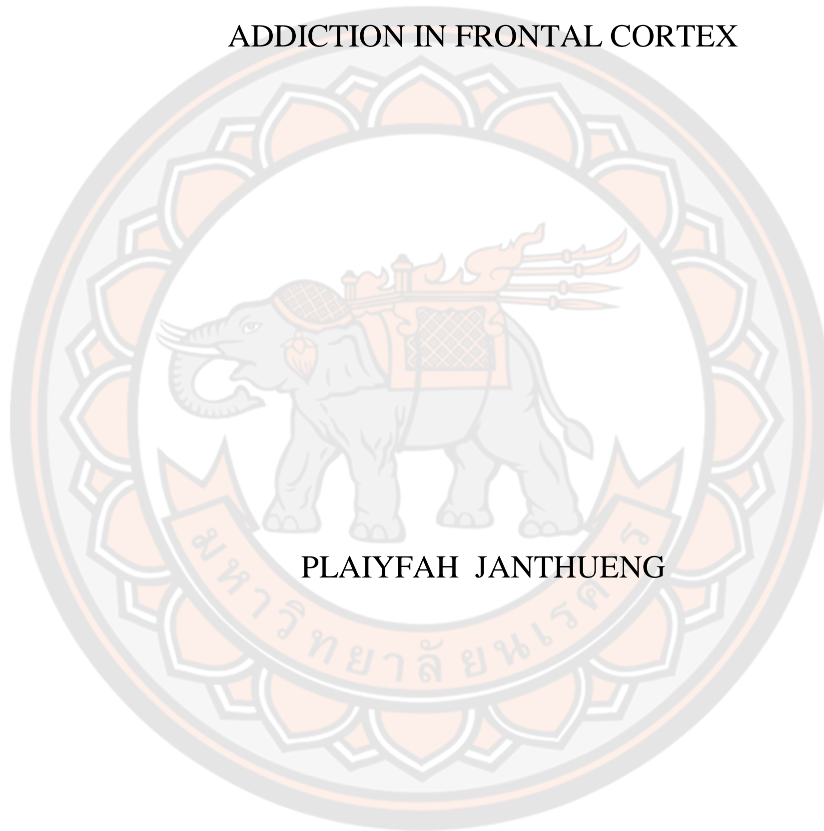
SHOTGUN PROTEOMICS ANALYSIS OF PROTEINS RESPONDING TO DRUG ADDICTION IN FRONTAL CORTEX



PLAIYFAH JANTHUENG

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Anatomy
2021

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By PLAIYFAH JANTHUENG

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Anatomy of Naresuan University

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ABSTRACT

Drug addiction is regarded as a major public health problem in Thailand. The most common addictive drugs are methamphetamine (METH) and over the counter medication such as dextromethorphan (DXM). Drug addiction has been reported as a behavioral abnormality, through the alteration of molecular and cellular levels in the brain, such as the frontal cortex. METH is an addictive psychostimulant with potent effects on the central nervous system (CNS), prolonged use can impair brain structures and functions. As well, DXM is an antitussive agent which is included in several over-the- counter cough and cold medications, prolonged use can cause psychoactive effects and hallucinogen-like addiction. Several studies have shown drug addiction's effect on the neurotransmitters including glutamate, GABA, dopamine and associated protein in the frontal cortex, resulting in cognitive, behavioral, and physiological abnormalities. So, the therapeutic approaches for drug addiction have been involved in these neurotransmitter systems, such diazepam have anxiolytics that are specific to the GABAergic system, but chronic use can develop into addiction. Nutraceuticals are an alternative abundant interest, that is pre-germinated brown rice (PGBR), high GABA contents. However, little is known about the underlying mechanisms of PGBR effects on neurobiological proteins. Therefore, the aim is to investigate the protein expression profiling in the rat frontal cortex after exposure to addictive drugs and addiction treatments. Male Sprague-Dawley rats were divided into 2 groups of addiction models, including METH and DXM addiction. In METH, the rats were treated with saline (CM)

and escalating binge dose of METH (0.1 to 4 mg/kg of METH (3 times /day), for 14 days and binge dose, 6 mg/kg (4 times /day) at day 15), respectively. In DXM, The animals were divided into six groups including control (CD), dextromethorphan (D), withdrawal (DW), diazepam (DD), synthetic GABA (DS), and pre-germinated brown rice (DR). The animals in the control group received saline i.p. for 15 days and treated with distilled water by oral administration for 60 days. Animals in the DW group were drug-withdrawal by receiving oral administration of distilled water. Animals in the DD group received 10 mg/kg diazepam. Animals in the DS group were administered with 0.8 mg/kg synthetic GABA. Animals in the DR group were treated with 5 mg/kg PGBR. After the last administration of the subgroup, animals were sacrificed, and the brain was collected. The alteration proteins in rat frontal cortex were investigated by proteomic technique (LC-MS/MS). An expression level of some protein was validated by western blotting technique. The proteomic result shows the alteration of neuronal signaling proteins expression in rat frontal cortex after drug addiction, drug withdrawal and drug treatment (diazepam, GABA and PGBR). Several biochemical functions appear to be enriched among these proteins, the glutamatergic and GABAergic pathway. These pathways were enriched in receptor, transporter, enzyme and associated proteins. Moreover, western blotting confirmed the associated candidate proteins, which refer to glutamatergic and GABAergic dysfunction after METH addiction. These findings highlight the identification of glutamatergic and GABAergic neurotransmitter systems and provide insight into the biological function involved in drug addiction and its treatment.

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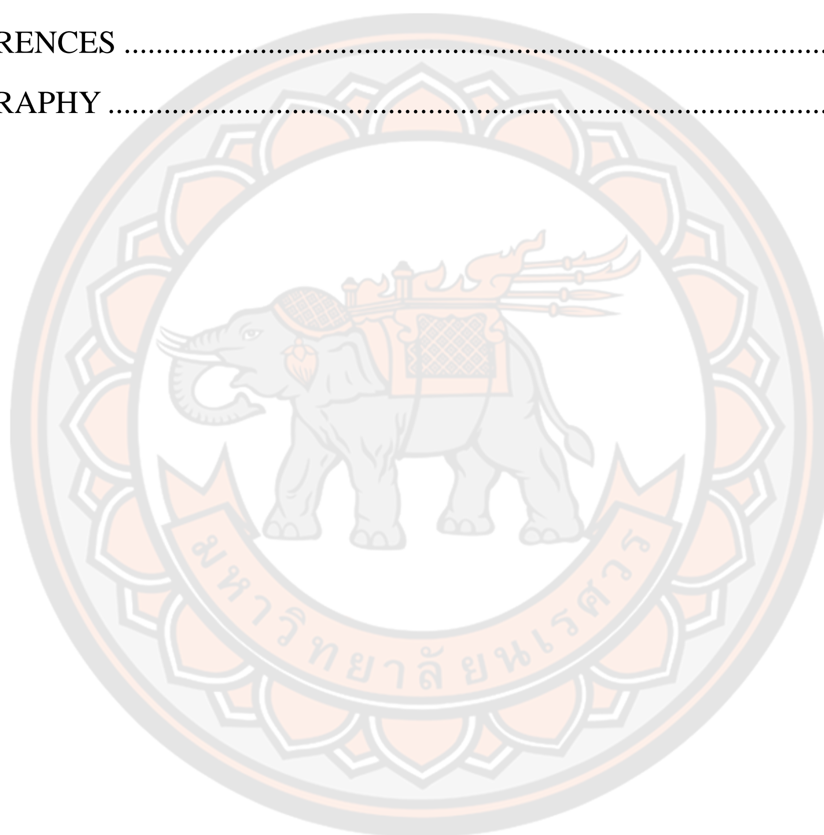


ABBREVIATION

5HT = 5-hydroxytryptamine receptor

µg = microgram

ABC = avidin-biotin complex

AMPA = alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ANOVA = Analysis Of Variance

BCA = Bicinchoninic Acid Protein Assay

BDNF = Brain-derived neurotrophic factor

BSA = Bovine Serum Albumin

°C = Celsius

Ca2+ = Calcium

Cacna1b = Calcium Voltage-Gated Channel Subunit Alpha1 B

Calb1 = calbindin

CaMKII = Calcium/calmodulin-dependent protein kinase II

CNS = central nervous system

CREB = cAMP-response element binding protein

CYP2D6 = Cytochrome P450 2D6

CYP3A4 = Cytochrome P450 3A4

Da = dalton

DAT = dopamine transporter

Dlgap1 = Disks large-associated protein 1

DMT = Dimethyltryptamin

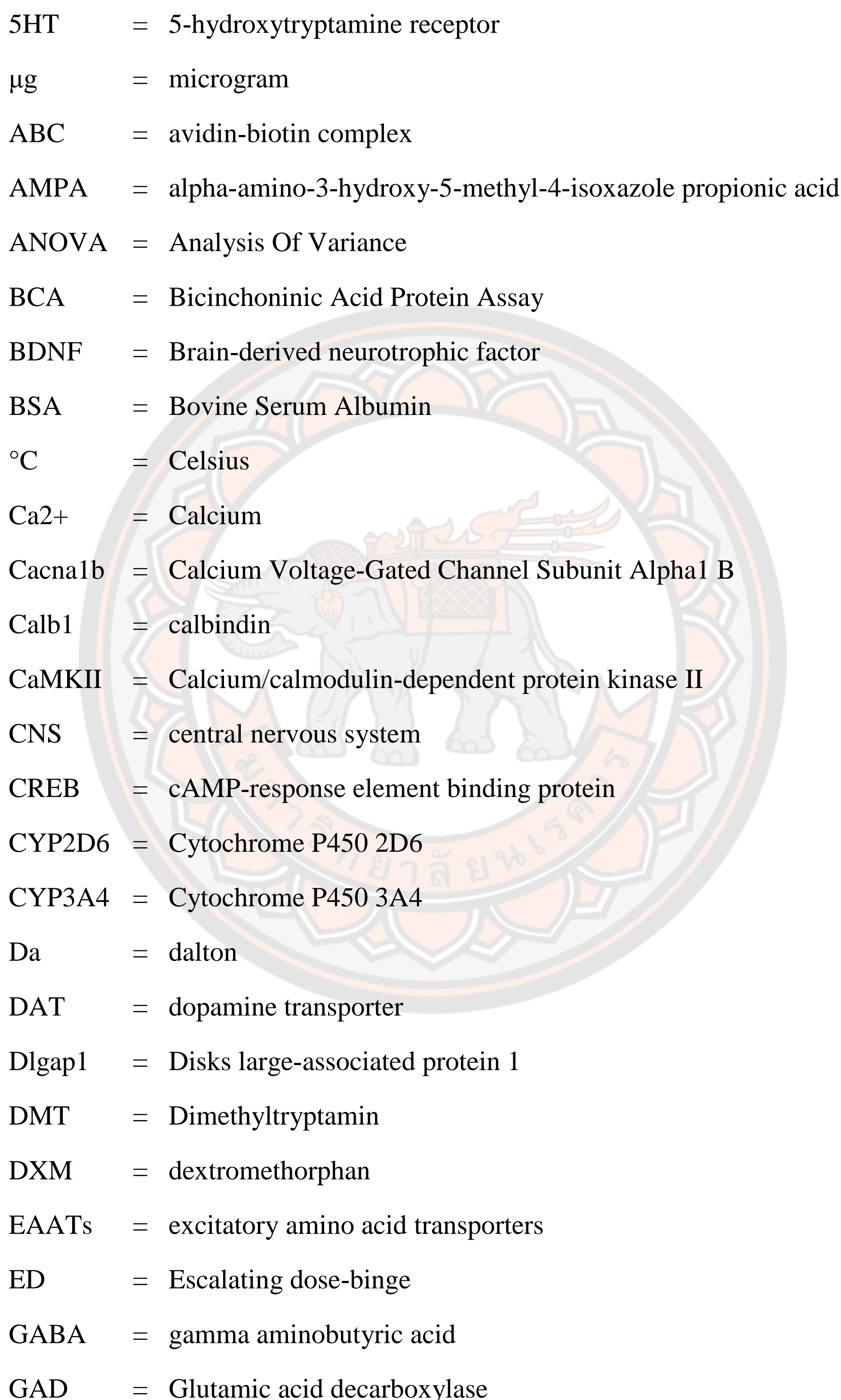
DXM = dextromethorphan

EAATs = excitatory amino acid transporters

ED = Escalating dose-binge

GABA = gamma aminobutyric acid

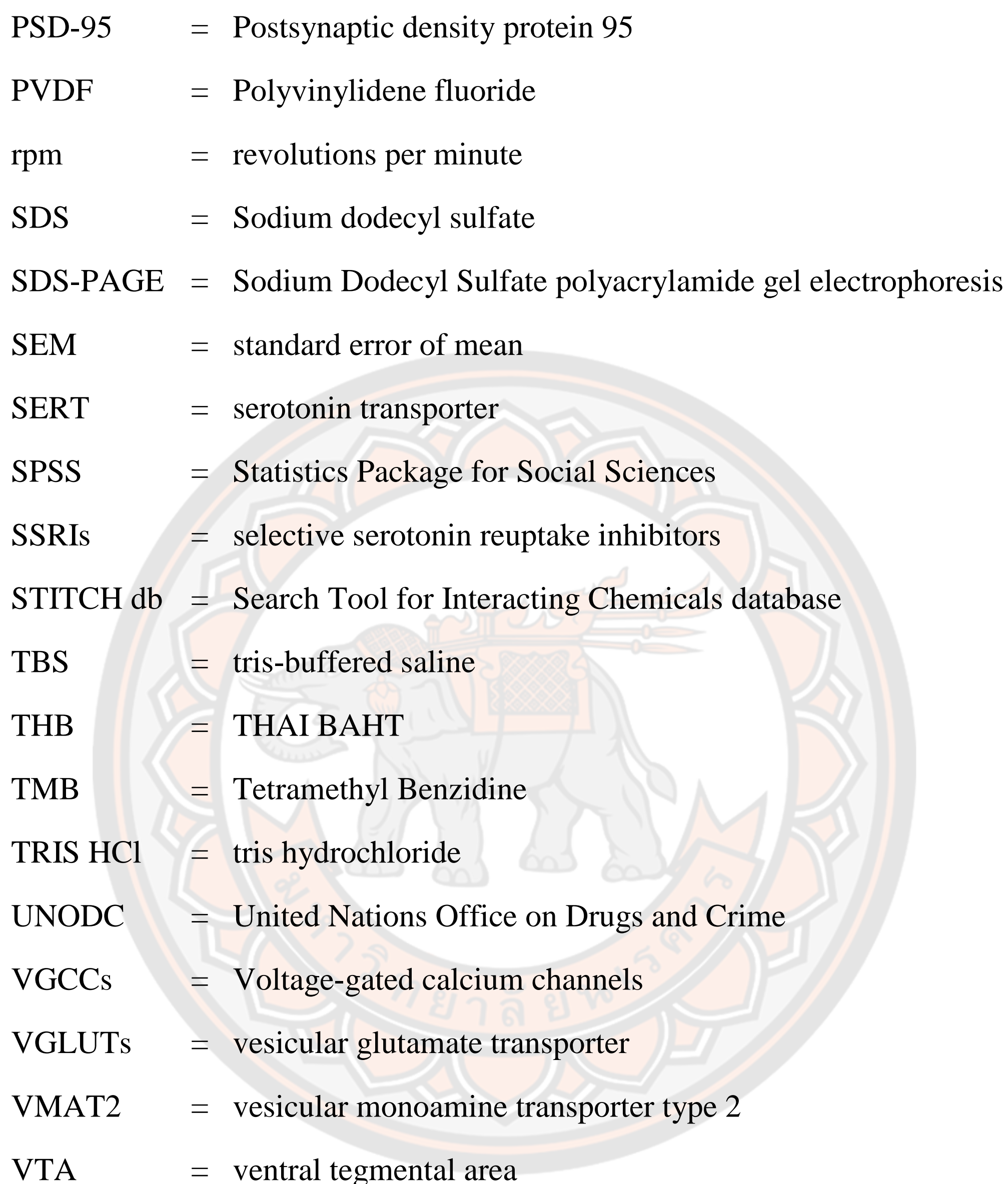
GAD = Glutamic acid decarboxylase



ABBREVIATION (Cont.)

GKAP	=	guanylate kinase-associated protein
GluR	=	Glutamate ionotropic receptor AMPA type
GO	=	Gene Ontology
Gria	=	Glutamate ionotropic receptor AMPA type
Grin	=	Glutamate ionotropic receptor NMDA type subunits
HBR Syrup	=	hydrobromide Syrup
kDa	=	kilodaltons
KEGG	=	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	=	liquid Chromatography with tandem mass spectrometry
M	=	Molar
MAOIs	=	monoamine oxidase
METH	=	methamphetamine
MeV	=	Multiple Experiment Viewer
mGluRs	=	metabotropic glutamate receptors
mM	=	millimolar
mRNA	=	Messenger ribonucleic acid
Na^{+}	=	Sodium
NaCl	=	Sodium chloride
NET	=	norepinephrine transporter
NMDA	=	N-methyl-D-aspartate receptor
NO	=	Nitric oxide
nNOS	=	Neuronal Nitric Oxide Synthase
NR	=	N-methyl-D-aspartate receptor
PCP	=	phencyclidine
PGBR	=	pre-germinated brown rice
Pld2	=	Phospholipase D2

ABBREVIATION (Cont.)



PSD-95 = Postsynaptic density protein 95

PVDF = Polyvinylidene fluoride

rpm = revolutions per minute

SDS = Sodium dodecyl sulfate

SDS-PAGE = Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis

SEM = standard error of mean

SERT = serotonin transporter

SPSS = Statistics Package for Social Sciences

SSRIs = selective serotonin reuptake inhibitors

STITCH db = Search Tool for Interacting Chemicals database

TBS = tris-buffered saline

THB = THAI BAHT

TMB = Tetramethyl Benzidine

TRIS HCl = tris hydrochloride

UNODC = United Nations Office on Drugs and Crime

VGCCs = Voltage-gated calcium channels

VGLUTs = vesicular glutamate transporter

VMAT2 = vesicular monoamine transporter type 2

VTA = ventral tegmental area

CHAPTER I

INTRODUCTION

Rationale and significance of the study

Drug addiction is regarded as a major public health problem. Drug addiction has been defined as a neuropsychiatric disorder characterized by behavioral changes and other responses resulting in compulsion to drug seeking and continued taking. Repeated uses of drugs induce stable changes in the brain at the molecular and cellular levels which may possibly underlie behavioral abnormalities (Zou et al., 2017). Furthermore, addiction can contribute to psychiatric disorders, such as schizophrenia, anxiety, mood, or impulse-control disorders. The most common addictive drugs are heroin, methamphetamine, tobacco and over the counter medication such as dextromethorphan. Psychostimulant drugs such as methamphetamine are a growing public health problem worldwide, that over half of the world resides in Southeast Asia, including Thailand. Methamphetamine use in Thailand has been reported more common among men than women and also found in children and youth (up to 25 years) which is a common and risky behavior due to curiosity (Angkurawaranon et al., 2018; Kanato et al., 2020). Long-term behavioral effects of methamphetamine have many negative consequences, including cognitive impairments, anxiety or depression, violent behavior, insomnia, repetitive movements and psychosis (Moszczynska & Callan, 2017). Methamphetamine use is directly associated with the release and blocks the reuptake of catecholamines in the central nervous system, including adrenaline, dopamine and serotonin (Courtney & Ray, 2014). In addition to the catecholamines, methamphetamine also leads to excessive glutamate release and decreases GABA (gamma-aminobutyric acid) (Hsieh et al., 2014). Chronic methamphetamine produces long-lasting alterations of these neurotransmitters, which may be leading to potentially of methamphetamine-induced neurotoxicity. The neurotoxic effects of METH include neuronal damage, neuronal apoptosis and neuroinflammatory response within the several brain regions (Yang et al., 2018), the frontal cortex as well. Not only methamphetamine is the drug problem in Thai youth but also over-the-counter cough

medicine dextromethorphan. Many teenagers feel that recreational use of dextromethorphan is safe to abuse because they are legal. In addition, it has an inexpensive price, easily accessible and a false perception that high-dose drugs are not dangerous. In contrast, other drugs of abuse such as ketamine and methamphetamine, which are not as easily accessible (Chomchai & Manaboriboon, 2012). Dextromethorphan is commonly used as an antitussive activity and is devoid of analgesic or addictive properties at therapeutic doses. Psychoactive and physical effects, including tachycardia, hypertension, agitation, ataxia, and psychosis have been reported following high-dose dextromethorphan (Banken & Foster, 2008; Bryner et al., 2006). Particularly, dextromethorphan and its major metabolite, dextrorphan, action is N-methyl-d-aspartate (NMDA) receptor antagonist, which high dose of dextromethorphan can produce hallucinations similar to ketamine and phencyclidine (Reissig et al., 2012). These properties of dextromethorphan can lead to high abuse and misuse potential. Other mechanisms of action of dextromethorphan are serotonin reuptake inhibitor and a sigma-1 receptor agonist (Nguyen et al., 2014; Silva & Dinis-Oliveira, 2020), both actions are efficient in inducing psychoactive effects.

There is much evidence that indicates addiction effects on several brain regions, which can cause cognitive, behavioral, and physiological abnormalities (Simon et al., 2001; Verdejo-García et al., 2006). Several studies showed that the drugs marked structural changes in hippocampus(Thompson et al., 2004), striatum and frontal brain regions (Ersche et al., 2011). The structural abnormalities also have related to behavioral problems of drug abuse. Specifically, the frontal cortex is a direct effect of drug abuse, including cocaine, cannabis and methamphetamine (Goldstein & Volkow, 2002). Interestingly, several neurochemical studies have shown drug addiction effect on the neurotransmitters including glutamate (Kerdsan et al., 2009; Kerdsan et al., 2012), amino acids γ-aminobutyric acid (GABA) (Brummelte et al., 2007), dopamine (Volkow et al., 2002) and associated protein (Veerasakul et al., 2016) in the frontal cortex. Furthermore, the altered proteins involved in oxidative stress, neuroinflammation and neurotrophic factor were also evidenced after drug exposure in the frontal cortex (A. Quinteros et al., 2019; Berríos-Cárcamo et al., 2020). So, over many years more research of therapeutic approaches for drug addiction have been utilized. GABAergic system is the major inhibitory neurotransmitter of the central

nervous system, which is the target of drug treatment, such as antidepressants, anticonvulsants, sedatives, and anxiolytics. Sedatives and anxiolytics are used to prevent and treat psychiatric symptoms in drug abusers. However, several GABAergic drugs, including benzodiazepines, barbiturates, neurosteroids and anesthetics, possess toxicity properties on cognitive function, physical health, and mental health (Henschel et al., 2008; Vargas, 2018). Nowadays, nutraceuticals are an alternative abundant interest due to potential nutritional, safety and therapeutic effects. Pre-germinated brown rice (PGBR), which is a new type of rice contains more nutritional components, such as GABA, γ-oryzanol, alpha-tocopherol (Vitamin E), pyridoxine (Vitamin B6) and thiamine (Vitamin B1) (Roboon et al., 2017). Particularly, GABA is found in high amounts in PGBR, and exhibits antidepressant and mood-stabilizing effects (Mamiya et al., 2007; Sakamoto et al., 2007). In addition, it was previously reported that PGBR induced enhancement of learning and memory ability in mice (Mamiya et al., 2004). However, there are few studies examining the effects of PGBR on brain functions. Therefore, it is good to investigate protein profiles in order to understand the underlying mechanisms after drug addiction administration and its treatment.

Proteomics is a tool in elucidating global changes in complex biological systems that involve large numbers and networks of proteins. The proteomic analysis has been performed to evaluate the effects of drug addiction on differential expression of proteins and functional biological processes. Several studies have demonstrated the association of drug exposures with differential expressions of proteins involved in oxidative stress, apoptosis, inflammation, and mitochondrial metabolism (Faure et al., 2009; Li et al., 2008). Similarly, the alterations in the expression of proteins in synaptic regulation, protein phosphatase signaling, mitochondrial function, and inhibitory GABAergic network were identified in methamphetamine administration (Wearne et al., 2015). Therefore, this study has investigated that drug addiction may affect alterations of proteins related to neurotransmitter signaling in rat frontal cortex, and the drug addiction treatment as well.

Purpose of the study

A general experiment objective

This experiment is designed to investigate the protein expression profiling in the rat frontal cortex after exposure to addictive drugs and addiction treatments.

Specific objectives

1. To investigate the protein expression profiling in the rat frontal cortex after exposure to addictive drugs.
2. To examine the comparative effects of drug addiction treatments in the rat frontal cortex of drug addiction.
3. To examine the alteration of identified protein expression in rat frontal cortex after exposure to drug addiction.

Hypotheses

A general experiment hypothesis

Drug addiction may affect the protein expression profiling in the rat frontal cortex.

Specific hypotheses

1. The effects of drug addiction may lead to changes in protein expression profiling of the rat frontal cortex.
2. Drug withdrawal and addiction treatments may lead to changes in the protein expression profiling of rat frontal cortex in addiction models.
3. The differential expression of neurotransmitter proteins in each group may be related to behavioral changes in the animal model of drug addiction.

Scope of the study

This study investigated the alteration of protein expression profiling in the rat frontal cortex of drug addiction models induced by methamphetamine and dextromethorphan by proteomic technique. Moreover, protein expression profiling of withdrawal and treatments (GABA, pre-germinated brown rice and diazepam drug) were also observed in the rat frontal cortex of DXM addiction model. The selected protein was then validated the proteomics results using the western blotting technique.

Keywords

Drug addiction, Methamphetamine, Dextromethorphan, Frontal cortex, Proteomics

Anticipated outcomes

1. To provide an understanding about the alteration of differentially expressed proteins in the rat frontal cortex of drug addiction.
2. To provide information about the possible mechanisms of the frontal cortex in methamphetamine and dextromethorphan addiction.
3. To support information about the proteomics data in the frontal cortex of methamphetamine and dextromethorphan addiction.
4. To provide an understanding of the pathways involved will lead to better treatment approaches to the addicted abusers.
5. To provide novel knowledge of pre-germinated brown rice for addiction treatment in the methamphetamine and dextromethorphan addiction.

Conceptual framework

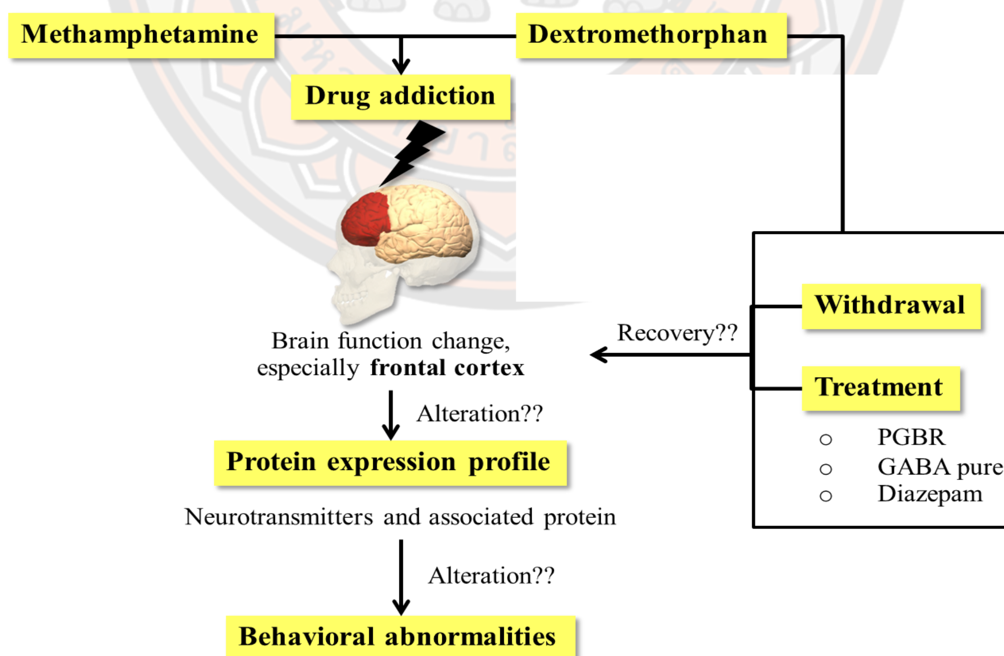


Figure 1 Conceptual framework of this study

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Drug addiction

Drug addiction or substance abuse refers to the misuse of medication without the use of drugs for treatment, but to be used for the change of mood, perception or to increase the bravery to do any activity. Addiction is a chronic mental disorder characterized by compulsive drug-seeking and drug-taking behavior continued use despite serious negative consequences (Nestler, 2001). When receiving the drugs for a period, the body will adjust from the effects of drugs as follows: drug dependence, drug tolerance, and drug withdrawal that these symptoms lead to drug addiction. Drug abuse can affect several aspects of a person's physical and psychological health that are long-lasting and permanent, certain drugs lead to drowsiness, insomnia, paranoia, or hallucinations. The classification of drugs used categorizes according to their expected major clinical effects into depressants, stimulants, hallucinogens, and volatile substances (Hill & Thomas, 2016). However, that some drugs have actions in more than one of these categories. Trends in drug use, cannabis is the most used illegal drug worldwide, followed by opioids and amphetamines (UNODC, 2021). Thailand is one of the one country in Southeast Asia in drug production and trafficking (Windle, 2017). Methamphetamine is the most used drug in Thailand (Macdonald & Nacapew, 2013; Saingam, 2018), among children and youth. In addition, found that the misuse of prescription drugs has been increased such as dextromethorphan, morphine, pseudoephedrine, diazepam, and others. Taking prescription drugs in a way that has not been recommended by a doctor can lead to drug addiction. Dextromethorphan, they represent the most abused substances among Thai youths (Chomchai & Manaboriboon, 2012).

Neurobiology of addiction

Drug addiction includes a relapsing cycle of intoxication, binging, withdrawal and craving that results in excessive drug use (Figure 2). Addiction appears to be a

combination of environmental factors and biological risk factors, like other neurobehavioral disorders, like schizophrenia or depression Drug addiction includes a relapsing cycle of intoxication, binging, withdrawal and craving that results in excessive drug use (Figure 2). Addiction appears to be a combination of environmental factors and biological risk factors, like other neurobehavioral disorders, like schizophrenia or depression (Crews & Boettiger, 2009) (Crews & Boettiger, 2009). All drugs of abuse trigger euphoric feelings consequent to increase of dopamine levels in the brain by mesolimbic system. Mesolimbic pathway has dopaminergic cells, play a pivotal role in the rewarding action of drugs of abuse. Mesolimbic pathway begins in the ventral tegmental area (VTA) and connects the nucleus accumbens, amygdala, hippocampus, and prefrontal cortex (Figure 3), that to be especially important to mediating pleasure, rewarding experiences and cognitive processes. When using an addictive drug or experiencing something otherwise rewarding, dopamine neurons in the VTA are activated. These neurons project to the nucleus accumbens via the mesolimbic dopamine pathway, and dopamine levels in the nucleus accumbens to rise. Suggesting that, these areas are important to addictive behavior. However, dopaminergic projections from the VTA travel to the prefrontal cortex as well; these fibers are involved in reward and motivational cognition (Adinoff, 2004; Volkow et al., 2013). The brain regions and biological mechanisms that underlie addiction that perturbation of cognitive control. As cognitive control is the pivotal role of the frontal cortex, there is interest to investigate the frontal cortex in the addiction.

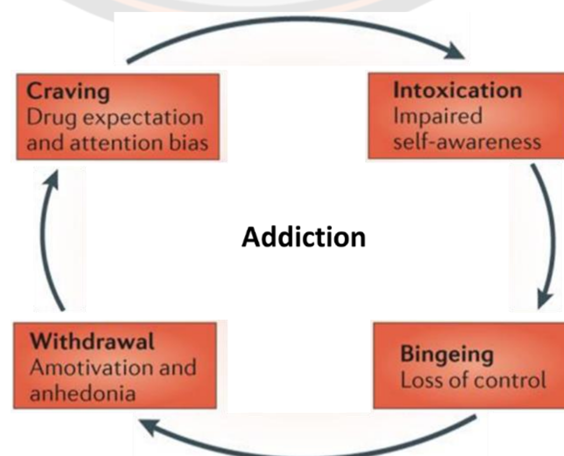


Figure 2 Behavioural manifestations of drug addiction (Goldstein & Volkow, 2002)

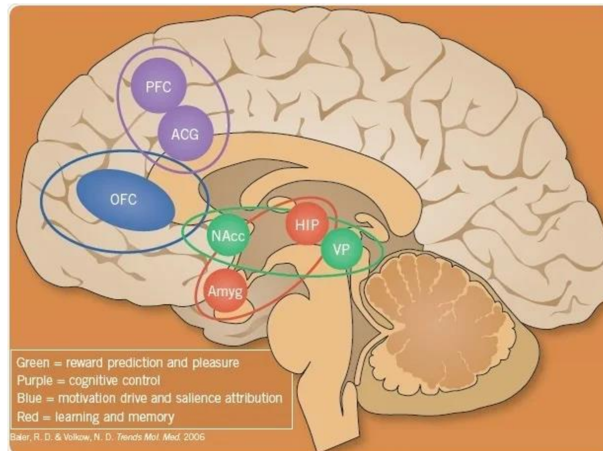


Figure 3 the brain circuits involved in addition. Image credit: Tocris Bioscience

Abnormalities in frontal cortex related with drug addiction

Frontal cortex, it is located at the front of the cerebrum that plays a critical role in the organization of behavioral, linguistic, and cognitive actions. In anatomy, it includes the parts of the brain from the frontal to the central sulcus. Therefore, if the frontal cortex is damaged, it will result in impairment in working memory, executive function, and emotional dyscontrol (Hoffmann, 2013; Stuss, 2011). Several studies in the brain's prefrontal cortex have been associated with impulsive action as a character of addictive behavior. Additionally, patients with damage to the prefrontal cortex are similarities in behavior to addicted individuals (Crews & Boettiger, 2009). Drug abuse alters the structure and function in frontal cortex (Mueller & Meyerhoff, 2021), producing cognitive abilities disruption that promote continued drug use and difficulty of abstinence (Goldstein & Volkow, 2011). The frontal cortex is responsible for cognitive control and depends on a GABA/glutamate balance. Drugs of abuse are critical to inducing or reducing one or the other, changing the glutamate and GABA balance (D'Souza, 2015). In addition, dopamine involvement in drug addiction is likely to mediate functional and structural changes in the frontal cortex (Goldstein & Volkow, 2002). The short-term drug administration has been traditionally associated with higher extracellular dopamine concentrations in frontal regions (Goeders & Smith, 1986). In human imaging studies have also revealed that the cues associated with drug-induced craving increase dopamine release in the prefrontal cortex, in human and animal models (Franklin et al., 2007; Milella et al., 2016).

Methamphetamine

Methamphetamine (METH) is a highly addictive central nervous system (CNS) stimulant drug, which causes a serious and growing worldwide problem associated with medical, social, and economic domains. In 1919, Akira Ogata synthesized METH using ephedrine, a compound of ephedra, with the pharmaceutical goals to CNS stimulation, bronchodilation, or nasal vasoconstriction (Vearrier et al., 2012). Globally, the production, distribution, and sale of METH is restricted or illegal in many countries, due to psychotropic substances. Southeast Asia is one of the largest synthetic markets, and consumption of METH, including Thailand. The drug was popularly used by workers, including male truck drivers, factory workers and farmhands, to work for longer periods and combat fatigue. In 1996, the Thai Ministry of Public Health changed the name to "Ya Ba" to give a negative implication to the drugs (Chomchai & Manaboriboon, 2012). The Thais population prevalence of drug use 82% in ages 15-64 years and use is higher among men than women. This drug is usually sold in tablet form (called "YA BA") and crystalline form (called "crystal meth" or "ice"), with the majority as d-methamphetamine form (Courtney & Ray, 2014; Macdonald & Nacapew, 2013). The routes of administration are intranasal sniffing, oral ingestion, inhalation, and injection. The plasma half-life of METH is approximately 10-12 hours and acute effects can persist to 8 hours, even though received only once (Cruickshank & Dyer, 2009). METH can be lipid-soluble due to the methyl group, which can distribute to many parts of the body, especially blood-brain-barrier. METH rapidly accumulates in the brain, and this is likely to increase its neurotoxicity (Turowski & Kenny, 2015).

The prominent effects of METH administration include heart rate, blood pressure, body temperature, loss of appetite, euphoria, arousal, and psychomotor activation (Cruickshank & Dyer, 2009). However, the effects are different depending on the dosage of drug administration. At the acute low doses (0.01-0.03 mg/kg), METH responses include hypolocomotion in mice whereas moderate (0.3-2.5 mg/kg) to high doses (5-10 mg/kg) induced hyperlocomotion (Singh et al., 2012). A chronic administration study of METH, at low, moderate, and high doses, evoked behavioral responses more than in acute studies (Peachey et al., 1976). Among METH users, dosing patterns tend to be initially used at lower doses before progressively increasing the dose (Clark et al., 2007), that is similarly to the escalating-binge dose model in

animals (Segal & Kuczenski, 1997). Importantly, the METH-induced alteration of neuropathological in frontal cortex and hippocampus have been reported in animal models, treated with escalating-binge dose (Kuczenski et al., 2007; Veerasakul et al., 2016). The evidence from several approaches suggests that prolonged METH exposure results in persistent changes in brain function.

The effect on neurotransmitter systems of methamphetamine primarily relates to the monoamine neurotransmitter systems, due to its structure, including dopamine, noradrenaline, and serotonin (Figure 4). Administration of METH increases the extracellular dopamine levels through prevents the reuptake of released dopamine and stimulates dopamine efflux. The extensive dopamine overflow after METH treatment appears critical to the subsequent neurotoxicity. Furthermore, the degeneration of dopamine nerve terminals was observed in animals exposed to METH, with reductions in dopamine transporter (DAT), tyrosine hydroxylase, and vesicular monoamine transporter-2 (VMAT2) protein levels (Lohr et al., 2015; Nordahl et al., 2003). In addition to dopamine, METH also impacts serotonin (5-HT), noradrenaline, and glutamate neurotransmitter systems through interactions with 5-HT transporters (Haughey et al., 2000), monoamine transporters, and N-methyl-D-aspartate (NMDA) receptors (Kerdsan et al., 2009). The mechanism of METH damage to other neurotransmitters is unknown, and the release of dopamine overflow is predicted to be an intermediate in the cause of another neurodegeneration (Courtney & Ray, 2014; Stephans & Yamamoto, 1994).

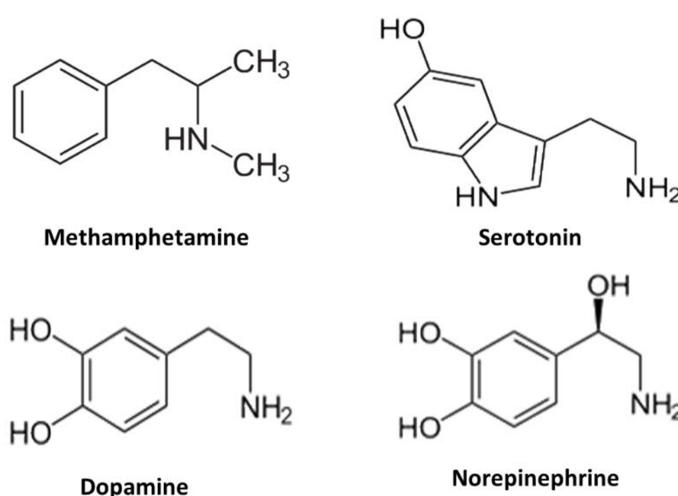


Figure 4 the chemical structures of METH and monoamine neurotransmitter

Dextromethorphan

Dextromethorphan (DXM) is a drug used in over-the-counter cough and cold medicines worldwide for over 50 years. It is a synthetic compound, which is the d-isomer of the levorphanol and codeine analogue (Siu & Drachtman, 2007). In Thailand, the first report of DXM misuse appeared in 2005, teenagers and young adults (age 12 to 25 years) have been popularly abusing of DXM for euphoria and hallucination (Bryner et al., 2006; Chomchai & Manaboriboon, 2012; Rosenbaum et al.; Schwartz, 2005). At the present, DXM is classified as a dangerous drug that can be received only when prescribed medication. DXM is a centrally acting agent cough suppressant, it is believed to suppress cough by acting on the medulla oblongata. It does not affect the central nervous system (CNS) when taken at therapeutic doses. At the high doses, it may produce adverse effects such as hallucinations, agitation, fatigue, drowsiness, especially addictive properties. The symptoms of the above are like dissociative drugs such as ketamine or phencyclidine (PCP) (Burns & Boyer, 2013; Chyka et al., 2007; Nguyen & Matsumoto, 2015).

DXM has three major metabolites: major active metabolite dextrorphan (DXO) and two minor 3-hydroxymorphinan, and 3-methoxymorphinan (Silva & Dinis-Oliveira, 2020). Both DXM and major metabolite DXO are responsible for the drug's psychoactive effects (Reissig et al., 2012). However, its pharmacology is not clear. DXM has been shown to bind to sigma opioid receptors (σ opioid receptor) in medulla oblongata to produce antitussive activity. In addition, DXM can risk to serotonin syndrome to be due to excessive stimulation at the 5HT2 receptor cause to neuronal serotonin release (Schwartz et al., 2008), which appear as altered mental status, seizures, hyperthermia, and death (Chyka et al., 2007; Rosenbaum et al.). High doses of DXM, an NMDA antagonist, which action results in neurobehavioral effects like dissociative agents (e.g. dysphoria, hallucinations, agitation, sedation) (Reissig et al., 2012). However, the excessive release of glutamate was evidenced by DXM or its metabolite treatment in rat cerebral cortex nerve terminals (Lin et al., 2009).

Pre-germinated brown rice (PGBR)

For Thai people, rice (Oryza sativa) is a daily dietary staple food and the source of carbohydrate and energy. Pre-germinated brown rice (PGBR), one kind of rice, is

slightly germinated by soaking brown rice in water as this reduces the hardness of brown rice and makes it easier to eat. PGBR is necessary for enhancing nutrients required for good health, and is evaluated as a functional food because it is good in digestion and absorption. Consumption of PGBR is increasing attention supported by evidence on effects reduction in the risk of some diseases such as obesity (Lim et al., 2016), cardiovascular diseases (Liang et al., 2020), type 2 diabetes (Bui et al., 2014; Liang et al., 2020) and neurodegenerative diseases (Mamiya et al., 2004). During germination, many nutrients in PGBR were increased nutritious (proteins, fat, and dietary fibre) and bioactive compounds (gamma-oryzanol, ferulic acid, and phytate) (Banchuen et al., 2009; Patil & Khan, 2011). The examples of biological activities in germinated rice as shown in Table 1. The γ-Oryzanol, which has a lot of beneficial effects, is a mixture of ferulic acid. It has been associated with various beneficial effects such as antioxidants, immune system, inflammation and neuroprotective (Sulaiman et al., 2021). The major phenolic acids of rice were ferulic acid, which has the capability to help to reduce blood pressure and protect skin (Patil & Khan, 2011). Phytates or phytic acid is the major storage form of phosphate in cereal grains, such as rice. Phytic acid has some anticancer and antioxidant functions because of its functions in reducing lipid peroxidation and inhibits hydroxyl radical formation (Shamsuddin, 2002). Besides, many studies reported that germination could induce the formation of new bioactive compounds, such as GABA (Cáceres et al., 2017). The protein in PGBR can break down into free amino acids, especially glutamic acid which can be changed into GABA compound (Jannoey et al., 2010) and can be carried into the brain through the BBB (Mamiya et al., 2007).

Table 1 Examples of biological activities in germinated rice

Nutrients	**Biological activities in germinated rice**
GABA	Hypotensive effect, accelerating metabolism in brain, preventing headaches or depressions aftereffects of cerebral arteriosclerosis and cerebral apoplexy, preventing climacteric disorder, preventing

Nutrients	Biological activities in germinated rice
	presenile derangement such as insomnia and mental irritation, Activating renal function
Dietary fiber	Relieving constipation, preventing cancer of colon, Regulating blood sugar levels
Ferulic acid	Scavenging super oxides, Suppressing melanogenesis
Phytic acid	Antioxidative effect, protecting cardiovascular disease, preventing platelet aggregation
Gamma-oryzanol	Antioxidative effect, preventing skin aging, modulating cholesterol values

Source: (Patil & Khan, 2011)

Proteomic techniques

The proteomics describes the study and characterization of proteomes that is characterized regarding the localization, interactions, and post-translational modifications and turnover, at a time. The proteome is the overall protein content of a cell, first used by Marc Wilkins in 1996 to mean the “protein complement of a genome”. The gene function can be comprehended by the proteomic methodology. This technique is particularly useful to study disease diagnosis; since the proteomic technique can characterize differentiate of expression, structure, functions, interactions and modifications that contribute to be identified the disease state (Aslam et al., 2017; Chandramouli & Qian, 2009; Faure et al., 2009). Mass spectrometry-based methods for the identification of proteins have become a standard in proteomic methodology (Delahunty & Yates Iii, 2005). The results are shown in the form of protein expression profile. Therefore, the proteomic approach is useful in the investigation of the molecular basis of drug addiction to consider the type of protein that effect on the brain can cause behavioral differences and to find further treatment.

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CHAPTER III

RESEARCH METHODOLOGY

This study is designed to investigate the differential protein expression in rat frontal cortex associated with drug addiction including methamphetamine and dextromethorphan compared with control. This study comprises four parts as follows: (1) animal models of drug addiction, (2) sample preparation for proteomic analysis and western blotting, (3) proteomic analysis of protein expression profile and (4) western blot analysis of protein expression changes. Protocols for experimental study were approved by the Naresuan Animal Ethics Committee for the use of experimental animals, reference number 62 02 014.

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 between 280-350 g were obtained from the National Laboratory Animal Center of Mahidol University, Nakhon Pathom, Thailand. The animals were maintained under conditions of controlled temperature (24 ± 1°C) and 12-hour light and dark cycle, with access to food and water. All animal procedures were carried out in compliance with Mahidol University Code of practice and the National Institutes of Health Guidelines for treatment of laboratory animals.

METH administration

This study used D-METH hydrochloride (Lipomed AG, Arlesheim, Switzerland) with the permission of the Ministry of public health. The drug was dissolved in saline and administered with intraperitoneal (i.p.) injection. The METH administration method was adapted from Segal et al. (Segal et al., 2003). Briefly, animals were divided into 2 groups (n=10 per group) as described; (1) control group (C), the animals were injected i.p. with saline 3 times a day for 14 days and 4 times at day 15, and (2) METH group, the animals were injected i.p. with escalating-binge dose

(three times per day) at the initial dose 0.1 mg/kg up to 4.0 mg/kg for 14 days. On day 15, animals were received 4 times of 6 mg/kg. The schedule for escalating and binge dese of METH injections is shown in Table 2. All animals were sacrificed by cervical dislocation. The brain tissues were collected and stored at -80°C until used.

Table 2 Schedule of METH administration

Day / Time	**METH dose (mg/kg)**		
	07:30	**10:30**	**13:30**
1	0.1	0.2	0.3
2	0.5	0.4	0.6
3	0.7	0.8	0.9
4	1.0	1.1	1.2
5	1.3	1.4	1.5
6	1.6	1.7	1.8
7	1.9	2.0	2.1
8	2.2	2.3	2.4
9	2.5	2.6	2.7
10	2.8	2.9	3.0
11	3.1	3.2	3.3
12	3.4	3.5	3.6
13	3.7	3.8	3.9
14	4.0	4.0	4.0
15	(4 x 6.0 mg/kg; 2 h intervals)		

Source: (Segal et al., 2003)

Animal model of dextromethorphan addiction

Animals

Male Sprague-Dawley rats weighing between 200-250 g were obtained from the National Laboratory Animal Center of Mahidol University, Nakhon Pathom, Thailand. The animals were maintained under conditions of controlled temperature (24 ± 1°C) and 12-hour light and dark cycle, with access to food and water. All animal procedures were carried out in compliance with Mahidol University Code of practice and the National Institutes of Health Guidelines for treatment of laboratory animals.

Dextromethorphan and substances administration

This study used dextromethorphan hydrobromide (C18H25NO.HBr.H2O) that was purchased from Sigma-Aldrich® Lot#090M1298V. Diazepam was obtained from Naresuan University Hospital. The pre-germinated brown rice (PGBR) was provided by the Laboratory of Faculty of Agriculture Natural Resources and Environment, Naresuan University. PGBR powder made from brown rice (strain of Oryza sativa var. glutinosa), which was obtained from Khek Noi, Khao Kho, Phetchabun (Thailand), was soaked for 24 hours until germinated. After that, PGBR were dried and produced to powder. The substances in PGBR were assessed by the Central Laboratory (Thailand) Co., Ltd., contain the major substances including GABA, Gamma oryzanol, vitamin E, vitamin B6 and vitamin B1 as shown in Table 3. Gamma aminobutyric acid, synthetic GABA was purchased from Sigma Chemical Company, St. Louis, USA. Amount of synthetic GABA was equal to the GABA found in PGBR.

Table 3 The major substances in PGBR

Substances	Volumes (mg/100g)
GABA	16.51
Gamma oryzanol	29.61
Alpha tocopherol (Vitamin E)	0.91
Pyridoxine (Vitamin B_6)	0.11
Thiamine (Vitamin B_1)	0.05

Source: (Roboon et al., 2017)

The animals were divided into six groups (Figure 5) including control (C), dextromethorphan (D), withdrawal (DW), diazepam (DD), synthetic GABA (DS), and

pre-germinated brown rice (DR). The animals in the control group received saline i.p. for 15 days and treated with distilled water by oral administration for 60 days. The animals in D, DW, DD, DS and DR groups received dextromethorphan i.p. for 15 days and D animals were then sacrificed. Animals in DW, DD, DS and DR were divided into 3 subgroups which received treatment for 15, 30, 60 day. Animals in the DW group were drug-withdrawal by receiving oral administration of distilled water. Animals in the DD group received 10 mg/kg diazepam. Animals in the DS group were administered with 0.8 mg/kg synthetic GABA. Animals in the DR group were treated with 5 mg/kg PGBR. After the last administration of the subgroup, animals were sacrificed, and the brain was collected.

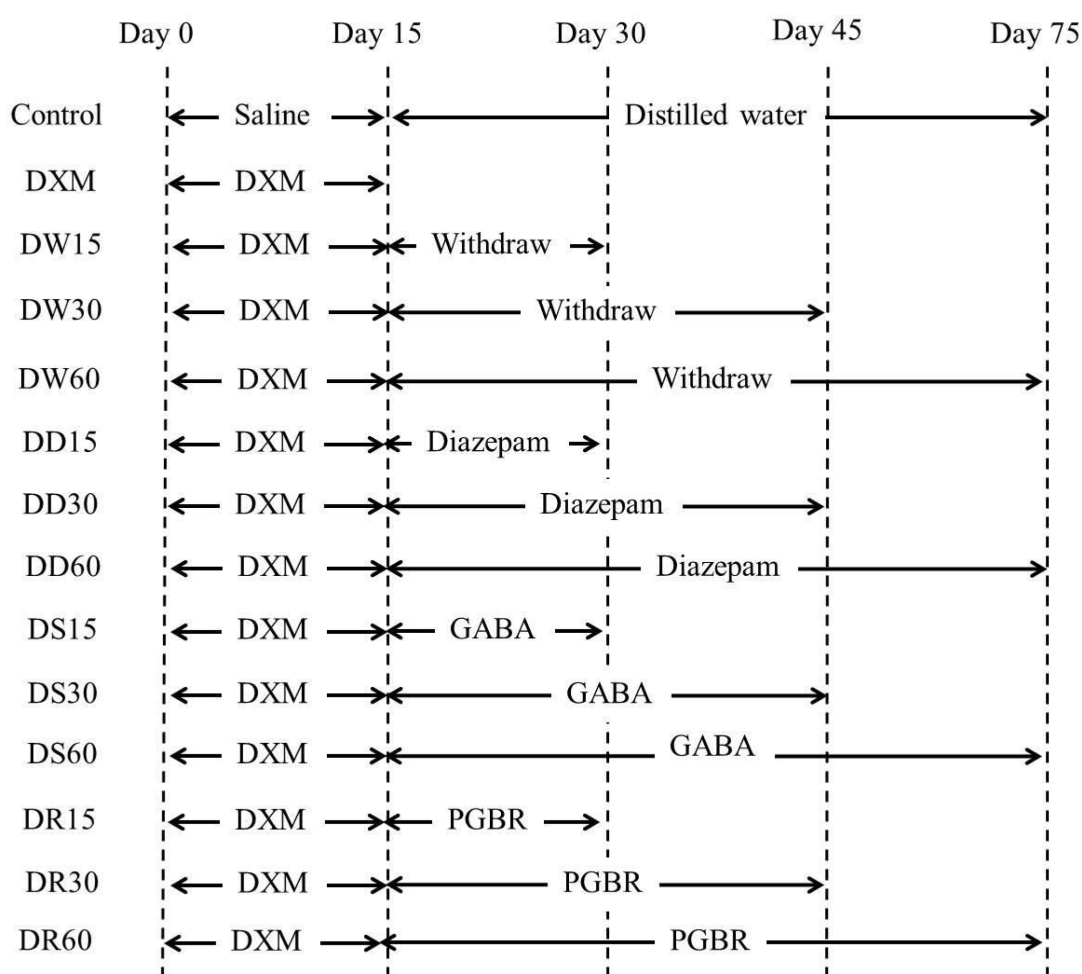


Figure 5 The schematic diagram of DXM and substances administration

Sample preparation for proteomic analysis and western blotting

Protein extraction

The frontal cortex is weighed (20 µg) and homogenized in 12 volumes of homogenate buffer (5 mM Tris-HCl, pH 8.0 and 25 mM NaCl). The homogenate was centrifuged at 14,000 rpm, 4°C for 10 minutes. The pellets were collected and re-homogenized in 4 volumes of lysis buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% SDS, 0.25% sodium deoxycholate and 1% protease inhibitor cocktail). Frontal cortex lysates were kept on ice for 1 hour to complete tissue lysis and were stored at -20°C until assay.

Protein concentration

Protein concentration was determined by the bicinchoninic acid (BCA) assay kit (Thermo Scientific, Pierce, Rockford, IL., USA) with bovine serum albumin (BSA) as the protein standard. The BSA standard had concentration between 0.25 to 1 mg/ml. The homogenate sample was diluted in the lysis buffer and measurements were performed in triplicate. The BCA working reagent, combination of BCA reagent A and B (50:1), was added to the sample and protein standard. The solution was mixed and incubated for 30 min at 37°C. Absorbance was read at a wavelength of 562 by the microplate reader (Labsystem iEMS Reader MF) with Transmit Software Revision 4.5. The protein samples of unknown concentration were determined with the standard curve of known protein concentration.

Proteomic analysis of protein expression profile

Protein digestion

Protein samples of each group were pooled (n = 3) for proteomic analysis by Liquid Chromatography with tandem mass spectrometry (LC-MS/MS). A total of 10 µg of lysate samples were reduced with 10 mM dithiothreitol in 10mM ammonium bicarbonate and were incubated at 56°C for 1 hour. After that, the alkylation with 30mM iodoacetamide in 10 mM ammonium bicarbonate and were incubated at room temperature for 1 hour. To perform in-solution digestion, the protein samples were digested with 50 mg trypsin (Promega, Madison, WI, USA) in 10mM ammonium bicarbonate overnight at 37°C. The peptide samples were transferred to the vial for injecting or kept at -20°C for further mass spectrometric analysis.

Liquid Chromatography with tandem mass spectrometry (LC-MS/MS)

The digested peptide solutions were analyzed by 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 is 0.1% formic acid and eluent B is 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 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 fragment mass spectra were acquired in data-dependent AutoMS mode with selecting most abundant precursor ions in 3 second cycles for fragmentation. The mass range of the MS scan is set to extend from 150 to 2200 m/z. The MS/MS data was submitted for a database search using the Mascot software (Matix Science, London, UK, (Perkins et al., 1999)). The data is searched against the NCBI database for protein identification. Database interrogation is; taxonomy (Rattus); enzyme (trypsin); variables 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 mas missed cleavages.

Proteins quantitation and identification

For protein quantitation, DeCyderMS Differential Analysis Software (DeCyderMS, GE Healthcare (Johansson et al., 2006; Thorsell et al., 2007)) was used. 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 maximum value of each group was used to determine the presence or absence of each identified protein. To indicate significant changes in protein expression, a fold-change of ≥±1.2 were set as cut-off values.

Data normalization and quantification of the changes in protein abundance between the control and treated samples was performed and visualized using MultiExperiment Viewer (Mev) software version 4.6.1 (Howe et al., 2010). Briefly, peptide intensities from the LC-MS analyses are transformed and normalized using a mean central tendency procedure. The database for Annotation, Visualization and

Integrated Discovery (DAVID 6.8, https://david.ncifcrf.gov/) was used to classify significant differential expressed proteins by their biological processes, molecular functions, or cellular components using gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway with the significant (Benjamini-Hochberg false discovery rate <0.05) were identified (Dennis et al., 2003). The identified proteins are simultaneously submitted to The Search Tool for Interacting Chemicals (STITCH) (http://stitch.embl.de) to search for understanding of cellular functions and Interactions between proteins and small molecules (Szklarczyk et al., 2016), and Pathway Studio™ (https://www.pathwaystudio.com/) to search for understand underlying biology of diseases, responses to drugs, and a wide range of biological processes.

Western blot analysis of protein expression changes

Protein preparation for SDS-PAGE

The protein samples in an equal volume to 2XSDS-PAGE sample buffer (0.5M Tris-HCl, pH 6.8, 20% sodium dodecyl sulfate, 0.12% Glycerol, 0.03% B-mercaptoethanol and 1% Bromophenol blue) was prepared and heat at 100°C for 5 min to denature proteins. The denatured protein sample was placed on ice for 3 min to stop reaction. Then, the sample was centrifuged at 3,000 rpm for 1 min. This protein supernatant was used for SDS-PAGE.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For AMPA3 detecting, the 20 µL of 40 µg proteins were separated in 4% stacking and 8% separating gel for 2 hours with 100-volt, 400 mA. For calbindin detecting, the 20 µL of 40 µg proteins were separated in 4% stacking and 12.5% separating gel for 2 hours with 90-volt, 400 mA. In this study, proteins were run and separated depending on protein size. Prestained SDS-PAGE standard (GeneDireX, Inc., USA) ranging from 3.5 to 245 kDa was run with sample as marker. Beta-actin was used as an internal control to confirm equal protein loading and sample transfer.

Protein blotting

After electrophoresis, separating proteins were transferred from the electrophoresis gel to polyvinylidene fluoride (PVDF) membranes (Amersham, Arlington Heights, IL, USA) with a wet transfer (2 hour, 120 V, 400 mA) for detected AMPA3 and semi-dry transfer (1.30 hour, 25 V, 120 mA) for detected calbindin.

Following, to confirm protein transfer, the gel was stained with Coomassie Brilliant Blue R-250 and was destined with destaining solution until clear background. The membrane was stained with Ponceau S solution (Sigma-Aldrich Co. LLC, St. Louis, Mo, USA) to confirm protein complete transfer and was washed with distilled water. The membrane could dry at room temperature and kept at 4°C until immunodetection.

Protein detection of AMPA3, calbindin and beta-actin

The band of protein was detected by an indirect immunoblotting method. The membranes were rehydrated by methanol and washed three times with 1XTBST (Tris-buffered saline, 0.1% Tween-20). Following, the membrane was blocked non-specific protein by 5% bovine serum albumin (BSA) or 5% nonfat dry milk in 1X TBST. The membrane was incubated with a primary for anti-AMPA3 (Gria3) (dilution 1:200, ab40845, Abcam, UK), anti-calbindin (dilution 1:2,000, C2724, Sigma-Aldrich Co. LLC., St. Louis, Mo, USA) and anti-beta-actin (dilution 1:1,000, 8H10D10, Cell Signaling Technology, Inc., Danvers, MA, USA). Then, the membrane was washed three times with 1X TBST and added a goat anti-rabbit (dilution 1:400, BA-1000, Vector Laboratories Inc., Burlingame, CA, USA) and goat anti-mouse (dilution 1:200, BA-9200, Vector Laboratories Inc., Burlingame, CA, USA) to bind with the primary antibody. Following three washes with 1X TBST, the membrane was incubated with avidin-biotinylated horseradish complex (ABC) kit (dilution 1:100, PK4000, Vector Laboratories Inc., Burlingame, CA, USA). The membrane was washed three times with 1X TBST and two times with 1X TBS. Latterly, the proteins on the membrane was visualized by peroxidase substrate, 3, 3’, 5, 5’-tetramethylbenzidine (TMB) (Promega Corporation, Madison, WI, USA) and stop reaction with distilled water. The detected membrane could dry and scanned into a computer for analysis. All protein bands were measured with integrated optical density by ImageJ software. The expression of AMPA3 and calbindin were normalized by beta-actin. The results were calculated and reported as percentage of control.

Statistical analysis

Data was expressed as mean ± standard error of the mean (SEM). Statistical analysis was used SPSS 17.0 (SPSS Inc., USA) by an independent sample t-test for

differences between groups. $P < 0.05$ was considered statistically significant for all data analyzed.

CHAPTER IV

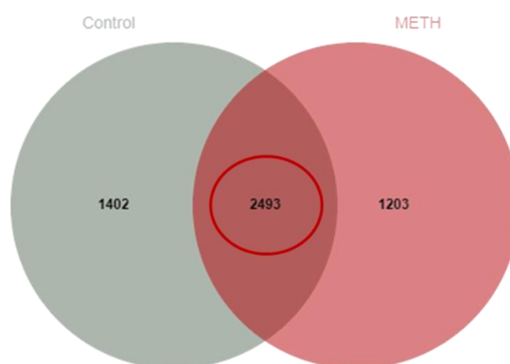
RESULTS

METH exposure leads to the alteration of the neurotransmitter system in rat frontal cortex

To identify the proteins related to the METH addiction, the proteomic technique (LC-MS/MS) was applied in this study. The LC-MS/MS data of protein expression showed 5,098 proteins in the control and the METH groups in which co-expression of 2,493 proteins were observed in both groups (Figure 6). Here, the co-expressed proteins were collected for analysis. Under the premise that the co-expressed proteins are more likely to be co-regulated by addiction conditions, major changes in co-expression may indicate changes in addiction mechanisms. Regarding 2,495 co-expression proteins, the 1,332 differentially expressed proteins were classified following the fold change standard ≥1.2 or ≤−1.2. To understand these proteins' functional and pathway with each other involved in the neurotransmitter system, the GO annotation in the cellular component and mapping in the KEGG pathway were performed. The results of GO annotation of these 1,332 proteins show that these proteins are involved in the composition of cell; cell, organelles, membranes and macromolecular complexes and so on (Figure 7). After GO analysis, a total of 137 consistently expressed proteins were identified from synapse-related proteins. To investigate the functional pathway of differentially expressed proteins in METH addiction, 137 proteins were mapped to the KEGG database in DAVID 6.8 (Figure 8). The top 20 identified pathways display high counts for protein abundance, the neuroactive ligand-receptor interaction was the most markedly enriched in differential protein. The neurotransmitter signaling pathway was also found in differentially expressed protein. Notably, the glutamatergic and GABAergic synapse were most markedly enriched in differentially expressed proteins. In line with our previous human and animal studies, the glutamatergic system (Iamjan et al., 2018; Kerdsan et al., 2009; Kerdsan et al., 2012) and GABAergic system (Veerasakul et al., 2016; Veerasakul, Watiktinkorn, Thanoi, Dalton, et al., 2017; Veerasakul, Watiktinkorn, Thanoi, Reynolds, et al., 2017) was also disrupted by METH. So, we chose the ‘glutamatergic and GABAergic system’ to analyze the

mechanism of METH on the frontal cortex. The protein expression values were extracted to heatmap and plotted to present the differential expressed proteins (Figure 9). In glutamatergic system was found the 11 of differences proteins when comparing the group samples, namely Dlgap1, Gria3, Grik2, Grin2a, Grm1, Grm5, Grm6, Itpr1, Pld2, Slc17a6 and Slc1a2. The number of up-regulation proteins was significantly lower than the down-regulated proteins (1vs10) after METH addiction. Meanwhile, the GABAergic system was identified equal of the number of up- and down-regulated proteins (4vs4), namely Gabbr2, Gabbr1, Gabrg3, Gabrg2, Nsf, Gabrg1, Cacna1b, and Gad1. Furthermore, the standard fold change in expression proteins was shown in Table 4, these analyses revealed significant changes in the expression of the glutamatergic and GABAergic identified proteins.

Targets of METH were obtained based on STITCH databases, which represent the different types of interactions between drugs and proteins by protein–protein interactions (PPIs) network (Figur10). The protein network was constructed and analyzed the differentially expressed proteins in glutamatergic and GABAergic systems together with METH, was 19 proteins. The PPIs network showed the glutamatergic system was the main target of METH addiction mechanism and can disrupt the GABAergic system through glutamatergic system. Each candidate protein influences the brain function from METH addiction. Interestingly, the calbindin (Calb1) was a part of the network in the GABAergic system. The Calb1 is one of calcium binding proteins, has a role in maintenance of calcium homeostasis within neurons (Fairless et al., 2019) and was identified as a subgroup of GABAergic interneurons (Reynolds et al., 2004). To better understand the molecular target of METH addiction, the PPIs network was performed by Pathway Studio to search for understanding addiction behavior (Figure 11). Similarly, of the candidate proteins associated with addiction behavior. Highlighting, the Pathway Studio’s PPIs network which found the regulatory of Calb1 was binding to Gria3 in addiction behavior condition. The glutamate receptor 3 (Gria3), subunit of glutamate receptor, has a role in excitatory synaptic transmission. Together, these findings further suggest that METH causes disturbances in the 'Glutamatergic-GABAergic system'.



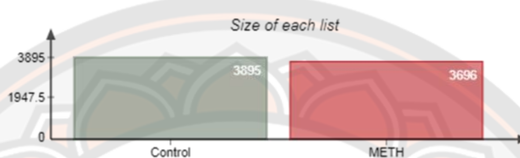


Figure 6 **Venn diagrams of unique and shared proteins of METH addiction in rat frontal cortex**

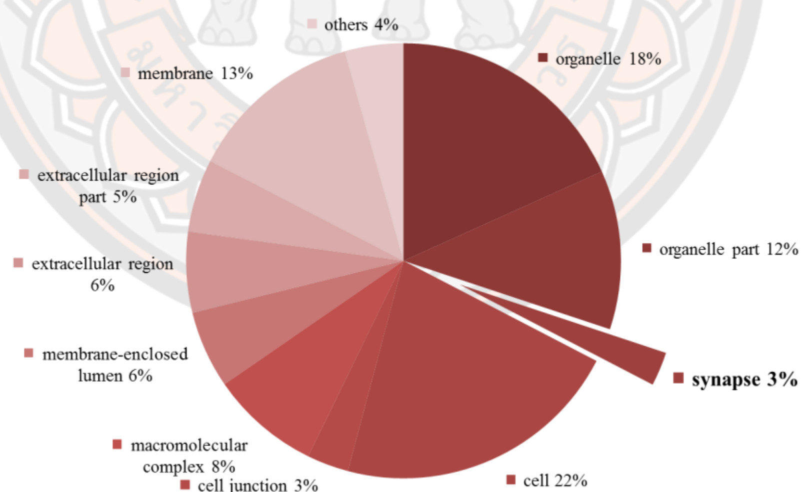


Figure 7 **Percentages of proteins enriched in different cellular components after METH addiction, according to Gene Ontology enrichment analysis.**

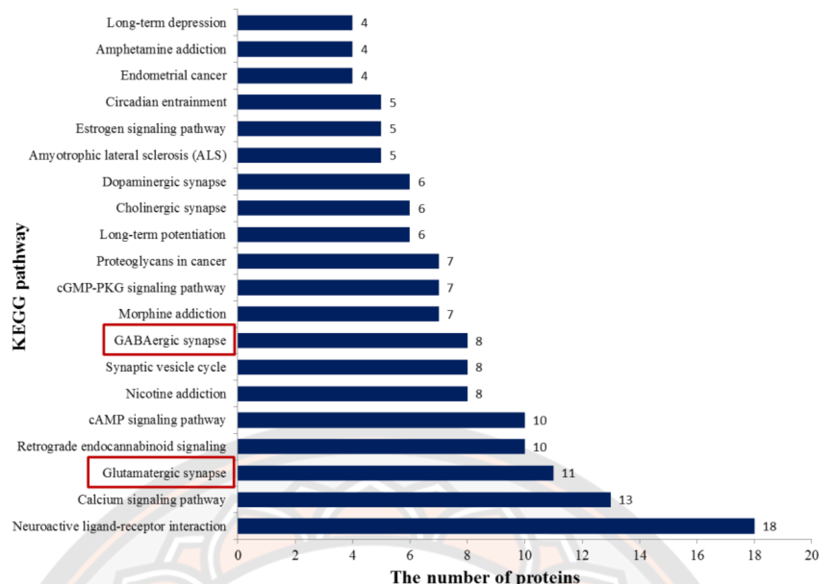


Figure 8 **Enriched KEGG pathway analysis of the identified synapse proteins after METH exposure, p-value < 0.05**

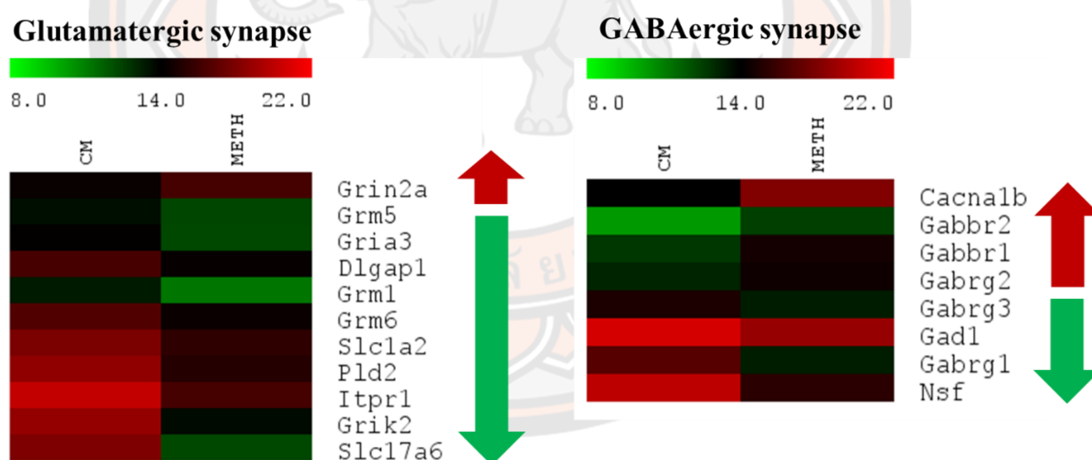


Figure 9 **Heat map showing differentially expressed proteins in METH addiction compared to the control. Green arrow represents down-regulation and red arrow represents up-regulation, performed in MeV software.**

Table 4 **The frontal cortex glutamatergic and GABAergic system proteins alterations after METH addiction.**

Accession Number	Gene name	FC METH/CM	Accession Number	Gene name	FC METH/CM
Glutamatergic synapse			**GABAergic synapse**		
Q00959	Grin2a	1.90	Q02294	Cacna1b	4.01
P31424	Dlgap1	-2.01	O88871	Gabbr2	2.16
P19492	Slc1a2	-2.41	Q9Z0U4	Gabbr1	1.95
P97836	Gria3	-1.80	P18508	Gabrg2	1.32
P23385	Grik2	-4.93	P28473	Gabrg3	-1.59
P35349	Grm1	-2.13	P18088	Gad1	-1.92
P31596	Grm5	-1.30	P23574	Gabrg1	-3.44
P70498	Grm6	-2.18	Q9QUL6	Nsf	-4.53
P29994	Itpr1	-3.93			
P42260	Pld2	-3.36			
Q9JI12	Slc17a6	-5.70			

FC = Fold change

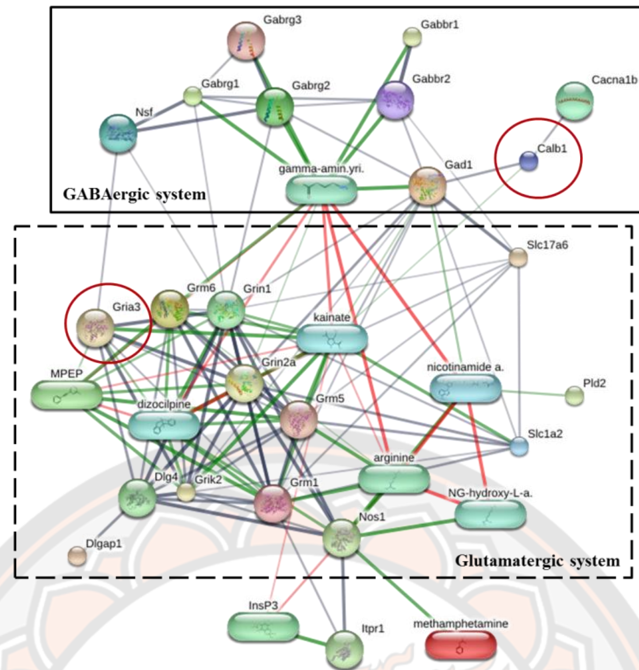


Figure 10 **STITCH 5.0 database predicted the interactions between METH and candidate protein. Modes of action are shown in different color lines.**

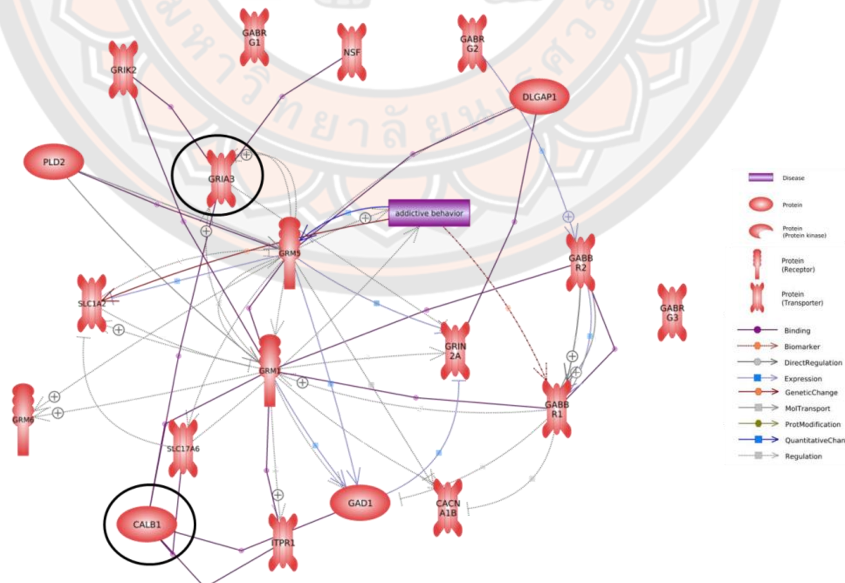


Figure 11 **The Pathway Studio database predicted the interactions between proteins and neuronal signaling systems response to METH addiction. Modes of action are shown in different type lines.**

To validate the results of the LC-MS/MS data was performed western blotting to examine the key functional proteins in neuronal signaling in the METH addiction. Interestingly, our previous studies in genetic variation of Gria3 gene associated with METH dependence (Iamjan et al., 2018) and this protein was also found in this proteomic study. Furthermore, the reduction of calbindin (calb1) demonstrated in the frontal cortex after METH administration (Veerasakul et al., 2016). Therefore, the glutamate receptor 3 (Gria3) and calbindin (calb1) were selected to verify the reliability of quantitative proteomics with molecular biotechnology. These data were consistent with the results of proteomic data.

Expression of AMPA3 in rat frontal cortex after METH exposure

The results showed that the mean value of relative AMPA3 protein intensity in control and METH groups were 1.3118 ± 0.470 and 0.7701 ± 0.2143, respectively as shown in Table 8 and Figure 12. There was a significant decrease in frontal cortex tissues after METH ($p = 0.006$) when compared to controls. This is consistent with results of proteomics that METH mediated the downregulation of Gria3 in the frontal cortex (Table 5).

Table 5 The mean value of relative AMPA3/ β-actin protein intensity in frontal cortex after METH exposure

Group	AMPA3/ β-Actin (mean ± SEM)
Control	1.3118 ± 0.4703
METH	0.7701 ± 0.2143**

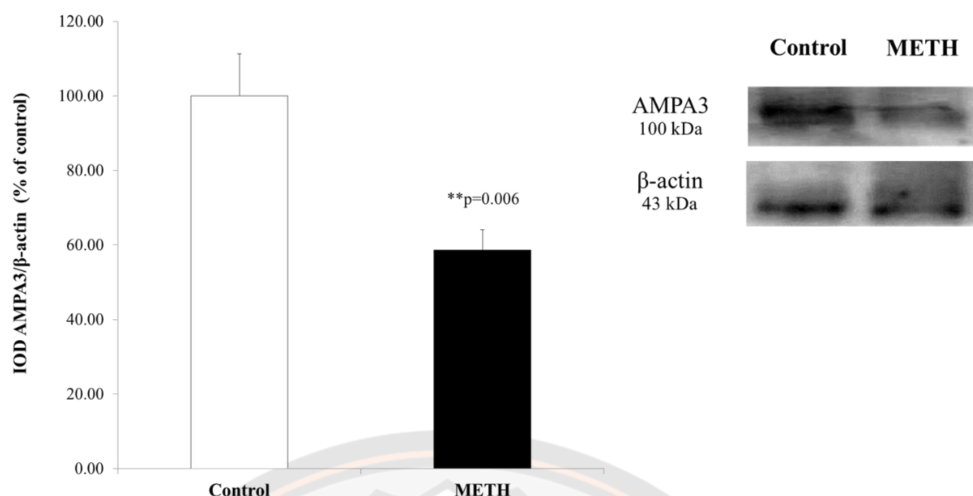


Figure 12 Quantitative analysis of AMPA3 expression after METH exposure in rat frontal cortex. $**p < 0.01$ compared to the control group

Expression of calbindin in rat frontal cortex after METH exposure

The results showed that the mean value of relative calbindin protein intensity in control and METH groups were 1.1548 ± 0.2923 and 1.0107 ± 0.2720, respectively as shown in Table 6 and Figure 13. There was no significant decrease in frontal cortex tissues after METH when compared to controls.

Table 6 The mean value of relative calbindin/ β-actin protein intensity in frontal cortex after addictive drugs exposure

Group	calbindin/ β-Actin (mean ± SEM)
Control	1.3619 ± 0.1027
METH	0.9896 ± 0.1316*

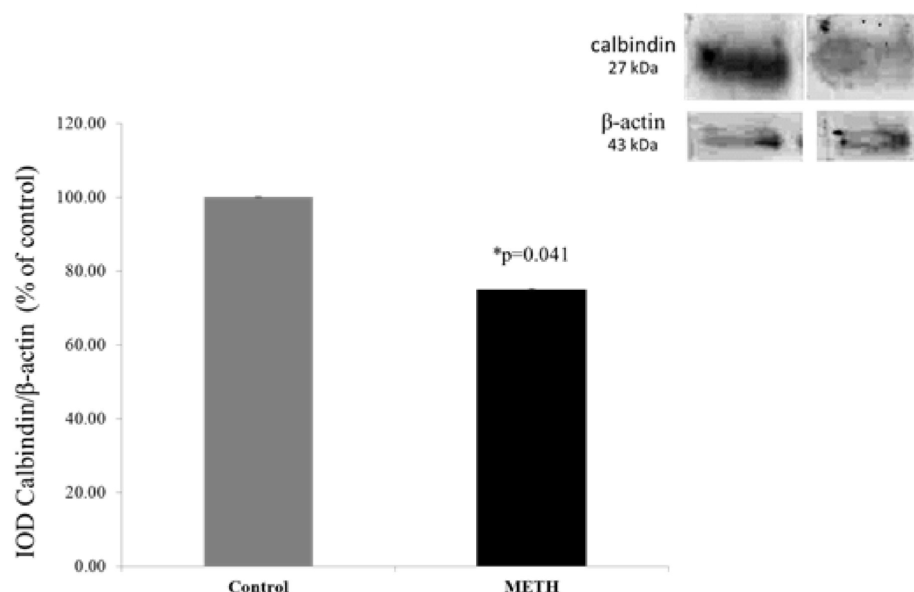


Figure 13 **Quantitative analysis of calbindin expression after METH exposure in rat frontal cortex. *p < 0.05 compared to the control group**

DXM exposure leads to the alteration of the glutamatergic and GABAergic system in rat frontal cortex

As previously, results show that the glutamatergic and GABAergic systems are the main mechanisms of METH addiction. Therefore, the DXM addiction takes an interest in the glutamatergic and GABAergic system as well. Figure 14 shows the 2,485 proteins in co-expression between control and DXM group. In total, 1,346 differentially expressed proteins were identified following the fold change standard. The GO analysis showed 142 proteins identified in synapse-related proteins (Figure 15) and collected to analyze the glutamatergic and GABAergic pathway with KEGG pathway (Figure 16). The protein expression values of glutamatergic and GABAergic systems were extracted to heatmap and plotted to present the differential expressed proteins (Figure 17). In glutamatergic system was found the 4 of up-regulation and 6 of down-regulation differences proteins when comparing the group samples, namely Grm4, Shank1, Grik2, Gria2, Grm8, Itpr1, Gria4, Grm7, Dlgap1, and Pld2. Similarly, in GABAergic system was found the 3 of up-regulation and 5 of down-regulation proteins after DXM addiction, including Gabrg1, Gad1, Cacna1b, Gabrg3, Gabbr1, Src, Hap1, and Gabre.

Furthermore, the standard fold change in expression proteins was shown in Table 7, these analyses revealed significant changes in the expression of the glutamatergic and GABAergic identified proteins.

The prediction of interactions formed between a DXM and its targeted protein via PPIs network based on STITCH databases (Figure 18). The PPIs network showed the glutamatergic system was the main target of the DXM addiction mechanism in the frontal cortex and can interfere with the GABAergic system through glutamatergic system. As a result, the Gria3 was altered in the frontal cortex after METH addiction, but not detected in DXM addiction. It may be that the Gria3 abundance is not enough to predict with this proteomics in DXM addiction condition. Nevertheless, the Gria3 was found directly regulated with DXM addiction and the other glutamatergic proteins, was observed in PPIs network as well. This result revealed Gria3 as one of the functional proteins in the glutamatergic system under METH and DXM addiction conditions. Likewise, in the GABAergic system, the calb1 cannot detect in DXM addiction but was found regulated with Gad1 in PPIs network. To more understand the molecular target of DXM addiction, PPIs network was performed by Pathway Studio with addiction behavior (Figure 19). The Pathway Studio database, the protein that connects to addiction behavior was Gabbr1 and regulated to the other proteins. The Gria3 and Calb1 were also one of them. Cumulatively, these findings show that the 'Glutamatergic-GABAergic system' was the one of DXM addiction mechanisms.

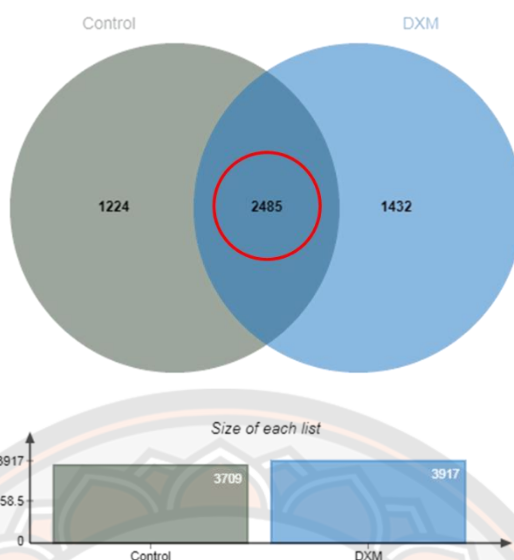


Figure 14 **Venn diagrams of unique and shared proteins of DXM addiction in rat frontal cortex**

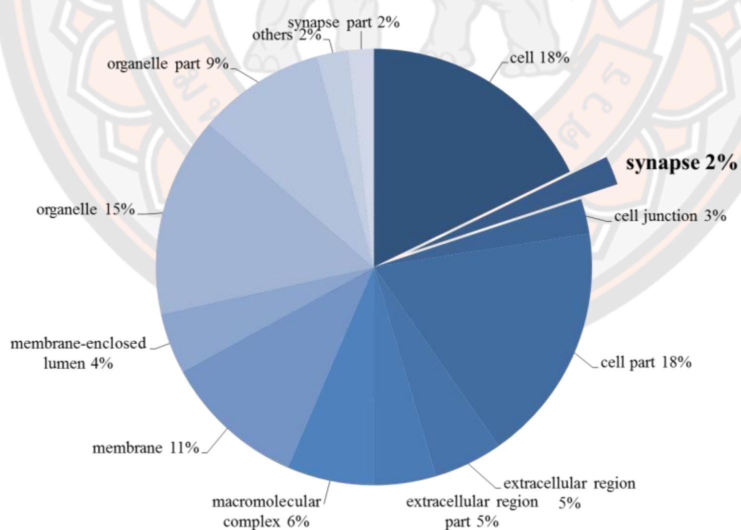


Figure 15 **Percentages of proteins enriched in different cellular components after DXM addiction, according to Gene Ontology enrichment analysis.**

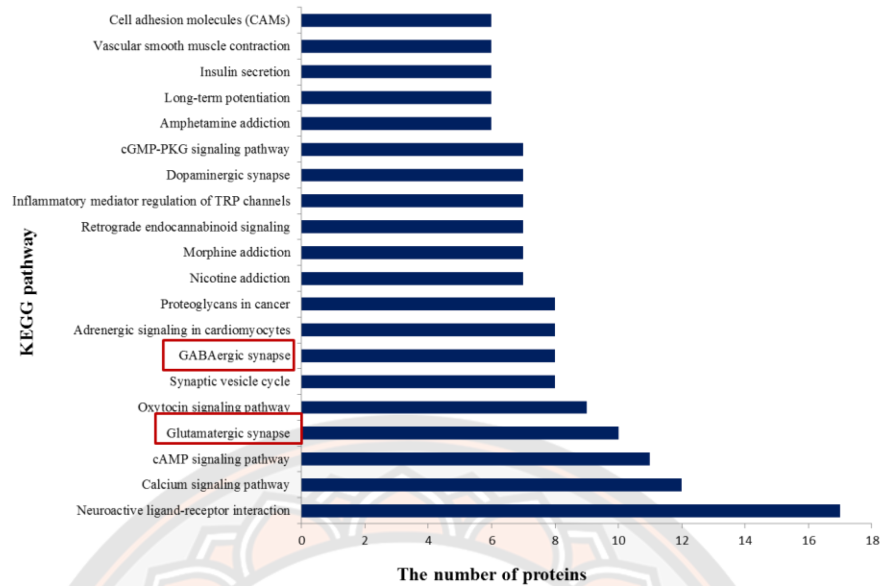


Figure 16 **Enriched KEGG pathway analysis of the identified synapse proteins after DXM addiction, p-value < 0.05**

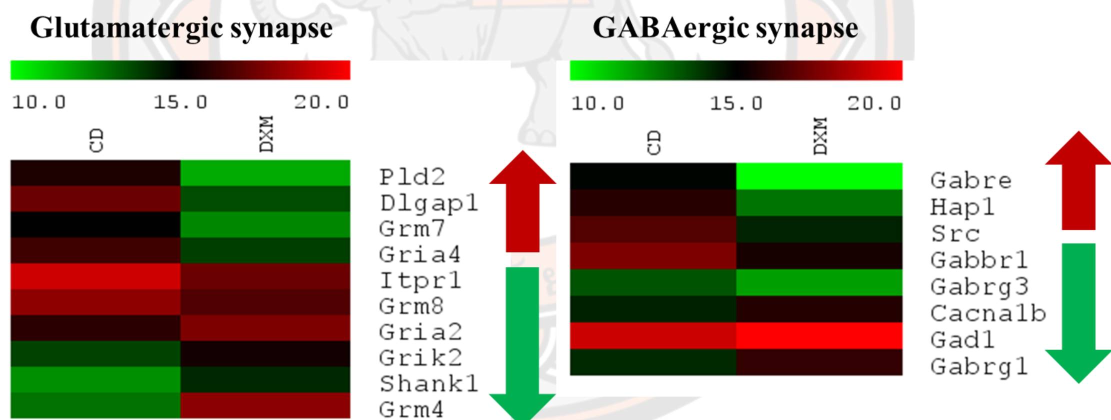


Figure 17 **Heat map showing differentially expressed proteins in DXM addiction compared to the control. Green arrow represents down-regulation and red arrow represents up-regulation, performed in MeV software.**

Table 7 The frontal cortex glutamatergic and GABAergic system proteins alterations after DXM addiction.

Accession Number	Gene name	FC DXM/CD	Accession Number	Gene name	FC DXM/CD
Glutamatergic synapse			**GABAergic synapse**		
P31423	Grm4	5.01	P23574	Gabrg1	1.82
Q9WV48	Shank1	2.01	P18088	Gad1	1.57
P42260	Grik2	1.74	Q02294	Cacna1b	1.36
P19491	Gria2	1.61	P28473	Gabrg3	-1.48
P70579	Grm8	-1.22	Q9Z0U4	Gabbr1	-1.99
P29994	Itpr1	-1.90	Q9WUD9	Src	-2.31
P19493	Gria4	-2.50	P54256	Hap1	-3.06
P35400	Grm7	-2.64	Q9ES14	Gabre	-4.90
P97836	Dlgap1	-3.63			
P70498	Pld2	-3.94			

FC = Fold change

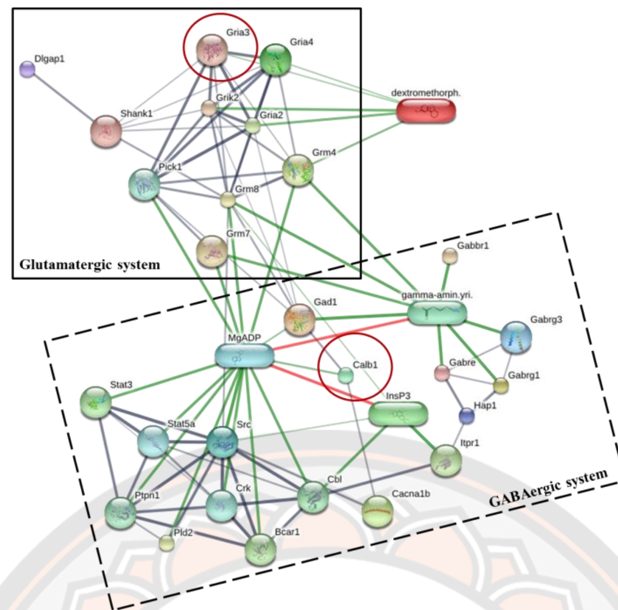


Figure 18 STITCH 5.0 database predicted the interactions between DXM and candidate protein. Modes of action are shown in different color lines.

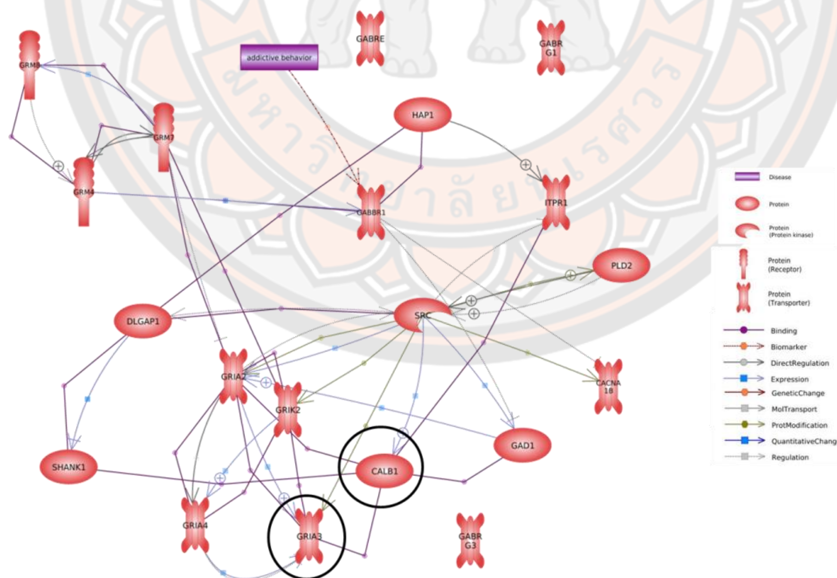
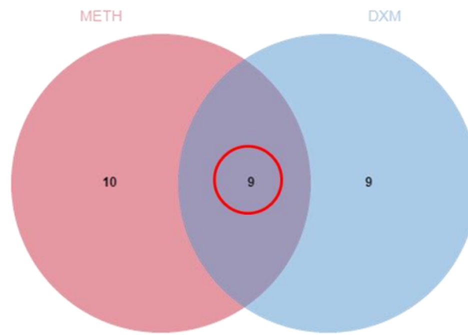


Figure 19 The Pathway Studio database predicted the interactions between proteins and neuronal signaling systems response to DXM addiction. Modes of action are shown in different type lines.

Drugs addiction leads to the alteration of the glutamatergic and GABAergic system in rat frontal cortex

From the METH and DXM addiction result, the glutamatergic and GABAergic system have important roles in the frontal cortex. To better identify protein patterns, we incorporate findings of METH and DXM addiction proteins changes in the glutamatergic and GABAergic system. The overlapping proteins proposed to drug addiction, 9 proteins were identified in both groups (Figure 20). These proteins were found that 4 proteins in glutamatergic system and 5 proteins in GABAergic system, namely Grik2, Itpr1, Pld2, Dlgap1, Gabrg1, Gad1, Gabrg3, Gabbr1, and Cacna1b, respectively. The protein expression values were extracted to heatmap plotted (Figure 21) and standard fold change (Table 8) to present the differential expressed proteins. The differentially expressed proteins in the same direction across drug addiction reveal the potential protein identified from drug addiction (Comes et al., 2018). Five of these proteins were differentially abundant in the same direction across all two drug addictions, including Itpr1, Pld2, Dlgap1, Gabrg3, and Cacna1b.

To support these 5 proteins for confirmation of addictive mechanistic and discovery of putative proteins, PPIs networks were performed to this. By mapping 5 proteins to STITCH database (Figure 22), a PPIs network constructed the prediction framework of drug addiction which represents METH and DXM substances. As previously reported, METH-induced Gria3 and Calb1 alteration in frontal cortex; these 2 proteins were selected to be part of this network. The STITCH network showed 5 candidate proteins not regulated directly with drug addiction, while regulated through the Gria3. It may be possible to have small research of these proteins under drug addiction conditions. To support the molecular target of drug addiction, PPIs network was performed by Pathway Studio to search for understanding addiction behavior (Figure 23). It was found that only the Itr1 protein involved with addiction behavior through the GABAergic system, in the same with Calb1 and Gria3. Despite this, the other candidate proteins were interesting to find the mechanism of drug addiction; it is still needed to study further.



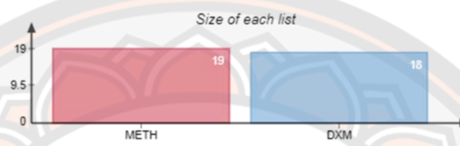
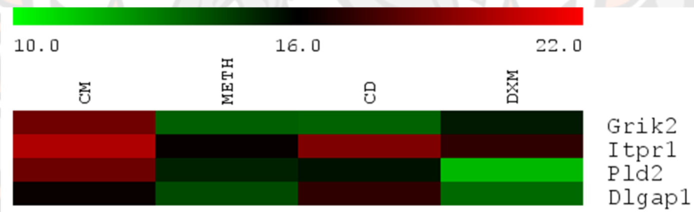


Figure 20 Venn diagrams of unique and shared proteins of drug addiction in rat frontal cortex

Glutamatergic synapse



GABAergic synapse

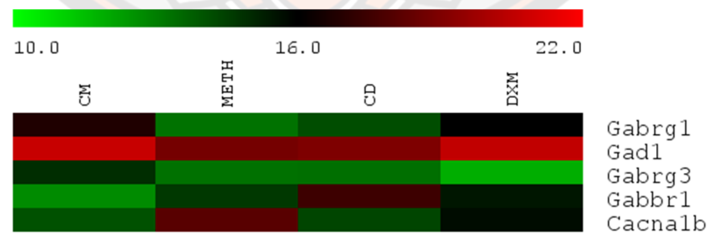


Figure 21 Heat map showing differentially expressed proteins in drug addiction compared to the control, performed in MeV software.

Table 8 **The frontal cortex glutamatergic and GABAergic system proteins alterations after DXM addiction.**

Gene name	FC METH/CM	FC DXM/CD	Gene name	FC METH/CM	FC DXM/CD
Glutamatergic synapse			**GABAergic synapse**		
Grik2	-4.93	1.74	Gabrg1	1.95	-1.99
Itpr1*	-3.93	-1.90	Gad1	-1.92	1.57
Pld2*	-3.36	-3.94	Gabrg3*	-1.59	-1.48
Dlgap1*	-2.01	-3.63	Gabbr1	-3.44	1.82
			Cacna1b*	4.01	1.36

FC = Fold change, *the proteins was regulated in the same direction from METH and DXM addiction.

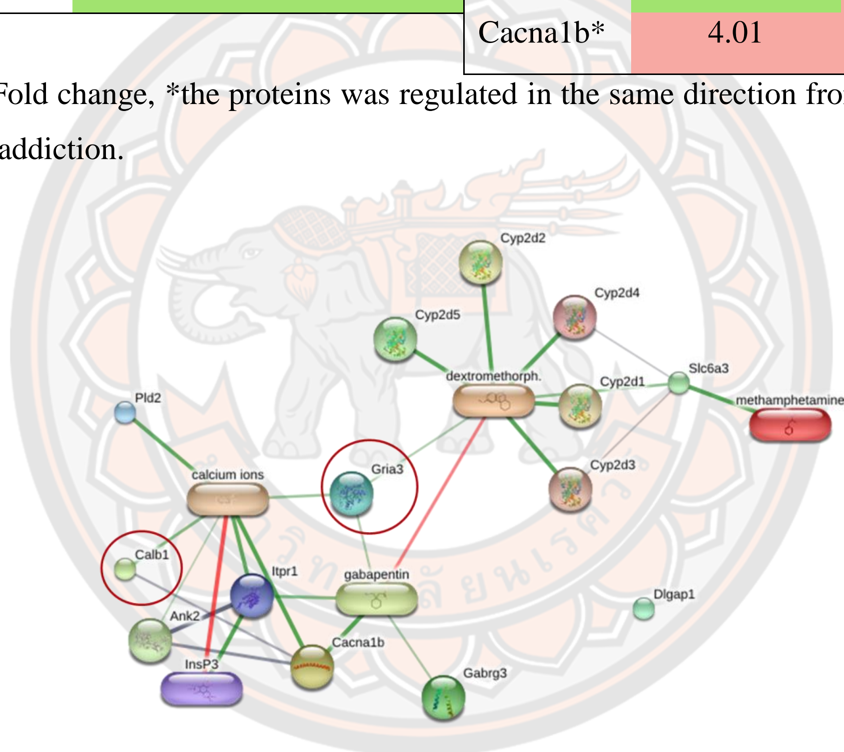


Figure 22 **STITCH 5.0 database predicted the interactions between DXM, METH and candidate protein. Modes of action are shown in different color lines.**

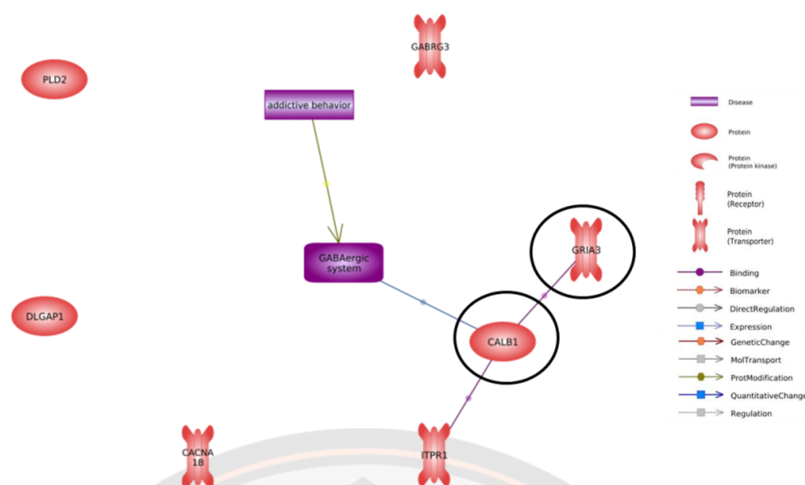


Figure 23 The Pathway Studio database predicted the interactions between addiction behavior and candidate proteins response to drug addiction. Modes of action are shown in different type lines.

The treatment of drug addiction leads to the alteration of the GABAergic system in rat frontal cortex

In the above proteomic studies of drug addiction identify proteins associated with glutamatergic and GABAergic systems. Interestingly, the GABAergic system is targeted as a potential for the treatment of drug abuse disorders (Addolorato et al., 2012). Only a few medications are approved for safe use, the natural products are another interesting alternative treatment for drug addiction. In the current proteomic studies have used a combination of treatment results, including diazepam, GABA and PGBR. Figure 24 shows the 2,574 proteins in co-expression among all groups. In total, 671 differentially expressed proteins were identified following the fold change standard. The GO analysis showed 64 proteins identified in synapse-related proteins (Figure 25) and collected to analyze the glutamatergic and GABAergic pathway with KEGG pathway (Figure 26). From the KEGG pathway only identified proteins in the GABAergic system, namely Gabbr1, Nsf, Prkcg, and Cacna1b. The protein expression values were extracted to heatmap plotted (Figure 27) and standard fold change (Table 9) to present the differential expressed proteins. Three of these proteins were differentially abundant in the same direction across all drugs addiction treatment,

including Nsf, Prkcg, and Cacna1b. The proteins were differentially abundant in the same direction, it reveals that corresponding protein system at the drug addiction treatment in frontal cortex.

The prediction of interactions formed between a treatment and its targeted protein via PPIs network based on STITCH databases (Figure 28). As previously drug addiction reported, Gria3 and Calb1 alteration in frontal cortex; these 2 proteins were selected to be part of this network. The PPIs network shows all four candidate proteins influence on the brain function from the treatment under addiction condition. The Gria3 and Calb1 were a part of this network, as well. Interestingly, the Gabbr1 and Nsf were closely to interaction with diazepam, while the Prkcg, and Cacna1b were closely to interaction with contents of PGBR. To support the molecular target of drug addiction treatment, PPIs network was performed by Pathway Studio to search for understanding addiction behavior (Figure 29). The Pathway Studio database, all the proteins were interacting with addictive behavior through the GABA. This result supported that the GABAergic system was the main mechanism of addiction treatment, the PGBR as well.

Figure 24 **Venn diagrams of unique and shared proteins of drug treatment in rat frontal cortex**

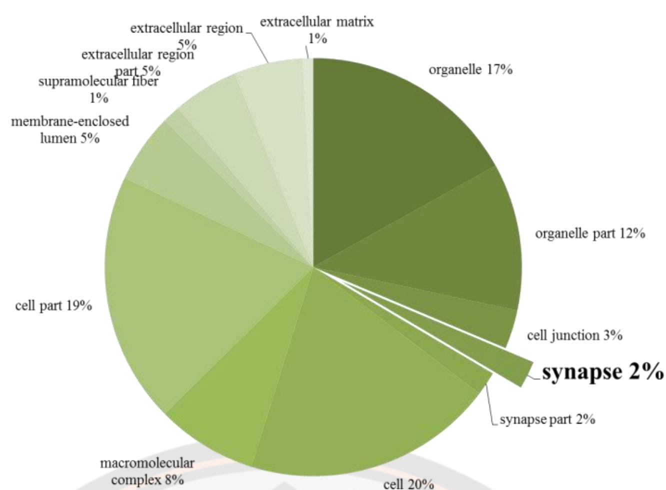


Figure 25 **Percentages of proteins enriched in different cellular components after drug treatment, according to Gene Ontology enrichment analysis.**

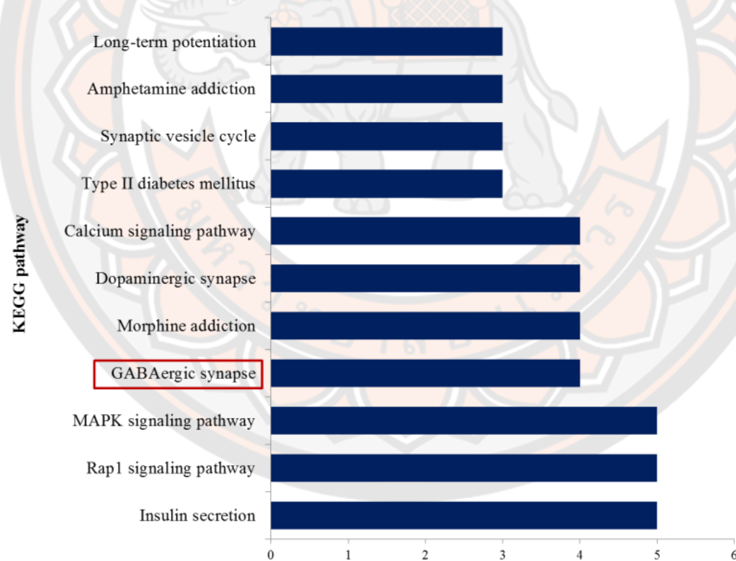


Figure 26 **Enriched KEGG pathway analysis of the identified synapse proteins after drug treatment, p-value < 0.05**

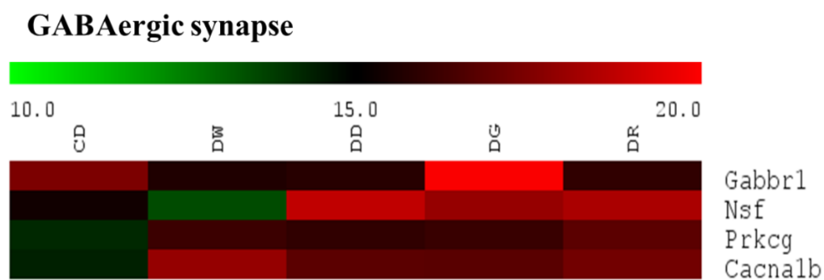


Figure 27 **Heat map showing differentially expressed proteins in drug treatment, performed in MeV software.**

Table 9 **The frontal cortex GABAergic system proteins alterations after drug treatment.**

Gene name	FC DW/CD	FC DD/CD	FC DG/CD	FC DR/CD
GABAergic synapse				
Gabbr1	-1.84	-1.68	2.47	-1.49
Nsf*	-1.83	3.43	2.61	3.00
Prkcg*	1.97	1.77	1.92	2.60
Cacna1b*	3.59	2.46	2.43	2.91

FC = Fold change, *the proteins was regulated in the same direction from each treatment.

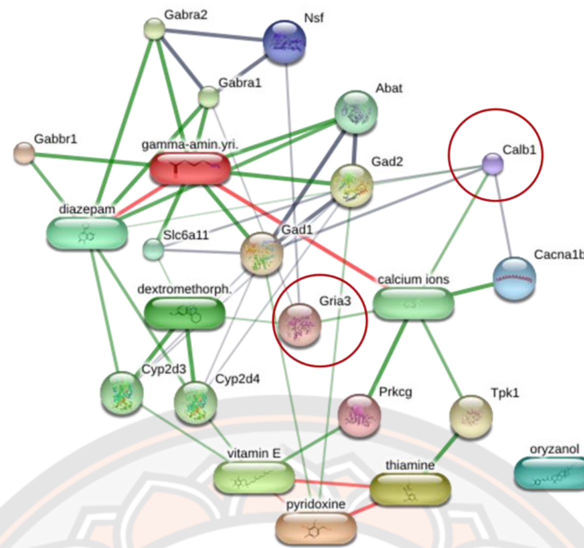


Figure 28 **STITCH 5.0 database predicted the interactions between DXM, PGBR content and candidate protein. Modes of action are shown in different color lines.**

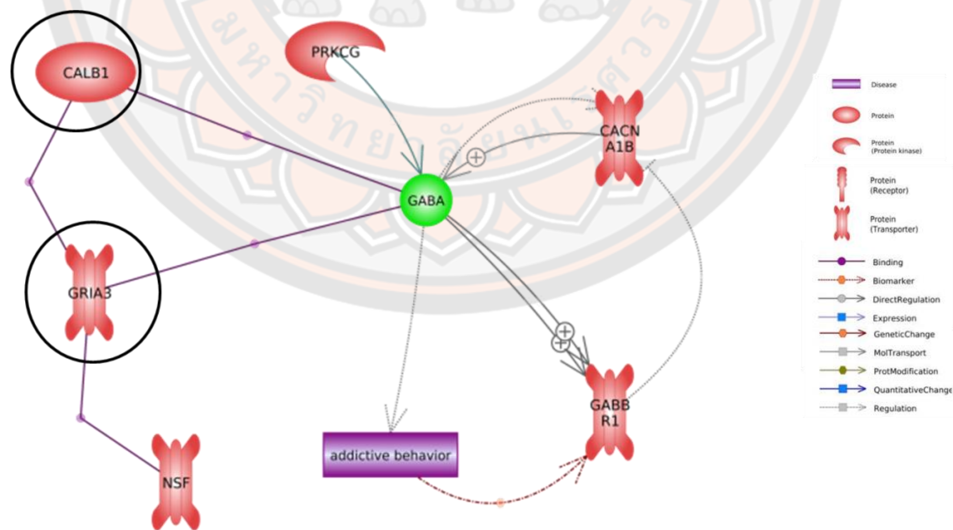


Figure 29 **The Pathway Studio database predicted the interactions between addiction behavior and candidate proteins response to drug treatment. Modes of action are shown in different type lines.**

CHAPTER V

DISCUSSION

Drug addiction-induced neurotoxicity remains a global problem and the molecular mechanisms underlying the neuronal signaling change in drug addiction are complex. Many researchers have identified the underlying biological mechanisms of drug addiction that have led to more understanding targeted drug action. Most drugs of abuse are capable of neurotoxic effects, which are mediated by neurotransmitter signaling, or by altering the activity of other neuronal signaling. These studies have shown that the addictive drugs can alter the level of neuronal signaling protein expression in the frontal cortex, including METH and DXM addiction. Moreover, the drug withdrawal and drug treatment were also investigated.

The alterations of glutamatergic and GABAergic systems in rat frontal cortex following drug addiction

In this study, proteomic approaches were performed in the METH addiction study. The results showed that 137 synaptic proteins were differentially expressed in the frontal cortex. Of these proteins, 11 differentiated proteins were observed in the glutamatergic system and 8 proteins in the GABAergic system.

It is known that METH administration induced increased glutamate extracellular levels in the CNS (Abekawa et al., 1994; Stephans & Yamamoto, 1994). The neurotoxicity and later neurodegeneration can then result from overstimulation of the glutamate ionotropic receptors, N-methyl-D-aspartate receptor (NDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPARs). These receptors are known as ligand-gated ion channels permeable to various cations, including Na^{+} and Ca^{2+}. The NMDA receptors are ligand-gated ion channels that mediate a Ca^{2+}-permeable component that are expressed in most central neurons, including the frontal cortex. Functional NMDARs are formed from the co-assembly of two NR1 and two NR2 (A-D) subunits. NR2A subunits are a basis for NMDAR subunits expressed in the postnatal mammalian brain (Monyer et al., 1994; VanDongen,

2008). From the current proteomic study, **NR2A (Grin2a) subunits** increase expression levels in the frontal cortex after ED-binge METH exposure in comparison with the rats treated with saline. Similarly, the upregulation of this protein was observed in the frontal cortex and hippocampus of the rat with acute METH but no difference in ED METH. While in the striatum, the NR2A levels were decreased in the acute METH group and increased in the ED METH group (Simoes et al., 2007; Simões et al., 2008). The different METH schedule administration had different effects in NR2A subunits in the brain. In this case, the augment of the NR2A subunit may be reflecting the effect of acute binge dose after ED METH. Nevertheless, the previous study supports the role of NR2A subunit in METH-induced behavioural sensitization, which the NR2A knockout mice displayed no behavioural abnormalities after chronic METH in nucleus accumbens (Miyazaki et al., 2013). However, The NR1 subunit had been examined in our research group (Kerdsan et al., 2009), which the up-regulation of NR1 subunit expression was found after acute and subacute METH administration in the striatum and the frontal cortex. Taken together, the overexpression of NMDA receptor observations, including NR1 and NR2A, provide support for the glutamatergic dysfunction after METH exposure. Likewise, AMPARs subunit composition is also regulated under different brain conditions and associated with to activate the NMDA receptors. As such, the alterations in the AMPA receptor protein can lead to dysregulation of NMDA receptor activation. The functional AMPA receptor subtypes that predominate are composed of Gria1/2-, Gria2/3- and Gria2-lacking containing receptors (Reimers et al., 2011). In this study the reduction of **AMPA3 or Gria3 receptors** was observed after METH administration in the frontal cortex. Consistently, our orevious study found, the Gria3 expression levels tend to decrease in the hippocampus in the ED-METH administration group (unpublished data, Iamjan). This information may convey the understanding that, the overactivation of ionotropic receptors (NMDARs and AMPARs) can be activated by METH administration and resulted in increased intracellular Ca^{2+} level, which promoting the production of nitric oxide (NO) by neuronal nitric oxide synthase (nNOS) (Yang et al., 2018). Thus, this contributes to reactive nitrogen species formation, which has been implicated in METH neurotoxicity. However, the previous genetic variation study in NOS1 gene, found reduced prefrontal expression of the gene, contributes to schizophrenia (Freudenberg et

al., 2015). Activation of nNOS depends on not only NMDAR-mediated calcium influx, but it is also associated with postsynaptic density protein 95 (PSD-95) coupling (O'Toole et al., 2016). The protein PSD95 interacts with **Disks large-associated protein 1 (Dlgap1)**, PSD95 was reported to be a predictor of cognitive deficits (Sultana et al., 2010). The decrease of PSD95 was also found in schizophrenia in the prefrontal cortex and hippocampus(Ohnuma et al., 2000). It may be the downregulation of DLGAP1 of this study, the downregulation of Dlgap1 found in this study may be reflected to the decrease of PSD-95. These results support the reduction of nNOS function in METH administration. Despite this, the inhibition nNOS has previously proved to impair learning and memory (Kelley et al., 2009; Li et al., 2018). It is consistent with our previous finding that the ED-binge METH rats showed the lack of short-term and long-term recognition memories (unpublished data, Veerasakul et.al). Hence, it might be ED-binge METH administration affecting learning and memory impairment through the reduction of nNOS activation. Alternatively, the increased intracellular Ca^{2+} level induced the other processes, including the activation of protein kinases. Protein kinases such as Ca^{2+}/calmodulin kinase II (CaMKII) have been reported to play a role in learning and memory (Zalcman et al., 2018). Moreover, the current proteomic result found the augment of CaMKII beta (Camk2b) regulated by ED-binge METH exposure in the frontal cortex. Similarly, It was found that chronic exposure to psychostimulants, increased CaMKII activity in striatum and nucleus accumbens (Lee & Messing, 2008), then may lead to learning and memory deficits (Zalcman et al., 2018). Interestingly, the activation of CaMKII were induced the Brain-derived neurotrophic factor (BDNF) expression through the activation of cAMP-response element binding protein (CREB) (Miyazaki et al., 2013; Tao et al., 1998; Wang et al., 2018). The BDNF plays a role in neuronal survival and differentiation, which is important for neuroplasticity of learning and memory (Yamada et al., 2002). In line with previous study, it has been reported that METH-treated animals showed augmented BDNF levels in the hippocampus (Kim et al., 2020). In the other psychostimulant drug, such as cocaine, found the increase of BDNF protein, and Bdnf mRNA expression in the prefrontal cortex (Pitts et al., 2016). However, the greater BDNF levels can also correlate with drug seeking response and loss of behavioral control (Corominas-Roso et al., 2013; Kim et al., 2020). Cumulatively, these findings

show that METH-induced glutamate accumulation overstimulates various downstream signal transduction pathways associated with Ca^{2+} influx. Nevertheless, the other components of glutamatergic neurotransmission, such as excitatory amino acid transporters (EAATs) and vesicular glutamate transporters (VGLUTs), could also be involved in the development of METH-induced toxicity.

EAATs play a critical role in controlling extracellular glutamate homeostasis by glutamate reuptake from the synaptic cleft. **EAAT2** is the main glutamate transporter found in the forebrain, it is abundantly expressed in astrocytes and presynaptic nerve terminals (Magi et al., 2019). In present study, the reduction of EAAT2 (Slc1a2) was observed in METH-treated rats. It is similar to a previous study, the repeated high doses of METH decrease EAAT2 in the nucleus accumbens and the prefrontal cortex (Althobaiti et al., 2016), Then, it is possible to decrease glutamate uptake in astrocytes (Lepore et al., 2011). Likewise, The METH-induced downregulation of **VGLUT2 (Slc17a6)** in the frontal cortex, which is observed in this proteomic study. Interestingly, the decreased VGLUT2 expression in dopamine neurons regulates behavioural activation induced by psychostimulant drugs (Alsiö et al., 2011). Together these findings illustrate a prominent role for glutamate neurotransmission in neurotoxicity after METH addiction.

Excess glutamate can cause damage to the GABAergic interneurons, causing dysregulation of the signals, which may relate to the psychotic symptoms that occur with METH addiction. The overregulation of NMDA receptors from repeated METH might lead to GABA interneurons damage (Hsieh et al., 2014). In METH-induced behavioural sensitization rat was reported to decrease the **Glutamate decarboxylase 1 (GAD67)** protein level in the nucleus accumbens (Zhang et al., 2006). As well, repeated amphetamine administration is followed by decrease in GAD67 mRNA expression and GABA release in rat striatum (Lindefors, 1993). In agreement with the present study which found decreased GAD67 (Gad1) protein levels in rat frontal cortex after METH administration, suggesting the effects on GABA synthesis and membrane GABA receptor stimulation. On the molecular level, the action of GABA is mediated by ionotropic (GABAA) and metabotropic (GABAB) receptors. Previous studies have revealed that addictive an addictive behaviour is directly correlated with synaptic GABAA receptors (Stephens et al., 2017). The GABAA receptors making up the

complex come from 3 related subunit families, named α (1–6), β (1–3) and γ (1–3). From this present result, the **GABAA γ2** was up-regulated while **GABAA γ1&2** were down-regulated after METH administration. Nevertheless, there is quite a bit of study about GABAA γ (1–3) receptor on drug addiction. As previously mentioned, the action of GABA is also mediated by GABAB receptors, playing a role in the action of different drugs of abuse (Filip & Frankowska, 2008). This proteomic result was found to increase of **$GABA_B$1 and $GABA_B$2** receptors (Gabbr1, Gabbr2) protein levels after METH administration in rat frontal cortex. Despite this, this results that the relative levels of receptors may not correspond to the GABA level. The loss of GABAB receptors was reported in acute METH injected mice (Padgett et al., 2012). Our results are consistent with previous results reported by Pratt and Bowery (1993) and Pibiri et al. (2005) (Pibiri et al., 2005; Pratt & Bowery, 1993), showing a chronic treatment of $GABA_B$ receptor antagonists increased the number of $GABA_B$ receptors in the brain. It has been suggested that down-regulation of the receptor by the chronic METH administration leads to a compensatory increase in $GABA_B$ receptor. Likewise, deficits in markers of GABAergic neurotransmission in the brain was found in METH administration (Veerasakul et al., 2016), containing parvalbumin, calbindin, and calretinin, define different subgroups of GABAergic neurons (Reynolds et al., 2004). It supports the dysfunction of GABAergic signaling in the frontal cortex after METH addiction.

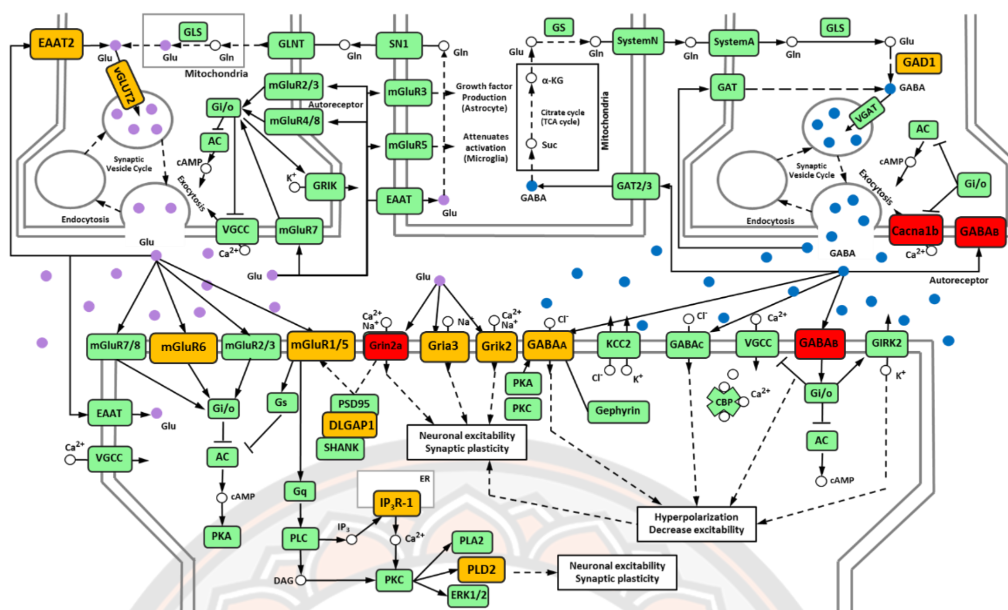


Figure 30 **the system of glutamatergic and GABAergic. Red box was down-regulating and the yellow box was up-regulating proteins after METH addiction. **The picture adapted from the KEGG pathway in DAVID 6.8.**

The downregulation of Gria3 after METH addiction in rat frontal cortex

Studies have shown that Gria3 (AMPA3) may play an important role in METH addiction. According to proteomic analysis and the western blotting validation, Gria3 expression was downregulated after METH addiction in the frontal cortex. The dysregulation of Gria3 affects Ca^{2+} permeability, together with Gria2 contributing to the abnormal activity of AMPA receptors (Meng et al., 2003). As well, the acute METH exhibits small decreases of Gria3-containing (Gria2/3) receptors in the nucleus accumbens and the striatum after 24 hours administration (Nelson et al., 2009). While, an increase in the expression levels of the Gria2 after acute METH in hippocampus (Simoes et al., 2007) and tendency to increase in the striatum (unpublished data, Iamjan). Alternatively, the Gria1 (gene and protein expression) was significantly increased in the prefrontal cortex (unpublished data, Iamjan) and the striatum (Furlong et al., 2018) after ED-METH. Therefore, based on prior information, METH administration could replace Gria1/2-containing receptors for Gria2/3-containing receptors, affecting the augment total levels of Gria1 and Gria2. Moreover, in human

study, genetic polymorphism of Gria3 rs502434 was found to be associated with METH dependence and METH-induced psychosis (Iamjan et al., 2018). According to cellular component analysis, the validation of Gria3 may facilitate the understanding of the mechanism of drug abuse such as METH.

Changes in calbindin levels after METH addiction in rat frontal cortex

Studies have shown that calbindin (Calb1) may play an important role in METH addiction. According to proteomic analysis and the western blotting validation, Calb1 expression tends to downregulated after METH addiction in the frontal cortex. It has been shown an up-regulation of calbindin mRNA in rat prefrontal cortex after repeated METH (Wearne et al., 2017) and schizophrenia (Fung et al., 2014). The deficit of calbindin immunoreactivity was observed after chronic METH in the frontal cortex (Veerasakul et al., 2016) and hippocampus (Kuczenski et al., 2007). METH has been shown to suppress inhibitory neurons and disrupt the GABAergic transmission signalling (Jiao et al., 2015). Calbindin can be used as a marker of a specific subtype of GABAergic neurons (Reynolds et al., 2004). This suggests that calbindin reduction might be representative of the GABAergic interneurons degenerate after METH addiction. These results support the notion that chronic METH use might promote more GABAergic neurotransmitter dysfunction.

The alterations of glutamatergic and GABAergic systems in rat frontal cortex following DXM addiction

In this study, proteomic approaches were used in the DXM addiction study and found that 142 synaptic proteins were differentially expressed in the frontal cortex. Of these proteins, 18 differentiated proteins were observed in glutamatergic and GABAergic systems.

It has been reported that DXM acts as antagonists of the NMDA receptor (Silva & Dinis-Oliveira, 2020), and inhibits glutamate neurotransmission by blocking calcium channels (Ohi et al., 2011). In addition to implicating NMDA receptors as a critical element in mediating the antitussive-like actions of DXM, alter AMPA receptor activity (Nguyen & Matsumoto, 2015). Also, the alteration **GluR2 (Gria2) and GluR4 (Gria4)** were regulated by DXM exposure in the present study. It remains unclear how DXM

alters AMPA receptor activity, as it does not directly bind to AMPA receptors. Interestingly, hallucinogens, such as psilocin, LSD and dimethyltryptamine (DMT), increase extracellular glutamate levels in the prefrontal cortex (Vollenweider & Kometer, 2010). In addition, it has been reported that the NMDA antagonist, such as ketamine, also regulates AMPA receptor activity by increasing surface expression of GluR1 and GluR2 subunits (Nosyreva et al., 2013). The increased glutamate release in the cortex is thought to stimulate cortical AMPA more than NMDA receptors, leading to increased expression of BDNF (Vollenweider & Kometer, 2010). Besides, sigma-1 receptors activity, one of the DXM actions, has been shown to increase the levels of GluR2 mRNA subunit and GluR2/3 subunit proteins expression in the prefrontal cortex (Guitart et al., 2000). Thus, one indirect mechanism by which DXM could activate AMPA receptors is through the activation of sigma-1 receptors. Alternatively, Group III mGluRs including **mGluR4, mGluR7, and mGluR8** were also altered by DXM exposure in the present study. The mGluR7 is highly expressed in the central nervous system, implicating in emotional learning and working memory. In a previous report, mGluR7 knockout mice were actually impaired in working memory (Goddyn et al., 2008). Moreover, the acute high DXM dose produced cognitive impairments in rats and humans (Ijomone & Biose, 2019). The mGluR7 has been reported to have low affinity for glutamate, therefore it can be activated by high levels of extracellular glutamate (Kryszkowski & Boczek, 2021). It is conflicting with the DXM effect on high glutamatergic release. It may be that one direct mechanism by DXM inhibits the mGluR7 receptor. Nevertheless, the mechanism of DXM on glutamatergic rays should be considered and investigated for more understanding.

In addictive behaviour, GABAergic/antiglutamatergic mechanisms play a role in the development of dependency (Mutschler et al., 2010). That means the GABAergic system is also involved in the DXM addiction. The single dose dextromethorphan was found to increase and decrease GABAergic and glutamatergic tone, respectively (Salavati et al., 2018). The molecular mechanism of DXM in GABAergic neurotransmitters is little known, and an action receptor or transporter might be involved. In the present study the upregulation of **GAD67 (Gad1)** protein levels was found in the frontal cortex after DXM administration. It has been reported that the 2-day ketamine treatment (NMDA antagonist) reduced the GAD67 and parvalbumin

prefrontal cortex (Zhang et al., 2009), a reduction in GAD67 would be expected to reduce GABA synthesis. Likewise, the two PCP injections reduced the levels of parvalbumin and GAD67 in the prefrontal cortex of rats (Amitai et al., 2012). The exact mechanism by which reduced GABA neurotransmitter function alters the effects of NMDA antagonists is unclear. However, consistently reduced GABA function may result in compensatory changes that bring GABA function increase in response to blockade of GABA function by NMDA receptor antagonists, including chronic DXM administration. As well, the alteration of **GABAA (Gabre, Gabrg1 and Gabrg3) and GABAB (Gabbr1)** were regulated by DXM exposure in present study. Therefore, further study is needed to more understand the mechanism of action of DXM.

Figure 31 **the pathways of glutamatergic and GABAergic systems. The proteins in the red box were down-regulation proteins and in the yellow box were up-regulating proteins after DXM addiction. **The picture is adapted from the KEGG pathway in DAVID 6.8.**

The proteins response in glutamatergic and GABAergic system in the frontal cortex after addiction exposure

The present studies have used a combination of METH and DXM results to identify proteins associated with mechanisms of drug addiction. The proteomic

approaches found that 52 synaptic proteins were differentially expressed in the frontal cortex. Of these proteins, 9 differentiated proteins were observed in glutamatergic and GABAergic systems. It is important to identify the marker present in drug addiction at the synapse, The proteins that were regulated in the same direction after METH and DXM exposure, were focused in the discussion part. From 9 proteins, 5 differential expressed proteins were found in same direction, including Cacna1b, Itpr1, Pld2, Gabrg3, and Dlgap1.

In the glutamatergic system, **Disks large-associated protein 1 (Dlgap1)** was down-reglated in the frontal cortex after drug addiction in the present study. The Disks large associated protein 1 gene encodes the protein DLGAP1 (also known as GKAP or SAPAP1), which are important proteins in the postsynaptic density, act as scaffold proteins and are involved in glutamate receptor signaling (Rasmussen et al., 2017). The network enables downstream signalling from the postsynaptic glutamate receptors, containing DLGAP1–4, PSD-95 and NMDA receptors, and it also plays a role in AMPA receptor scaling (Rasmussen et al., 2017). Nevertheless, the alteration of DLGAP1 might lead to the glutamate signaling dysfunction, through the glutamate receptor. Besides, altered NMDA has also been implicated in many neuropsychiatric disorders, including schizophrenia, mood disorders and substance induced psychosis (Lakhan et al., 2013). Decreased PSD95 expression in the prefrontal cortex was also found in schizophrenia (Ohnuma et al., 2000). Similarly, genetic variants of DLGAP1 have been associated with neuropsychiatric disorders including schizophrenia (Li et al., 2013); Alzheimer's disease (Roselli et al., 2011) and major depressive disorder (Mathias et al., 2016). Furthermore, Dlgap1 knockout mice disrupt the protein interactions in the postsynaptic density of the cortex, and contribute to social deficits (Coba et al., 2018). Together, these findings further suggest the association of DLGAP1-DLG4-NMDA pathway with neuropsychiatric disorders, together with substance-induced psychosis.

Furthermore, the Dlgap1-Dlg4-NMDA can regulate the IP3R via the mGluR1/5 in the postsynaptic neuron. The present study found the downregulation of IP3R1 (Itpr1) in drug addiction. **Inositol trisphosphate receptors type 1 or IP3R-1 (Itpr1)** is an intracellular ion channel that mediates the release of calcium ions from the endoplasmic reticulum (ER) in response to hormones, growth factors, and

neurotransmitters (Egorova & Bezprozvanny, 2018). The IP3R-1 is the major neuronal member of the IP3R family; it is the predominant isoform in the central nervous system neurons in the control of key physiological functions, such as learning, memory and behaviour (Bosanac et al., 2002). Here, IP3 resulting from activation of mGluRs provided by the rush induced Ca2+ influx from NMDARs, synergistically binds IP3Rs to cause Ca^{2+} release from ER (Taylor & Laude, 2002). The previous study found the reduction of mGluR5 after repeated METH administration (Mao & Wang, 2001) and a single cocaine exposure (Bellone & Mameli, 2012) in the brain. It is consistent with the present METH result that found the downregulation of mGluR5 proteins after METH addiction (Table 6). The mGluR5 is coupled with phospholipase C (PLC) through G proteins, leading to the formation of diacylglycerol (DAG) and the production of inositol trisphosphate (IP3), which results protein kinase C (PKC) activation (Huang et al., 2019). The PKC has been implicated in the activation of extracellular-signal-regulated kinases (ERKs), Phospholipase A2 (PLA2) and Phospholipase D (PLD) (Ledonne & Mercuri, 2020). Furthermore, the Phospholipase D2 (Pld2) was down-regulated after drug addiction in the rat frontal cortex.

Phospholipase D2 (Pld2) is an enzyme of the phospholipase superfamily, which has been implicated in regulation of exocytosis, endocytosis, and neurotransmitter release. The PLD signaling, activated by neurotransmitter signalling, increased in the amygdala and decreased in the hippocampus which may result in altered anxiety states and increased locomotor response in cocaine-conditioned animals, respectively (Krishnan, 2016). Moreover, the opioid receptors, such as the μ- and δ-opioid receptors, were regulated by Pld2, which has implications for drug addiction (Koch et al., 2003; Koch et al., 2004). In addition, PLD appears to metabolize ethanol into phosphatidylethanol (PEtOH), which modulates alcohol's hyperactive response. Interestingly, Chung et al. reported lack of PLD blocks ethanol-mediated hyperactivity in fruit fly, supporting that PLD mediates behavioural responses (Chung et al., 2019). Our studies have observed the hyperlocomotion in rats after METH and DXM (unpublished data, Iamjan, unpublished data, Veerasakul). It may be that the reduction of Pld2 mediates the drug-induced hyperactivity response. Together, these findings further suggest that the drug addiction provided to decrease the mGluR

stimulation, which was evidenced by the reduction of Dlgap1, IP3R1 and Pld2 in the rat frontal cortex, mediates abnormal behavioural responses.

In the GABAergic system, this study found the reduction of **GABA receptor subunit gamma-3 (Gabrg3)** after the drug addiction in the frontal cortex. The GABA receptor subunit gamma-3 is a subunit of the GABAA receptor for the GABA neurotransmission, associated with aspects of addictive behaviour in humans and animal models (Stephens et al., 2017). Previously, chronic alcohol consumption caused the increase of Gabrg3 mRNA levels in mice, involved in behavioural sensitization to ethanol (Buck, 1996; Enoch et al., 2012). Moreover, alcohol dependence is associated with genetic variation in GABRG3 (Dick et al., 2004). In light of GABAA receptors are poorly known in addictive mechanisms, Gabrg3 as well. Nevertheless, the GABAergic neuronal marker, calbindin, was found deficits in the frontal cortex after METH (Veerasakul et al., 2016) and morphine administration. Together, these two proteins suffice to suggest that substance abuse caused the GABAergic dys-regulation.

The last, the **voltage-dependent N-type calcium channel subunit alpha-1B (Cacna1b)** was up-regulated in the frontal cortex of drug addiction. Influx of Ca^{2+} through presynaptic voltage-gated calcium channels (VGCCs) into presynaptic terminals promotes synaptic vesicle exocytosis and neurotransmitter release. The VGCCs are multi-subunit complexes formed by α1 subunits with subordinate β, α2, and γ subunits. Moreover, it has been suggested that VGCCs are implicated in drug-related plasticity and addictive behaviour. Furthermore, the strong association between N-type VGCCs and addiction was evident at the increased cacna1b mRNA expression level by repeated METH (González et al., 2016). Cacna1b encodes α1B subunits of the N-type calcium channels, which are predominant in presynaptic terminals in neurons. At the behaviour, deletion of α1B subunits of the N-type calcium channels mice reduced ethanol intake (Newton et al., 2004), increased locomotor activity and memory impairment (Nakagawasai et al., 2010), these findings suggest that N-type calcium channels may involve addictive-like behavior. Our results indicate that N-type channels up-regulated to METH and DXM drugs in the frontal cortex, suggesting a result of N-type calcium channels in drug addiction.

In summary, the findings of this study provides initial evidence for the protein's response to drug-addiction. These results also provide basic knowledges for diagnosing

and developing of the medication of addiction drugs. In addition, the research studies on the proteomic characterization of drug-addicts revealed that the Cacna1b, Itpr1, Pld2, Gabrg3, and Dlgap1 were candidate proteins.

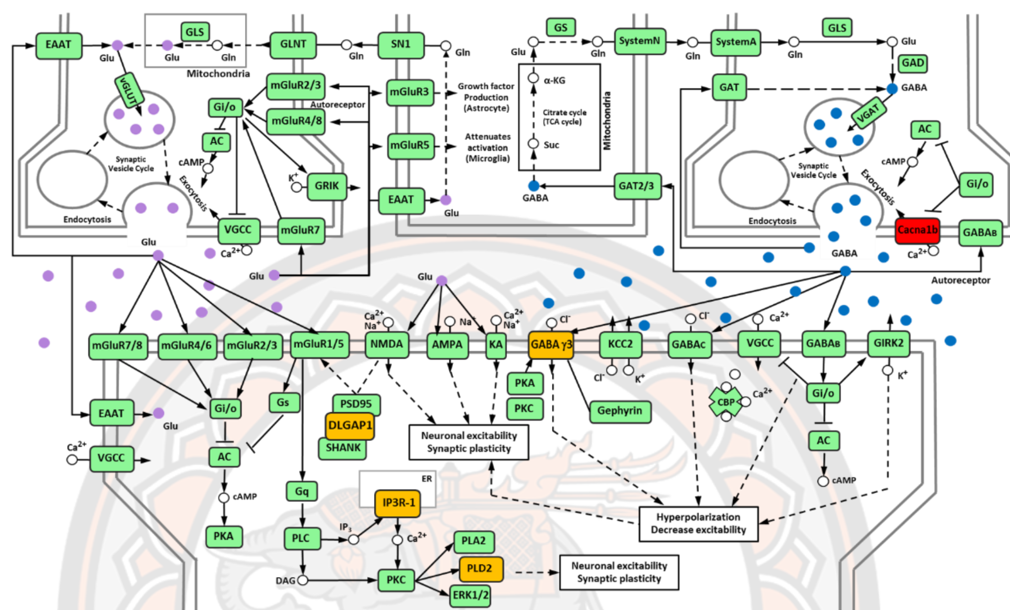


Figure 32 **the pathways of glutamatergic and GABAergic systems. The proteins in the red box were down-regulation proteins and in the yellow box were up-regulating proteins after drug addiction. **The picture is adapted from the KEGG pathway in DAVID 6.8.**

The role of GABAergic systems proteins in the treatment of drug addiction in rat frontal cortex

For a long time, the treatment for addiction to drugs is not concerned about physical and mental well-being. It is focused mainly on a single outcome, the absence of addiction. Treatment medications for addiction tend to be similar to the drug of abuse that affects the same brain system. One of them, diazepam, is a medicine of the benzodiazepine family that acts as a sedative and anxiolytic, used to treat anxiety, alcohol withdrawal, and seizures. Its mechanism of action is by increasing the effect of the GABA neurotransmitter, binding to a unique site on the alpha-gamma subunit complex (Calcaterra & Barrow, 2014). Although diazepam is clinically effective for addiction treatment, unfortunately, they have abuse potential, with a developing drug

dependence, tolerance, and withdrawal syndrome like another drug abuse (Licata & Rowlett, 2008). The chronic diazepam failed to produce an anxiolytic (Rodgers et al., 1992) and increase on diazepam withdrawal (Allison & Pratt, 2006; Talarek et al., 2018). Moreover, Pádua-Reis et al. have reported that diazepam treated mice had reduction in anxiety levels as well as, while also inducing sedative effects (Pádua-Reis et al., 2021). The beneficial effect of PGBR may be the result of the interaction between GABA and other components such as vitamin B1, B6, E and gamma-oryzanol (Roboon et al., 2017). It has been reported that the intake of germinated brown rice (GBR) improves the hyperglycemia, sleeplessness, inhibits development of cancer cells and assists the treatment of anxiety disorders (Patil & Khan, 2011). In the present proteomic studies, we have used a combination of treatment results, including diazepam, GABA and PGBR, to identify proteins associated with mechanisms of drug treatment. The proteomic approaches found that 64 synaptic proteins were differentially expressed in all of the treatment. Of these proteins were observed in the GABAergic system, 4 proteins namely Gabbr1, Nsf, Prkcg, and Cacna1b. Intriguingly, 3 proteins regulated in the same direction after drug treatment, accepted Gabbr1.

From the conventional drug, diazepam increases apparent affinity of the GABAA receptor which it is important in the potentiation of GABAergic inhibition responses (Mihic et al., 1994). Interestingly, the protein kinase C gamma type (Prkcg), an enzyme member of the **protein kinase C (PKC)** family that can be mediating the effects of this drug on the GABAA receptors (Kumar et al., 2006; Kumar et al., 2010), was found up-regulation after drugs treatment. This suggests that the exhibit high rates of GABAA receptor activity mediate the GABAergic inhibition dramatically increased. This conceptually, high extrasynaptic GABA may show high activity GABAA receptors, for instance PGBR. Our previous study found the GABA levels in PGBR treated that were higher than diazepam treated and equivalent to GABA treated under drug-addicted in the rat frontal cortex (unpublished data, Veerasakul). In addition, the **N-ethylmaleimide-sensitive fusion protein (Nsf)** was found up-regulated in rat frontal cortex after treatment. The Nsf is involved in a variety of intracellular vesicle transport and fusion events, including GABAA receptor because its interact with the GABAA receptor-associated protein (GABARAP) to transporting GABAA receptors to the plasma membrane in neurons (Leil et al., 2004). Besides, the **voltage-dependent N-**

type calcium channel subunit alpha-1B (Cacna1b) was up-regulated in the frontal cortex after treatment. As a previous mention, Cacna1b partially controls GABA release, through Ca^{2+} influx in presynaptic neurons. Clinical studies have shown that blockage of N-type calcium channels increase anxiety in humans (Blazon et al., 2021). Taken together, the up-regulation of Prkcg, Nsf and Cacna1b revealed that the treatment enhances the GABAergic inhibition activity in the rat frontal cortex.

In summary, the findings of this study provides initial evidence for the treatment of drug-addicts for a basic knowledge for development of the treatment of addictive drugs. Moreover, in present proteomic study in GABA enriched-PGBR, the functions related to neurotransmitter did not worsen in the test group compared with conventional drug, and from these results, can affirm that PGBR were suitable for drug addiction treatment.

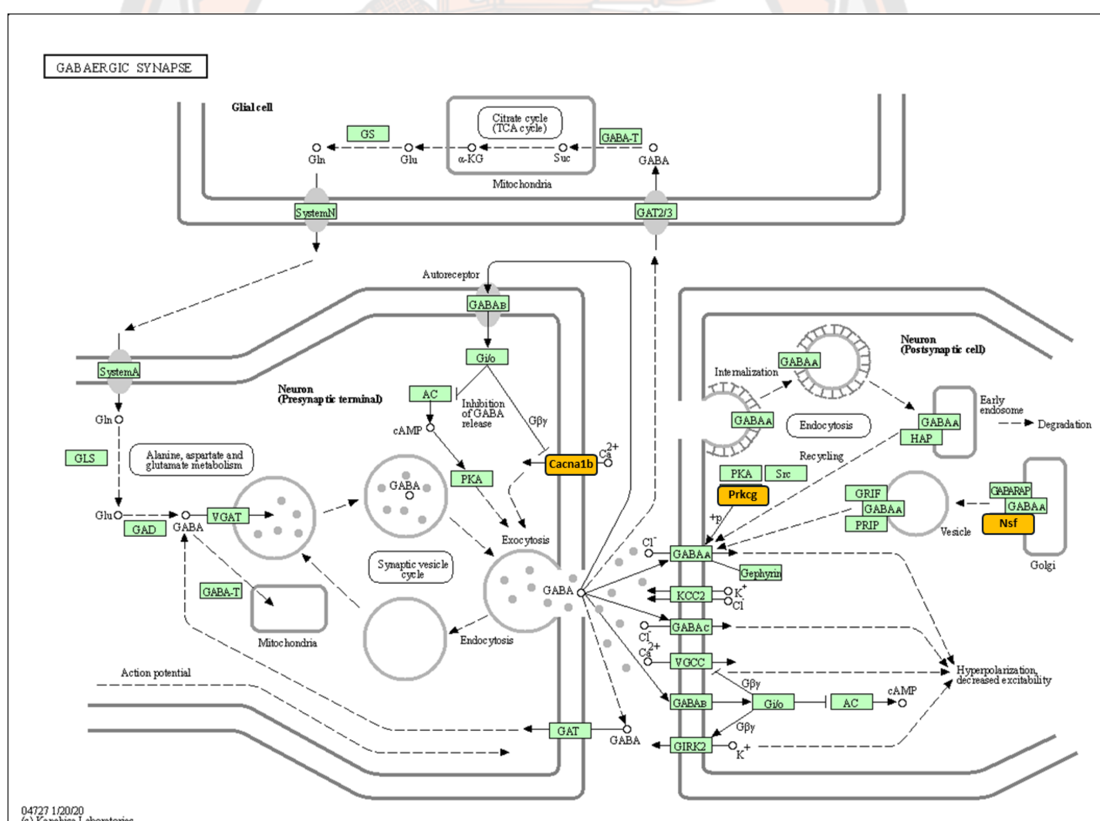


Figure 33 **the system of GABAergic. Yellow box was up-regulating proteins after treatment of drug-addicts. **The picture adapted from the KEGG pathway in DAVID 6.8.**

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CHAPTER VI

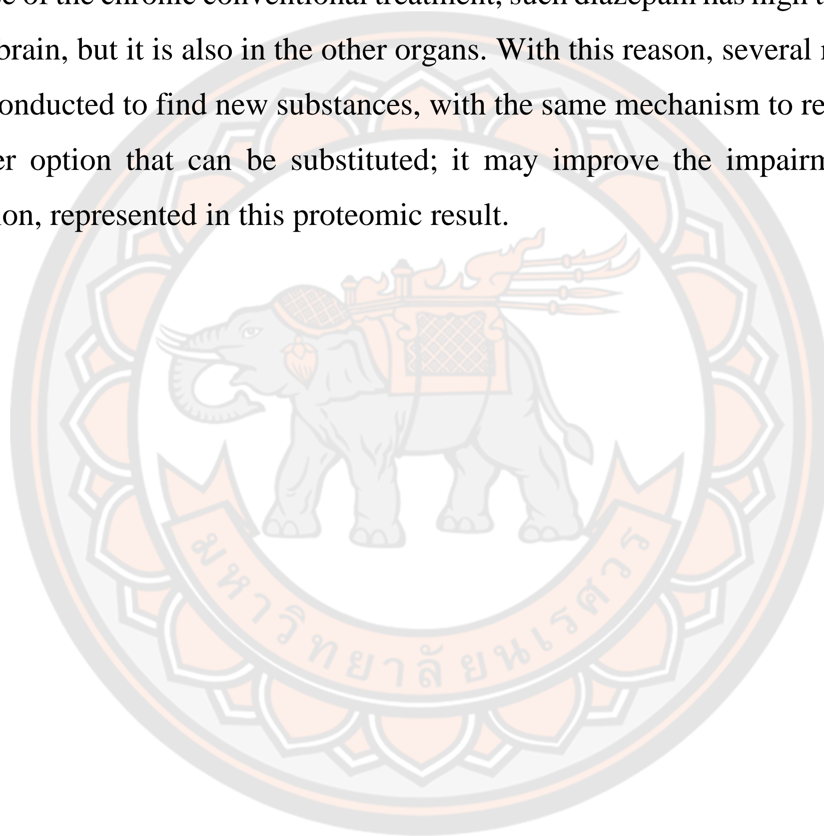
CONCLUSION

This proteomic study found protein alterations in glutamatergic and GABAergic neurotransmitter systems after drug addiction in the frontal cortex, including Gria3 receptor and calbindin. Importantly, the result demonstrated that the level of Gria3 was downregulated in METH addiction, which may explain the roles in the development of drug dependence. Moreover, our previous study suggests that up-regulation of GluR1 in METH-induced hyperlocomotion and stereotypes. These imply that decrease of Gria3 effect to increase functional impact of GluR1 leads to METH-induced behavioral abnormalities. While, several human studies suggest the alteration of Gria3 gene implicated in many psychiatric disorders, such as schizophrenia and bipolar disorder. Along with this result, the alteration of calbindin was found after METH administration in the frontal cortex and can be reflected in the GABAergic dysfunction after METH exposure. Nevertheless, it has been complicated between calbindin mRNA and protein levels after METH-treated in the brain. It may be methamphetamine toxicity that interrupts the mRNA translation in the brain, resulting in the lowering of calbindin protein. Thus, METH triggers GABAergic neurotransmission dysfunction on calbindin markers further for better understanding.

By comparing the proteins expression profiles in response to drug addiction, the proteomic results found four proteins associated with the exposure to multiple drug, containing Voltage-dependent N-type calcium channel subunit alpha-1B (Cacna1b), disks large-associated protein 1 (Dlgap1), GABA receptor subunit gamma-3 (Gabrg3), Inositol trisphosphate receptors type 1 (Itpr1) and phospholipase D2 (Pld2). Similarly, it is indicated that their chemical properties and the receptors interacting with different drugs may cause similar changes in cellular activities in neurons. Further research is necessary to demonstrate that substances of abuse may directly or indirectly interfere with these proteins, which may be related to regulation of neurotoxicity response to drug exposure. Furthermore, the expression of neuronal signaling proteins, including glutamatergic and GABAergic signalling, showed alteration after drug withdrawal.

These alteration proteins were suggested to decrease glutamatergic and GABAergic function, implicated to anxiety-like behaviour. This implies that the drug withdrawal (stop drug taking) cannot recover from neurotransmitter dysfunction after drug abuse, even though long-term withdrawal.

Furthermore, the proteomic analysis revealed that the drug treatment, containing diazepam, GABA and PGBR, that perform the same targets and pathways to treat drug addiction via regulation of GABAergic signaling proteins. However, because of the chronic conventional treatment, such diazepam has high toxicity not only in the brain, but it is also in the other organs. With this reason, several researches have been conducted to find new substances, with the same mechanism to replace. PGBR is a better option that can be substituted; it may improve the impairment from drug addiction, represented in this proteomic result.



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