31981690	-1.51	-1.57	-1.64	0.001	0.001	0.001
NADH dehydrogenase (ubiquinone) complex I	gi|157817227	-1.32	-1.35	-1.41	0.003	0.003	0.001
synaptotagmin-7	gi|160948571	-1.61	-1.42	-1.90	0.001	0.002	0.002
usherin	gi|145699097	-1.55	-1.65	-1.67	0.004	0.003	0.001
clathrin heavy chain 1	gi|51491845	-1.40	-1.53	-1.66	0.005	0.002	0.001
malate dehydrogenase	gi|31982186	1.50	1.60	1.68	0.001	0.006	0.006
coiled-coil domain-containing protein 138	gi|1046877484	1.42	1.51	1.53	0.002	0.003	0.006

Table 7 (cont.)

Protein name	Accession number	Fold change (log 2)			P-value		
		DR15_1	DG15_2	DD15_3	DR15_1	DG15_2	DD15_3
cAMP-specific 3',5'-cyclic phosphodiesterase 4D	gi|57015263	1.52	1.57	1.63	0.004	0.002	0.003
syntaxin binding protein 3A	gi|148670038	1.35	1.37	1.44	0.001	0.001	0.002
beta-2-glycoprotein 1	gi|160358825	1.34	1.38	1.42	0.001	0.001	0.002
sodium/glucose cotransporter 1	gi|261824023	1.34	1.39	1.46	0.003	0.008	0.003
E3 ubiquitin-protein ligase listerin	gi|198041681	-1.64	-1.55	-1.41	0.001	0.003	0.002
78 kDa glucose-regulated protein precursor	gi|254540168	-1.49	-1.59	-1.60	0.001	0.003	0.004
G-protein coupled receptor 98	gi|1046874075	-1.40	-1.46	-1.56	0.001	0.004	0.004
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	gi|395406791	-1.55	-1.49	-1.63	0.001	0.001	0.001
WD repeat and SOCS box-containing protein 1	gi|1046849453	1.68	1.69	1.71	0.001	0.002	0.001
creatine kinase B-type	gi|122065316	1.38	1.48	1.57	0.003	0.001	0.002
calcium/calmodulin-dependent protein kinase type 1B	gi|884859030	-1.41	-1.52	-1.73	0.001	0.001	0.004
citrate synthase	gi|13385942	-1.27	-1.29	-1.14	0.006	0.019	0.007
gap junction gamma-2 protein	gi|674039090	-1.43	-1.43	-1.47	0.001	0.003	0.003
60S ribosomal protein L31-like	gi|672049066	-1.31	-1.49	-1.48	0.002	0.002	0.002
prostaglandin E2 receptor EP1 subtype	gi|507579036	-1.37	-1.44	-1.61	0.003	0.001	0.004

Table 8 List of significantly identified protein after different treatments for 30 days

Protein name	Accession number	Fold change (log 2)			P-value		
		DR30_1	DG30_2	DD30_3	DR30_1	DG30_2	DD30_3
Cell-cell signaling							
excitatory amino acid transporter 1	gi|24233554	1.65	1.61	1.59	0.001	0.001	0.001
calbindin	gi|6753242	-1.61	-1.82	-2.13	0.002	0.001	0.001
protein shisa-6	gi|123794581	-1.50	-1.46	-1.34	0.002	0.001	0.003
neuronal acetylcholine receptor subunit alpha-10	gi|12018340	1.53	1.58	1.64	0.003	0.006	0.001
Signal transduction							
plexin-B3	gi|209529650	-1.59	-1.65	-1.87	0.003	0.003	0.004
epidermal growth factor receptor	gi|46560582	-1.35	-1.41	-1.47	0.003	0.006	0.008
serine/threonine-protein kinase SIK1	gi|148747268	-1.36	-1.57	-1.64	0.002	0.007	0.001
NUAK family SNF1-like kinase 2	gi|110815882	1.53	1.58	1.62	0.009	0.002	0.001
phosphatidylethanolamine-binding protein 1	gi|84794552	1.56	1.64	1.71	0.006	0.006	0.001
Others							
heat shock 70 kDa protein 1-like	gi|124339838	-1.46	-1.35	-1.33	0.001	0.001	0.003
synaptotagmin-7	gi|160948571	-1.73	-1.85	-1.88	0.001	0.002	0.002
clathrin heavy chain 1	gi|51491845	-1.63	-1.56	-1.53	0.002	0.002	0.002
usherin	gi|145699097	-1.50	-1.51	-1.58	0.002	0.002	0.002
leucine carboxyl methyltransferase 1	gi|564330837	-1.41	-1.44	-1.32	0.003	0.003	0.009
calcium/calmodulin-dependent protein kinase type 1B	gi|884859030	-1.41	-1.49	-1.77	0.005	0.003	0.004

Table 8 (cont.)

Protein name	Accession number	Fold change (log 2)			P-value		
		DR30_1	DG30_2	DD30_3	DR30_1	DG30_2	DD30_3
E3 ubiquitin-protein ligase listerin	gi|198041681	-1.35	-1.34	-1.48	0.003	0.004	0.003
prostaglandin E2 receptor EP1 subtype	gi|507579036	-1.42	-1.50	-1.64	0.001	0.003	0.003
78 kDa glucose-regulated protein	gi|254540168	-1.38	-1.53	-1.60	0.001	0.001	0.001
G-protein coupled receptor 98	gi|1046874075	-1.35	-1.53	-1.58	0.002	0.001	0.001
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	gi|157817227	-1.48	-1.66	-1.68	0.001	0.001	0.002
malate dehydrogenase, mitochondrial	gi|31982186	1.49	1.53	1.54	0.002	0.002	0.002
coiled-coil domain-containing protein 138	gi|1046877484	1.46	1.45	1.54	0.002	0.001	0.001
cAMP-specific 3',5'-cyclic phosphodiesterase 4D	gi|57015263	1.63	1.74	1.78	0.002	0.002	0.002
syntaxin binding protein 3A	gi|148670038	1.40	1.44	1.49	0.001	0.002	0.001
beta-2-glycoprotein 1 precursor	gi|160358825	1.38	1.38	1.43	0.002	0.003	0.001
sodium/glucose cotransporter 1	gi|261824023	1.41	1.48	1.52	0.009	0.003	0.002
WD repeat and SOCS box-containing protein 1	gi|1046849453	1.57	1.67	1.68	0.001	0.001	0.002
creatine kinase B-type	gi|122065316	1.55	1.60	1.67	0.001	0.002	0.001
fructose-bisphosphate aldolase C	gi|344259126	1.46	1.53	1.53	0.002	0.004	0.001
NADH dehydrogenase (ubiquinone) complex I	gi|395406791	-1.55	-1.51	-1.35	0.003	0.004	0.002
gap junction gamma-2 protein	gi|674039090	-1.46	-1.51	-1.51	0.002	0.002	0.002
60S ribosomal protein L31-like	gi|672049066	-1.41	-1.51	-1.63	0.003	0.005	0.005
citrate synthase	gi|13385942	-1.35	-1.39	-1.63	0.003	0.010	0.006
scavenger receptor class F member 2	gi|157786746	-1.42	-1.54	-1.64	0.001	0.002	0.004

Table 9 List of significantly identified protein after different treatments for 60 days

Protein name	Accession number	Fold change (log 2)			P-value		
		DR60_1	DG60_2	DD60_3	DR60_1	DG60_2	DD60_3
Cell-cell signaling							
excitatory amino acid transporter 1	gi|24233554	1.73	1.69	1.74	0.003	0.002	0.002
neuronal acetylcholine receptor subunit alpha-10	gi|12018340	1.53	1.62	1.67	0.002	0.003	0.001
calbindin	gi|6753242	-1.89	-1.96	-1.68	0.001	0.001	0.002
protein shisa-6	gi|123794581	-1.46	-1.41	-1.57	0.004	0.001	0.001
Signal transduction							
epidermal growth factor receptor	gi|46560582	-1.49	-1.57	-1.70	0.002	0.001	0.003
plexin-B3	gi|209529650	-1.40	-1.62	-1.55	0.004	0.004	0.004
NUAK family SNF1-like kinase 2	gi|110815882	1.47	1.52	1.60	0.008	0.007	0.005
phosphatidylethanolamine-binding protein 1	gi|84794552	1.65	1.74	1.72	0.002	0.002	0.002
serine/threonine-protein kinase SIK1	gi|148747268	-1.61	-1.48	-1.34	0.002	0.001	0.004
Others							
NADH dehydrogenase (ubiquinone) complex I	gi|395406791	-1.40	-1.41	-1.44	0.002	0.001	0.002
heat shock 70 kDa protein 1-like	gi|124339838	-1.46	-1.42	-1.37	0.002	0.002	0.001
usherin	gi|145699097	-1.43	-1.47	-1.68	0.005	0.002	0.002
synaptotagmin-7	gi|160948571	-1.46	-1.60	-1.53	0.002	0.003	0.002
leucine carboxyl methyltransferase 1	gi|564330837	-1.53	-1.46	-1.49	0.002	0.003	0.003

Table 9 (Cont.)

Protein name	Accession number	Fold change (log 2)			P-value		
		DR60_1	DG60_2	DD60_3	DR60_1	DG60_2	DD60_3
calcium/calmodulin-dependent protein kinase type 1B	gi|884859030	-1.44	-1.53	-1.53	0.002	0.003	0.007
E3 ubiquitin-protein ligase listerin	gi|198041681	-1.50	-1.44	-1.61	0.003	0.002	0.002
prostaglandin E2 receptor EP1subtype	gi|507579036	-1.64	-1.63	-1.44	0.001	0.001	0.002
78 kDa glucose-regulated protein	gi|254540168	-1.38	-1.36	-1.53	0.001	0.001	0.001
G-protein coupled receptor 98	gi|1046874075	-1.39	-1.40	-1.45	0.006	0.003	0.003
malate dehydrogenase	gi|31982186	1.54	1.56	1.65	0.004	0.003	0.002
coiled-coil domain-containing protein 138	gi|1046877484	1.42	1.47	1.53	0.005	0.001	0.004
cAMP-specific 3',5'-cyclic phosphodiesterase 4D	gi|57015263	1.67	1.71	1.74	0.001	0.002	0.000
syntaxin binding protein 3A	gi|148670038	1.38	1.49	1.55	0.003	0.001	0.002
beta-2-glycoprotein 1	gi|160358825	1.40	1.42	1.48	0.002	0.002	0.002
sodium/glucose cotransporter 1	gi|261824023	1.41	1.50	1.52	0.003	0.002	0.003

Table 9 (cont.)

Protein name	Accession number	Fold change (log 2)			P-value		
		DR60_1	DG60_2	DD60_3	DR60_1	DG60_2	DD60_3
WD repeat and SOCS box-containing protein 1	gi|1046849453	1.61	1.66	1.70	0.002	0.002	0.002
creatine kinase B-type	gi|122065316	1.55	1.50	1.64	0.002	0.001	0.002
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	gi|157817227	-1.48	-1.73	-1.64	0.001	0.001	0.001
gap junction gamma-2 protein	gi|674039090	-1.43	-1.41	-1.66	0.001	0.002	0.001
scavenger receptor class F member 2	gi|157786746	-1.40	-1.46	-1.50	0.004	0.001	0.002

Expression of calbindin in rat cerebellum

The differentially expressed protein, calbindin, detected by LC-MS/MS was selected for validation using western blotting analysis.

Expression of calbindin in rat cerebellum after addictive drugs exposure

The results showed that the mean value of relative calbindin protein intensity in control and DXM groups were 1.2589 ± 0.0225 and 3.7398 ± 0.0544, respectively as shown in Table 10 and Figure 21. This value in control and METH groups were 1.4271 ± 0.0326 and 3.6233 ± 0.0730, respectively. There was a significant increase in cerebellar tissues after METH (p = 0.03) and DXM (p = 0.04) exposure when compared to controls.

Table 10 The mean value of relative calbindin/ β-actin protein intensity in cerebellar tissues after addictive drugs exposure

Group	Calbindin/ β-Actin (mean ± SEM)
Control	1.2589 ± 0.0225
DXM	3.7398 ± 0.0544*
Control	1.4271 ± 0.0326
METH	3.6233 ± 0.0730*

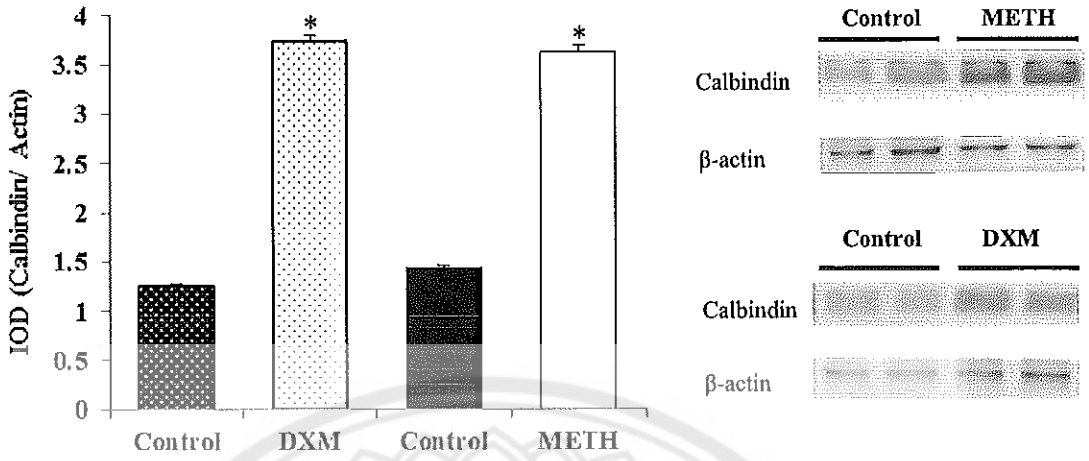


Figure 21 Quantitative analysis of calbindin expression after addictive drugs exposure in rat cerebellum. Bar graphs represent the mean ± SEM

Note: $*p < 0.05$ in comparison with control group by t-test

Expression of calbindin in rat cerebellum after drug withdrawal

There was no statistically significant difference in calbindin expression in DW15 ($p = 0.209$), DW30 ($p = 0.331$) and DW60 ($p = 0.489$) groups when compared with DXM group as presented in Table 11 and Figure 22. However, calbindin protein expression was significant difference in DW15 (p = 0.000), DW30 (p = 0.000) and DW60 (p = 0.000) groups when compared with control group, respectively.

Expression of calbindin in rat cerebellum after PGBR, GABA standard and diazepam treatments for 15, 30 and 60 days

Expression of calbindin in rat cerebellum after treatments for 15 days

Following the 15 days treatment, a significant difference in the level of calbindin protein was decreased in DD15 ($p = 0.001$) groups when compared with DXM group. However, calbindin protein expression was no significant difference in DR15 ($p = 0.085$) and DG15 ($p = 0.079$) groups when compared with DXM group, respectively (Table 12 and Figure 23).

Expression of calbindin in rat cerebellum after treatments for 30 days

Following the 30 days treatment, a significant decrease of calbindin protein was observed in DR30 ($p = 0.000$), DG30 ($p = 0.000$) and DD30 ($p = 0.000$) groups

when compared with DXM group (Table 13 and Figure 24).

Expression of calbindin in rat cerebellum after treatments for 60 days

Following the treatment 60 days, statistical analysis revealed a significant reduction of calbindin protein in DR60 ($p = 0.000$), DG60 ($p = 0.000$) and DD60 ($p = 0.000$) groups when compared with DXM group (Table 14 and Figure 25).

Table 10 The mean value of relative calbindin/ β-actin protein intensity in cerebellar tissues after drug withdrawal

Group	Calbindin/Actin (mean ± SEM.)
Control	1.2589 ± 0.0225
DXM	3.7398 ± 0.0544
DW15	3.6182 ± 0.0698
DW30	3.6460 ± 0.0966
DW60	3.6732 ± 0.0419

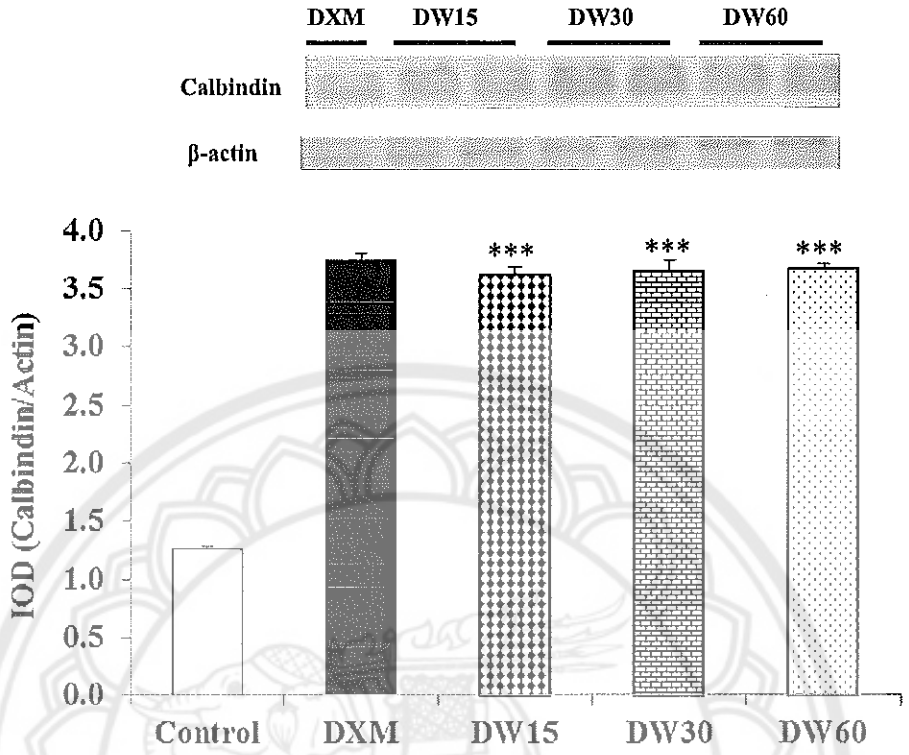


Figure 22 Quantitative analysis of calbindin expression after drug withdrawal in rat cerebellum. Bar graphs represent the mean ± SEM. The significant difference was conducted by one-way ANOVA with Dunnett test

Note: ***$p < 0.001$ vs Control

Table 12 The mean value of relative calbindin/ β-actin protein intensity in cerebellar tissues after treatments for 15 days

Group	Calbindin/Actin
Control	1.2589 ± 0.0225
DXM	3.7398 ± 0.0544***
DR15	3.5462 ± 0.0691
DG15	3.5431 ± 0.0396
DD15	3.4006 ± 0.0588##

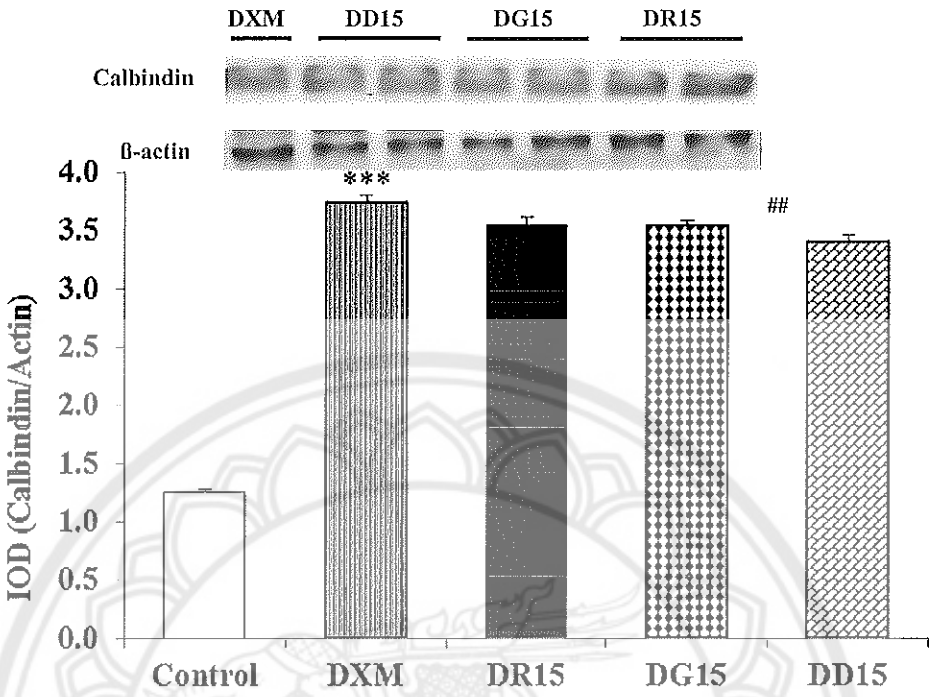


Figure 23 Quantitative analysis of calbindin expression after treatments for 15 days in rat cerebellum. Bar graphs represent the mean ± SEM. The significant difference was conducted by one-way ANOVA with Dunnett test

Note: $^{***}p < 0.001$ vs Control, $^{\#\#}p < 0.01$ vs DXM

Table 13 The mean value of relative calbindin/ β-actin protein intensity in cerebellar tissues after treatments for 30 days

Group	Calbindin/Actin
Control	1.2589 ± 0.0225
DXM	$3.7398 \pm 0.0544^{***}$
DR30	$2.7312 \pm 0.0356^{\#\#\#}$
DG30	$2.8428 \pm 0.0406^{\#\#\#}$
DD30	$2.9532 \pm 0.0313^{\#\#\#}$

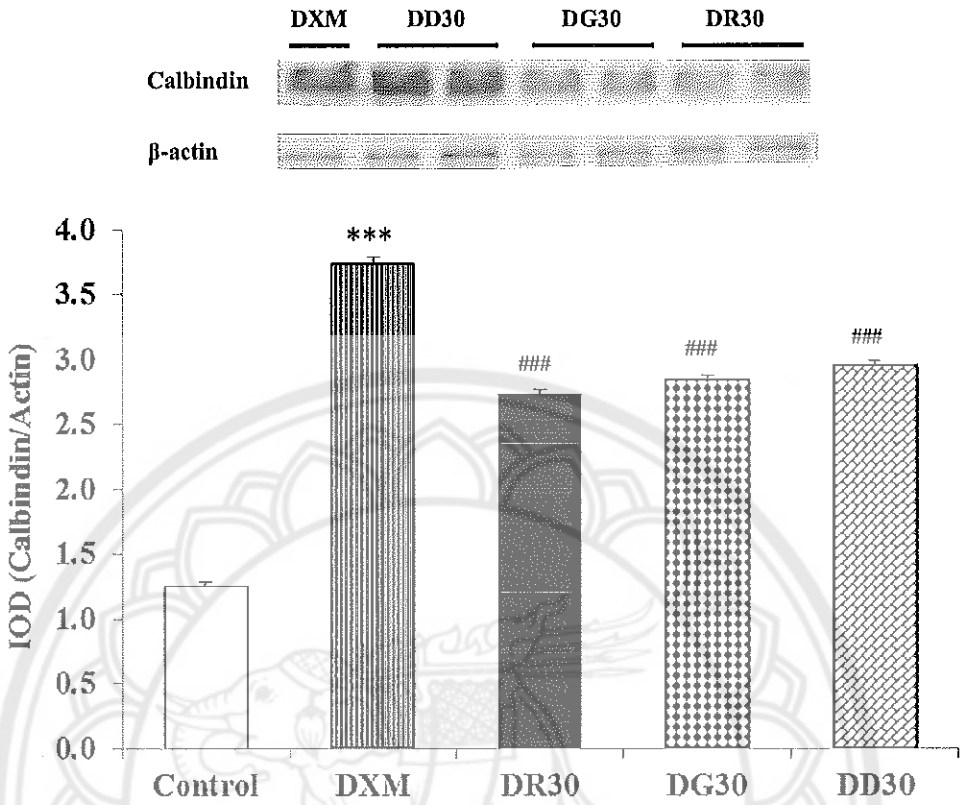


Figure 24 Quantitative analysis of calbindin expression after treatments for 30 days in rat cerebellum. Bar graphs represent the mean ± SEM. The significant difference was conducted by one-way ANOVA with Dunnett test

Note: $^{***}p < 0.001$ vs Control, $^{###}p < 0.001$ vs DXM

Table 14 **The mean value of relative calbindin/ β-actin protein intensity in cerebellar tissues after treatments for 60 days**

Group	Calbindin/Actin
Control	1.2589 ± 0.0225
DXM	3.7398 ± 0.0544***
DR60	2.2098 ± 0.0300###
DG60	2.4051 ± 0.0332###
DD60	2.7492 ± 0.0453###

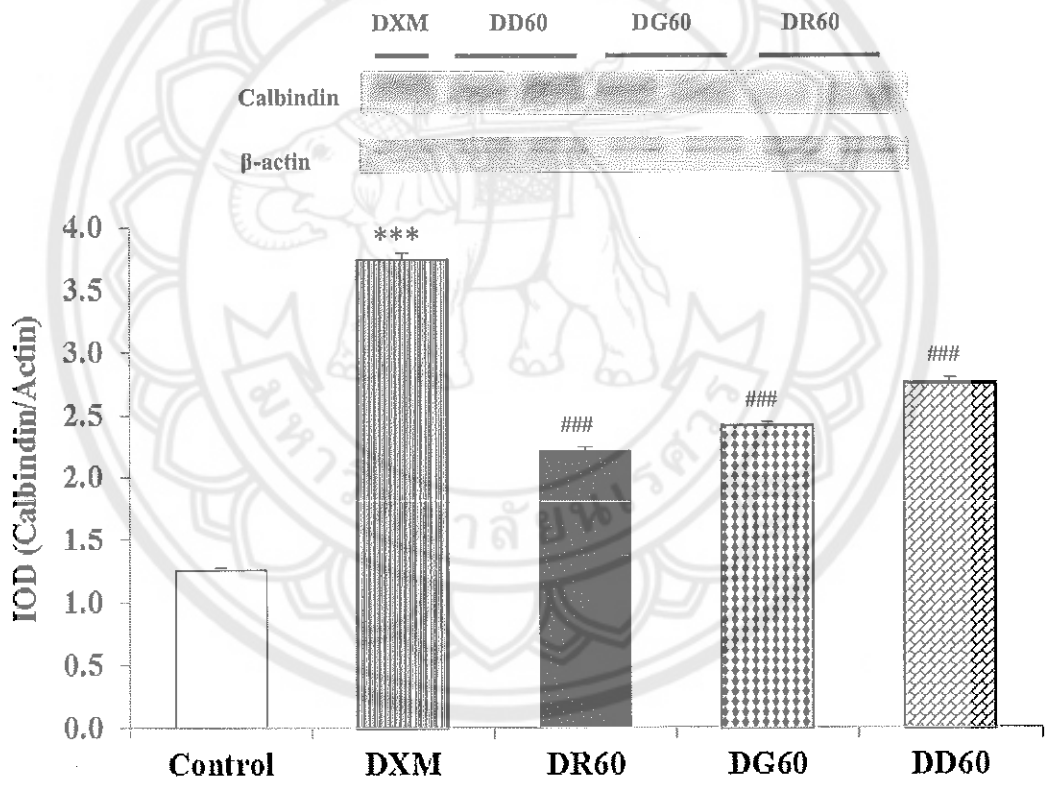


Figure 25 **Quantitative analysis of calbindin expression after treatments for 60 days in rat cerebellum. Bar graphs represent the mean ± SEM. The significant difference was conducted by one-way ANOVA with Dunnett test**

Note: ***$p < 0.001$ vs Control, ##$p < 0.01$ vs DXM

CHAPTER V

DISCUSSION

Alteration of protein expression profiles in rat cerebellum after drug addiction, drug withdrawal and treatments

In the current study, changes in global cerebellar proteome following exposure to addictive drugs, drug withdrawal and treatments (PGBR, GABA standard and diazepam drug) were identified using LC-MS/MS. As evident from the data, the known functions of differentially identified proteins are associated with important biological functions such as cell-cell signaling, signal transduction and others. Biological significance of some of the up-regulated proteins and down-regulated proteins of cell-cell signaling and signal transduction are discussed.

Cell-cell signaling and associated proteins are calbindin, excitatory amino acid transporter 1, shisa 6 and neuronal acetylcholine receptor subunit alpha-10. ***Calbindin (CB)***, a calcium binding protein, is located on GABAergic interneurons within several brain regions, including cerebellum (Bastianelli, 2003). CB may play a role in the maintenance of proper calcium (Ca2+) homeostasis and that its Ca2+ buffering ability may be important for neuronal survival. The up-regulation of CB was found in rat cerebellum after exposure to METH and DXM. As such, upregulation of this protein suggests increased intraneuronal cytosolic free Ca2+ concentration and may be a consequence of neuronal destruction. This view is supported by previous data which indicate that loss of CB-containing GABAergic interneurons was investigated in rat brains (i.e. frontal cortex and hippocampus) of METH addiction (Veerasakul et al., 2016) and schizophrenic brain patients (Benes, & Berretta, 2001). Surprisingly, the down-regulation of CB in the cerebellum was observed after treatments (PGBR, GABA standard, diazepam) in rat model DXM addiction. However, the stop to DXM taking (withdrawal) remained the over-expression of CB in rat cerebellum. The increase of CB in rat cerebellum observed in this study supports the hypothesis that METH and DXM addiction are neurotoxic by causing increases in free intracellular Ca2+ and that the decrease in CB levels after PGBR, GABA

standard, diazepam treatments is a protective response to elevated intracellular free Ca2+. ***Excitatory amino acid transporter (EAAT)*** plays an important role to maintain extracellular glutamate by reuptake glutamate from synaptic cleft, and alteration of this protein in METH addiction of rat brains (i.e. frontal cortex, hippocampus and striatum) has been reported (Kerdsan et al., 2012). Several studies have reported that drug addiction elevated extracellular glutamate concentration and contributed to the neurotoxic effect (Mark, Soghomonian, & Yamamoto, 2004; Shoblock, Sullivan, Maisonneuve, & Glick, 2003). Such patients with schizophrenia-induced neuronal degeneration may include effects on glutamatergic neurons and bring about a reduction of EAAT1 in prefrontal cortex (Bauer, Haroutunian, Meador-Woodruff, & McCullumsmith, 2010). In our chronic DXM regimen, this includes a down-regulation of EAAT1 in rat cerebellum. The results provide a support glutamatergic dysfunction with abnormalities involving a glutamate transporter that contributes to maintaining appropriate synaptic glutamate levels. Moreover, the up-regulation of EAAT1 was found in the cerebellum after PGBR, GABA standard, diazepam drug and withdrawal treatments in a rat model of DXM addiction. This provides information in a neuroprotective effect for addiction treatment. However, our study observed an up-regulation of EAAT1 in a rat model of METH addiction. An upregulation of EAAT1 may be a compensatory response to glutamate transporter to maintaining extracellular glutamate level. Consistent with this observation, METH has been reported to induce an up-regulation of EAAT3 in rat hippocampus (Kerdsan et al., 2012) and EAAT2 in rat striatum (Shirai, Shirakawa, Nishino, Saito, & Nakai, 1996). ***Shisa6*** is a single transmembrane protein that directly interacts with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor (Klaassen et al., 2016). An up-regulation of AMPA have been reported in rat treated with amphetamine and METH (Herrold, Persons, & Napier, 2013; Wang et al., 2017). Our data shows an increase in the Shisa6 following METH and DXM addiction. It is possible that protein alteration might be up-regulated to promote more AMPA synaptic activity and contributed to abnormalities of glutamate excitatory signaling. Furthermore, the down-regulation of Shisa6 was found in PGBR, GABA standard, diazepam drug and withdrawal in a rat model of DXM addiction. Thus, this may represent a protective mechanism to down-regulate this protein that may be helpful to maintain neuronal connection via

excitatory signaling in the brain. Evidence suggests that ***neuronal acetylcholine receptors (nAChRs)*** interact with methamphetamine that involved in locomotor activity (Camarasa, Rates, Pubill, & Escubedo, 2009). Upregulation of nAChR α4β2 subtype was found in mice lateral septum, caudate putamen, and nucleus accumbens using quantitative receptor autoradiography following chronic nicotine exposure (Besson et al., 2007). Nicotine is highly addictive and acts as a receptor agonist at most nAChRs, except nAChRα10 subunit where it acts as a receptor antagonist. Therefore, the down-regulation of nAChRα10 obtained from proteome in this study which may involve in addictive process. Interestingly, nAChRα10 expression was found upregulation in PGBR, GABA standard and diazepam drug treated rat. These results may provide the adaptive mechanism of addictive process via cholinergic pathway through influence on nAChRα10 protein expression.

Signal transduction and associated proteins are plexin-B3, G protein-coupled receptor kinase 4, epidermal growth factor receptor, serine/threonine-protein kinase SIK1 and NUAK family SNF1-like kinase 2. ***Plexin (PLXN)***, a main receptor for semaphorin, was found to be implicated in psychiatric disorder by disrupting limbic and cortical connectivity (Rünker et al., 2011). From the present proteome study, PLXNB3 showed upregulation in METH and DXM samples, while the down-regulation of this protein found in PGBR, but not changes in GABA standard, diazepam drug and withdrawal treated rats. In agreement with the results, upregulation of PLXNB3 gene expression profiling was found in prefrontal cortex of schizophrenic patients (Gilabert-Juan et al., 2015). Other PLXN subtypes, PLXNA2 was upregulated in rat striatum following cocaine treatment (Lynch, Girgenti, Breslin, Newton, & Taylor, 2008). These data suggest that the PLXNB3 overexpression may contribute to susceptibility to develop addictive disorder, while down-regulation of this protein may possible suppress the relevant addictive behavior. ***G protein-coupled receptor kinase 4 (GRK4)*** was up-regulated following METH and DXM administration. GRK is a family of protein kinase that regulates the activity of G protein-coupled receptors by phosphorylating. The alterations of the GRK6 system was found that induced DRD2 up-regulation and impairment dopamine signaling pathway (Oda et al., 2015). The observations in this study suggest that the alteration of GRK4 may contribute to addictive process through dopaminergic signaling pathway. ***Epidermal growth factor***

receptor (EGFR) is a transmembrane protein that is a receptor for EGF family and exerts neurotrophic activity in the central nervous system. The upregulation of EGFR was found in forebrain regions of postmortem schizophrenic patients (Futamura et al., 2003). The overexpression of EGFR was found to be related with an increasing of locomotor responses after repeated cocaine and methamphetamine (Mizuno, Malta, Nagano, & Nawa, 2004). This proteome study was observed the up-regulation of EGFR induced by METH and DXM exposure. Therefore, it is possible linked between EGFR activation and drug addiction, including, behavioral abnormalities and cognitive dysfunction. Furthermore, a reduction of EGFR was found in PGBR, GABA standard, diazepam drug treatments. This may provide a protective effect to alleviate behavioural abnormalities via an EGFR dependent mechanism. ***Serine/threonine-protein kinase SIK1 (SIK1)***, also known as salt inducible kinase 1, has been reported that its activation can regulate the neuronal activity both in *vivo* and *vitro* studies (Feldman et al., 2000; Liu et al., 2012). A previous study has revealed to repress of SIK1 in controlling of cAMP response element-binding protein (CREB)-mediated transcription (Katoh et al., 2004). Overexpression of SIK1 was found in rat striatum followed by cocaine exposure (Dietrich, Takemori, Grosch-Dirrig, Bertorello, & Zwiller, 2012; Sontag et al., 2014). The up-regulation of SIK1 was found in rat cerebellum following METH and DXM exposure, while the down-regulation of SIK1 was observed in PGBR, GABA standard and diazepam drug treated rats. The results provide evidence that SIK1 is mediated through the CREB pathway and probably involved in instigating drug addiction. ***NUAK family SNF1-like kinase 2 (NUAK2)*** is also called SNF1/AMPK-related kinase (SNARK) which its function is a regulation of cellular energy homeostasis. The evidence suggests that energy deficit contribute to an important regulatory element in drug addiction (Samikkannu, Atluri, & Nair, 2016; Thanos, Michaelides, Benveniste, Wang, & Volkow, 2008). Down-regulation of NUAK2 was observed in METH and DXM rats in this study. These results suggest that the down-regulation of NUAK2 in the cerebellum may be an influence of energy deficit and metabolic dysfunction by inducing METH and DXM exposure leading to addictive progression. In addition, up-regulation of this protein was found in PGBR, GABA standard and diazepam drug. Thus, its down-regulation may play a protective effect on drug addiction.

Other identified proteins are usherin and synaptotagmin 7. ***Usherin (USH)***, a protein involved in inner ear and retinal basement membrane integrity, is encoded by USH2A gene that the mutations in this gene lead to hearing and visual impairment in Usher syndrome (Adato et al., 2005; Liu et al., 2007). Previous studies reported mental disorders associated with Usher syndrome, suggesting mechanism of association between Usher syndrome and mental disorders, most commonly schizophrenia (Domanico, Fragiotta, Cutini, Grenga, & Vingolo, 2015; Domanico, Fragiotta, Trabucco, Nebbioso, & Vingolo, 2012). The present proteome study was shown the up-regulation of Usherin in METH and DXM treated rats. It is a possible role of Usherin protein that may involve in psychiatric like behavior, in particular addictive behavior. ***Synaptotagmin (SYT)*** is a calcium sensor that trigger vesicle to plasma membrane fusion. The up-regulation of SYT7 was observed in the present study of METH and DXM treated rats. Supporting the results of a previous study, SYT7 protein expression was escalated in the mice nucleus accumbens after cocaine exposure (Zhang et al., 2004). The results suggest that SYT7 may contribute to METH- and DXM- induced neuroadaptive changes including behavioral sensitization. On the other hand, the down-regulation of SYT7 was observed in PGBR, GABA standard, diazepam drug and withdrawal treated rats. These changes may possible alleviate the dysfunction of SYT7 through GABAergic signaling pathway.

The selected-calbindin protein for validation by western blot analysis

Amongst the interesting differentially-expressed protein was calbindin, selected protein for validating the mass spectrometry-identified using western blot analysis.

Changes in calbindin protein levels after addictive drug administration

In this study, the results show a significant increase of calbindin, calcium binding protein in rat cerebellum following METH and DXM administration, suggesting that overexpression of calbindin in the cerebellum maybe a consequence of GABAergic dysfunction induced by METH and DXM administration. This finding is consistent with a previous report that found increases in calbindin expression of rat prelimbic and orbitofrontal cortices after repeated METH administration (Wearne, Parker, Franklin, Goodchild, & Cornish, 2017). Calbindin is an important modulator

of calcium ion binding and releasing which may protect the cells and regulate their synaptic plasticity. An increasing of calbindin levels induced by excitotoxin exposure has been found to induce of neuronal cell death (Meier, Ho, & Sapolsky, 1997; Phillips et al., 1999). Additionally, an elevation of neuronal cell death has been observed in rat prefrontal cortex (Abekawa, Ito, Nakagawa, Nakato, & Koyama, 2008) and mice striatum (Zhu, Xu, & Angulo, 2006) after METH administration. The upregulation of calbindin not only suggest a compensatory mechanism, but also indicates a rise in intracellular calcium ion concentration, followed by neuron death. These finding suggests that upregulation of calbindin may indicate the disruption of calcium ion homeostasis due to METH- and DXM- induced neurotoxicity and leads to neuronal death. Dumas and coworker (2004) suggested that increased expression of calbindin in dentate gyrus granule cells of hippocampus impaired of spatial memory. Taken together with a previous study, calbindin knockout mice displayed enhanced synaptic transmission and showed cognitive impairment (Jouvenceau et al., 1999). In addition, our research team observed the behavioural changes in rats of METH (Iamjun et al, 2019) and DXM (Veerasakul et al, 2016) models including hyperlocomotion and behavioral stereotypies (repetitive head movements, sniffing, rearing and licking). Therefore, the current data provide additional evidence that the up-regulation of calbindin expression in rat cerebellum may be critical in mediating of the addictive drug process which could have consequences on behavioral abnormalities.

Changes in calbindin protein levels after drug withdrawal

In the present study, no significant decrease in calbindin expression protein was found in each experimental group after withdrawn to DXM administration for 15, 30 and 60 days. However, a regimen of DXM-induced hyperlocomotion of rats in our research team observed a decrease in locomotor scores after drug withdrawal for 15 days and reversed effect after drug withdrawal 30 and 60 days (Veerasakul et al., 2016). As well as in according to previous results from our laboratory, DXM-induced behavior-like anxiety observation showed slightly increase percentage of open arm time in the plus maze test for evaluating anxiety behavior after drug withdrawal for 15 days (Veerasakul et al., 2016). These findings indicate that stop drug taking (drug

withdrawal) remains behavioral abnormalities and could have no a reverse effect on return the calbindin expression protein to homeostasis in rat cerebellum.

Changes in calbindin protein levels after PGBR, GABA standard and diazepam treatments

The results of the present study demonstrated that DXM-induced overexpression of calbindin protein in the cerebellum reversed the effect by diazepam treatment for 15 days. Nevertheless, the treatment with PGBR, GABA standard and diazepam for 30 and 60 days showed the attenuation effect on DXM-induced upregulation of calbindin protein in the cerebellum. Interestingly, attenuation effect of PGBR is the greatest among different treatments. This indicates that if the rise in calcium ion is prevented by removing calcium ion from the extracellular environment resulting from PGBR treatment. Therefore, this finding suggested the attenuation effects of PGBR on calbindin protein change induced by DXM. The PGBR also showed a strong anti-anxiety response and a reduction of locomotor activity in DXM-induced anxiety-like behavior (Veerasakul et al., 2016). Along the same line, Mamiya, & coworkers (2007) exposed effect of PGBR on the antidepressant in depressed mice. PGBR treatment produced greater effects on the neurochemical and behavioral attenuation than treat of synthetic GABA and diazepam. Moreover, the diazepam treatment has produced several adverse effects include dizziness, ataxia, depression and confusion (Calcaterra, & Barrow, 2014). It is interesting that PGBR may be an alternative treatment for addictive drug treatment. In conclusion, the effects of PGBR treatment can attenuate the calbindin calcium binding protein and this effect may support the possible role in alleviation of the addictive behaviors through GABAergic signaling pathway.

CHAPTER VI

CONCLUSION

In conclusion, the proteomics results in the present study identified differentially expressed cell-cell signaling-associated proteins (calbindin; CB, excitatory amino acid transporter 1; EAAT1, protein shisa-6; Shisa6 and neuronal acetylcholine receptor subunit alpha-10; nAChRα10) and signal transduction-associated proteins (plexin-B3; PLXNB3, G protein-coupled receptor kinase 4; GRK4, epidermal growth factor receptor; EGFR serine/ threonine-protein kinase SIK1; SIK1 and NUAK family SNF1-like kinase 2; NUAK2) in rat cerebellum of drug addiction. Seven proteins were up-regulated in METH addiction including CB, EAAT1, Shisa6, PLXNB3, GRK4, EGFR and SIK1, while two proteins were down-regulated including nAChRα10 and NUAK2. For DXM addiction, four proteins were up-regulated including CB, Shisa6, PLXNB3 and EGFR while three proteins were down-regulated including EAAT1, nAChRα10 and NUAK2.

This may provide the evidence to support the contribution of these proteins in addictive process. The validation of selected protein, calbindin calcium binding protein, has done for confirming protein changes which the results can be reflected to the involvement of GABAergic interneurons following METH and DXM exposure. Moreover, our previous studies suggested that the effects of METH and DXM addiction contribute to behavioral changes including hyperlocomotion and anxiety-like behaviors in rats. Therefore, changes in expression of the calbindin may provide a number of key targets on the behavioral abnormalities. The analysis of proteomics results and behavior-related addiction needs to be elucidated further.

The results from the current study showed the expression of cell-cell signaling and signal transduction proteins, including calbindin were no statistically significant differences between DXM withdrawal and DXM-administered rats. This implies that drug withdrawal (stop drug taking) cannot recover changes of these proteins expression after DXM addiction. In addition, the symptoms of drug withdrawal, especially anxiety-like behaviour were observed after drug withdrawal in

a rat model of DXM addiction in our research team. Thus, the anxiety-like behaviour in drug withdrawal rats may reflect the persisting at high levels of calbindin expression.

Furthermore, changes in cell-cell signaling and signal transduction proteins, including calbindin are attenuated by PGBR, GABA standard, diazepam drug treatments. The effects of these treatments have also observed to be alleviated DXM-induced hyperlocomotion and anxiety-like behaviour in rats. Thus, treatments may be a potential role in the protective effect on the attenuation of behavioural abnormalities.

As mentioned above, this study provides evidence that the protein expression profiles of cell-cell signaling and signal transduction changes in the cerebellum which are involved in drug addiction, drug withdrawal and addiction treatments. Moreover, a selected-protein, calbindin to confirm the expression changes included increase level of calbindin in drug addiction, as well as reduced calbindin levels in PGBR, GABA standard and diazepam drug treatments. As such, further studies are needed to investigate with particular proteins of interest to further understand how these changes which may help to strengthen the present findings and may also develop new targets for addiction treatments.

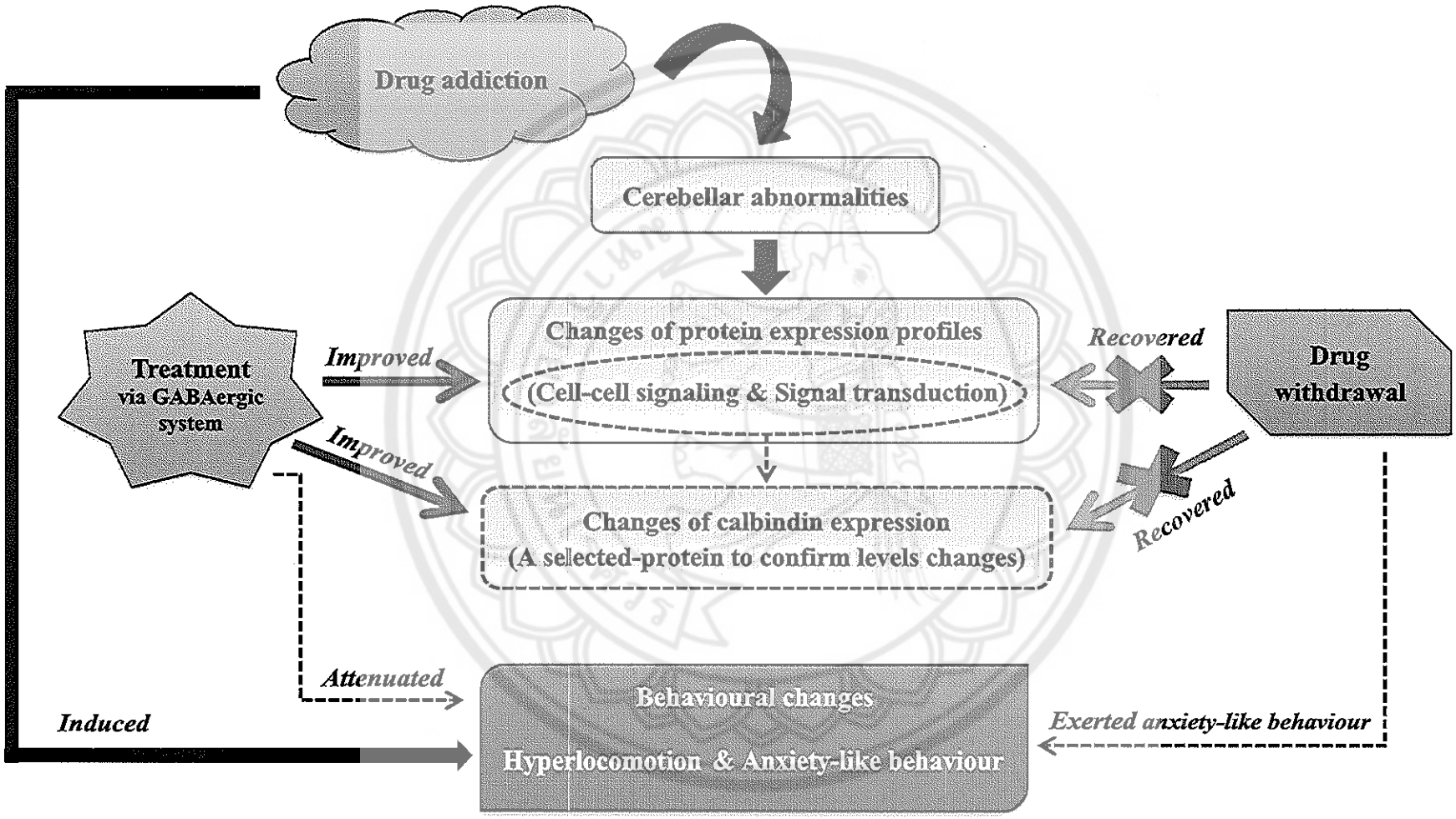


Figure 26 Schematic diagram for the conclusion of this study

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APPENDIX

APPENDIX A VENN DIAGRAM IDENTIFYING THE OVERLAPPED AND UNIQUE PROTEINS OF DRUG ADDICTION MODEL

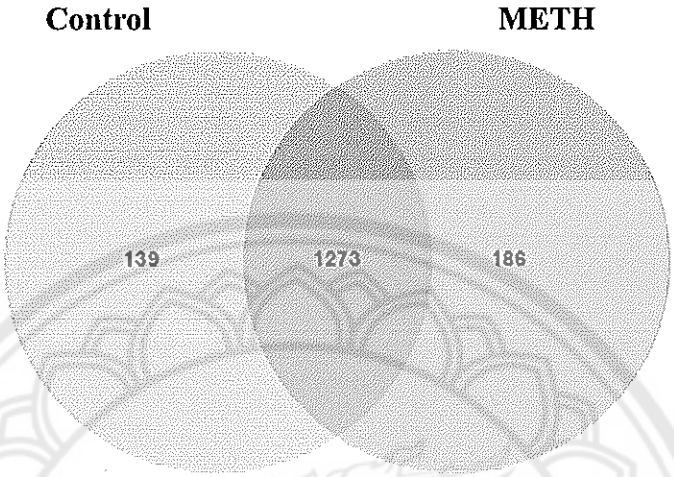

Figure 27 Venn diagram showing 1,273 overlapped proteins between control and METH groups

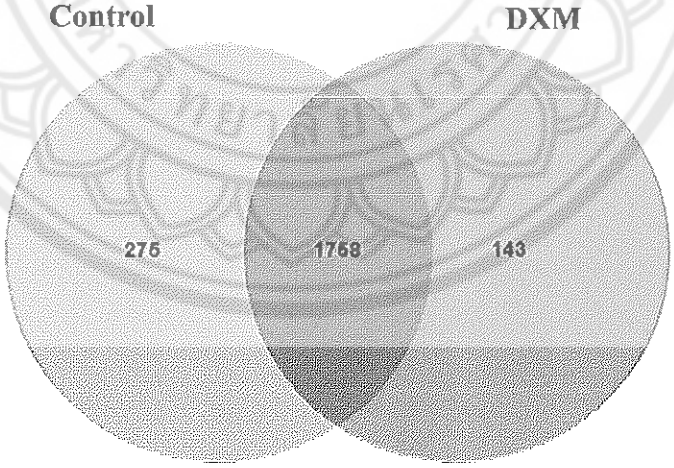

Figure 28 Venn diagram showing 1,758 overlapped proteins between control and DXM groups

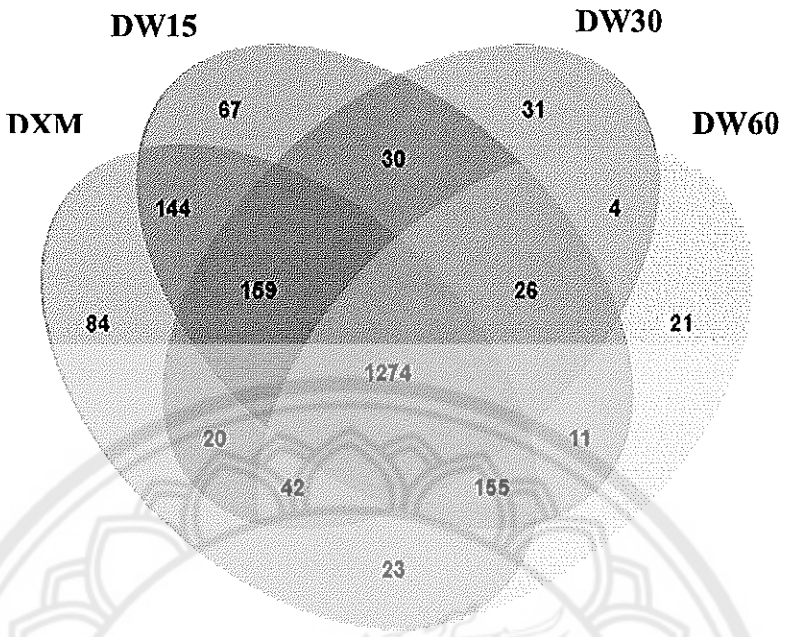

Figure 29 Venn diagram showing 1,724 overlapped proteins among DXM, DW15, DW30 and DW60 groups

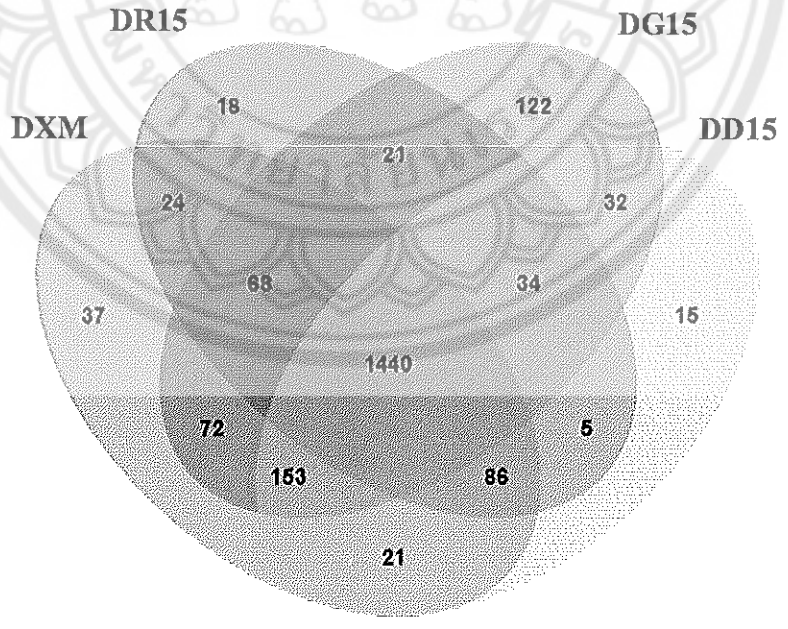

Figure 30 Venn diagram showing 1,440 overlapped proteins among DXM, DR15, DG15 and DD15 groups

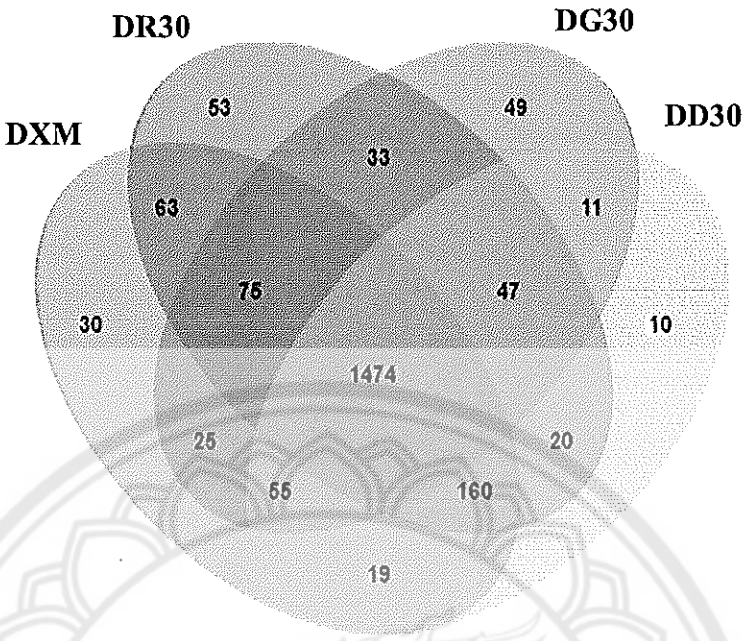

Figure 31 Venn diagram showing 1,474 overlapped proteins among DXM, DR30, DG30 and DD30 groups

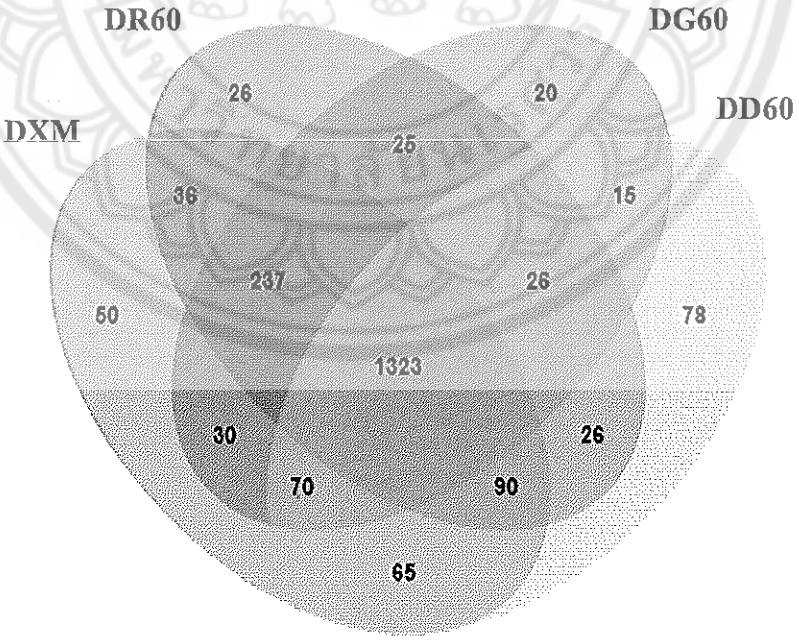

Figure 32 Venn diagram showing 1,323 overlapped proteins among DXM, DR60, DG60 and DD60 groups

APPENDIX B CERTIFICATE OF APPROVAL FROM ANIMALS ETHIC COMMITTEE

เอกสารรับรองโครงการ
คณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์
มหาวิทยาลัยนเรศวร (ดกส.)

ชื่อโครงการ	การวิเคราะห์การตอบสนองของโปรตีนต่อการติดยาเสพติดในสมองส่วนซีรีเบลลัม ด้วยเทคนิคช็อทกันโปรตีโอมิกส์ Shotgun proteomics analysis of proteins responding to drug addiction in rat cerebellum
เลขที่โครงการ	NU-AEE600605
เลขที่เอกสารรับรอง	61 02 001
ประเภทการรับรอง	แบบยกเว้น
ชื่อหัวหน้าโครงการ/ผู้ยื่นขอฯ	รศ.ดร.สุพิสา ทาน้อย
สังกัดหน่วยงาน /คณะ	วิทยาศาสตร์การแพทย์
วันที่รับรอง	18 มกราคม 2561
วันสิ้นสุดการรับรอง	18 มกราคม 2564

ขอรับรองว่าโครงการวิจัยนี้ ได้รับการรับรองด้านจรรยาบรรณการใช้สัตว์
จากคณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์
มหาวิทยาลัยนเรศวร (ดกส.)

.....

(รองศาสตราจารย์ ดร.รัตติมา จีนาพงษา)
ประธานคณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์ (ดกส.)
มหาวิทยาลัยนเรศวร

APPENDIX C LISTS OF SOLUTIONS AND BUFFERS

Brain homogenate buffer

5 mM	Tris-HCl, pH 8.0
25 mM	NaCl

Lysis buffer

50 mM	Tris-HCl, pH 8.0
0.15 M	NaCl
0.1%	SDS
0.25%	Sodium deoxycholate
1%	Protease inhibitor cocktail, add before use

2X SDS-PAGE sample buffer

250 mM	Tris-HCl, pH 6.8
2%	SDS
10%	Glycerol
20 mM	DTT
0.01%	Bromophenol blue

4X Stacking buffer

0.5 M	Tris-base pH 6.8
0.4%	SDS

4% Stacking gel (2.5 ml)

1.5725 ml	Distilled water
0.625 ml	4X stacking buffer
0.25 ml	40% Acrylamide
50 µl	10% APS
2.5 µl	TEMED

4X Separating buffer

1.5 M	Tris-base pH 8.8
0.4%	SDS

12.5% Separating gel (5 ml)

2.1335 ml	Distilled water
1.25 ml	4X Separating buffer
1.5625 mL	40% Acrylamide
50 µl	10% APS
4 µl	TEMED

1X Running buffer, pH 8.3

25 mM	Tris-base
192 mM	Glycine
0.1%	SDS

1X Transfer buffer, pH 8.3

48 mM	Tris-base
39 mM	Glycine
0.37%	SDS
20%	Methanol

1X TBS buffer, pH 7.6

20 mM	Tris-base
150 mM	NaCl

Coomassie Blue staining solution

50%	Methanol
10%	Acetic acid
0.25%	Coomassie Blue R-250

Destain of Coomassie Blue solution

10%	Methanol
5%	Acetic acid