Research Article

Proteomics analysis of rat testis reveals changes of proteins involving the signal transduction after methamphetamine exposure

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Abstract

Methamphetamine (METH) is an addictive drug potentially affecting the male reproductive system. It causes poor sperm quality and an increase in apoptotic cells within the seminiferous tubule. METH administration can also result in the changes of dopamine, norepinephrine, and GABA in rat testis. These findings provide the hypothesis that METH might associate with the changes of proteins involving biological process in spermatogenesis. Therefore, the aim of this study is to investigate the expression of the signal transduction proteins underlying biological processes in the testis of METH-administered rats using proteomics analysis. Male Sprague-Dawley rats in a control group were received normal saline for 15 days, whereas those rats in an ED-binge METH group were received an escalating dose of METH for 14 days following a binge dose of METH on day 15. Proteins were extracted from the rat testis, which were pooled from three samples in each group. The liquid chromatography-tandem mass spectrometry was performed to identify the protein profiles. The total of 383 proteins were identified in the testis of both groups. 38 proteins were mapped in the signal transduction sub-class underlying biological processes including 19 proteins with up-regulated expression and 19 proteins with down-regulated expression. Moreover, there were 8 proteins responding to METH exposure. In conclusion, our findings will be useful for better understanding of METH on protein expression resulting in the impairment of spermatogenesis in the testis.

Keywords: Methamphetamine, Proteomics, Signal transduction, Testis, Rat

Introduction

Drug addiction is characterized by persistent drug-seeking, drug-taking behaviors, and drug use [1], [2]. Methamphetamine (METH) addiction is often abused in people worldwide. The potential adverse effects of METH are found not only on the central nervous system (CNS) but also on the male reproductive system. METH causes the changes of neurotransmitter in the brain such as dopamine (DA), norepinephrine (NE), gamma-aminobutyric acid (GABA), and glutamate as well as the impairments of brain structure and function [3], [4], [5], [6]. Moreover, METH has been reported the adverse effects on the hypothalamic-pituitary-gonadal (HPG) axis, which plays a role in the testicular function, such as the change of gonadotropin-releasing hormone (GnRH) and testosterone concentrations [7]. Several previous studies have suggested that METH administration results in poor sperm quality such as the decrease in sperm concentration, motility, and normal sperm morphology as well as the increase in apoptotic activity in seminiferous tubules in testis [8], [9], [10]. Additionally, a previous study in our research team has also reported the change of sex steroid hormone receptors such as estrogen and progesterone receptors after METH exposure [11]. Interestingly, METH-administrated rats have been revealed the alteration of DA, NE, and GABA in the testis, which might underlie the impairment of spermatogenesis and testosterone production after METH exposure [12], [13].

Spermatogenesis is a complex cellular process including the proliferation of spermatogonial stem cells (SSCs) in the seminiferous tubules of the testis, and spermatogenic differentiation into mature sperm. These processes are maintained by the coordination of specific proteins in each stage of germ cell development and regulated by hormones in HPG axis such as GnRH, luteinizing hormone (LH) and follicle stimulating hormone (FSH) [14], [15]. Therefore, we hypothesized that these changes might be associated with the signal transduction pathways underlying testicular dysfunction including spermatogenesis and sex steroid hormone production.

Proteomics is a widely used technology for the identification and quantification of proteomes in cell, tissue, organism. Recently, proteomics has been used to evaluate the differential protein expression and functional output of protein in biological system on male reproductive system such as oxidative stress response, reproduction, and cellular process [16], [17]. Although the proteomics has been employed to evaluate the toxicity from other substances on male reproductive system, the proteomics study of the differential protein expression in testis because of METH exposure remained unclear. Therefore, this study aimed to investigate the changes of protein expression focusing on proteins involving the signal transduction underlying biological process in the testis of METH-administered rats using shotgun proteomics analysis.

Materials and methods

Chemicals

This study used D-METH hydrochloride (Lipomed AG, Arlesheim, Switzerland) with permission of the Ministry of Public Health, Thailand. METH was dissolved in saline (0.90% w/v of NaCl) [18].

Animals and METH administration

Male Sprague-Dawley rats ageing 5 weeks (280-350 g) from the National Laboratory Animal Center, Mahidol University, Nakorn Pathom, Thailand were used as experimental animals. They were housed in cages, temperature at 24 ± 1˚C under a 12/12 hours dark-light cycle with free access to food and water. The experimental protocol was approved by Animal Research Committee of Naresuan University, Thailand (Approval number: 62 02 012). All animal handlings were carried out by the Center for Animal Research Naresuan University following the National Institutes of Health (USA) Guidelines for the treatment of laboratory animals.

METH administration methods were adapted from Segal et al. and used as previously described [13], [19]. Briefly, all rats were divided into two groups including control and escalating dose-binge METH (ED-binge METH) groups. The rats in control group were injected intraperitoneally (i.p.) with 0.9% normal saline for 15 days, whereas those in ED-binge METH group were injected (i.p.) with the increasing doses of METH from 0.1 to 4.0 mg/kg, three times daily (3 hour intervals) for 14 days, and a binge dose of METH, four injections (i.p., 2 hour intervals) of 6.0 mg/kg METH, on the last day. At the end of the treatment, rats were sacrificed by cervical dislocation after anesthesia. Testes were removed immediately after sacrifice and kept at -80˚C until being used.

Protein extraction and digestion

Tissue of testis (30 mg) was homogenized in 5 mM Tris-HCl containing 25 mM NaCl, pH 8.0, and centrifuged at 14000 rpm for 10 min. After that, the pellet was collected and homogenized again in lysis buffer containing 50 mM Tris-HCl, 0.15 M NaCl, 0.1% SDS, 0.25% Sodium deoxycholate and 1% Protease inhibitor cocktail. Protein concentrations in the tissue lysates were measured by the bicinchoninic acid (BCA) assay. Protein samples (n=3) in each group were pooled for LS-MS/MS analysis. A total of 10 ug proteins were incubated with 10 mM dithiothreitol in 10 mM ammonium bicarbonate at 56˚C for an hour for the reduction. Then, the alkylation was performed by incubation with 30 mM iodoacetamide in 10 mM ammonium bicarbonate at room temperature for an hour in the dark box. To perform in-solution digestion, the proteins were incubated overnight with 50 ng trypsin in 10 mM ammonium bicarbonate at 37˚C.

LC-MS/MS and protein identification

The peptides were dried and resuspended in 0.1% formic acid. The peptide solution was centrifuged at 10,000 rpm for 10 min, and then transferred to vial tubes. The resuspended peptide was injected three times into the LC-MS/MS system. The protein quantification was analyzed by using the DeCyder MS Differential analaysis software (GE Healthcare), and then submitted for a database search by using the Mascot software. For protein identification, a protein database search was performed against the NCBI database. The Protein Analysis Through Evolutionary Relationships database (PANTHER) was used to classify the function of protein such as the biological process. The UniProt Knowledgebase (UniProt KB) was used to create the information of

the identified proteins [20]. Comparison of protein quantification was analysed by using the MultiExperiment Viewer (MeV, Version 4.9) software and shown as heatmap. The STITCH (http://stitch.embl.de/) 5.0 database was used to analyse protein-chemical interactions and generate the network [21].

Results

The total of 2,501 proteins in the Rattus database were identified including 1,389 proteins in control group, 729 proteins in ED-binge METH group, and 383 proteins in both groups (**Figure 1**). Only 383 proteins which were expressed in both groups were focused and classified by using the PANTHER software. They were categorized according to the biological process function in the regulation of molecular activity. Our results showed that three classes of the biological process were mostly found including cellular process (45%), biological regulation (26%), and metabolic process (23%), see in **Figure 2**. The identified proteins in the cellular process were mainly focused underlying their functions and identified in multiple sub-classes (e.g. cellular metabolic process, cellular component organization, cell cycle, cell communication, and signal transduction), as shown in **Figure 3.**

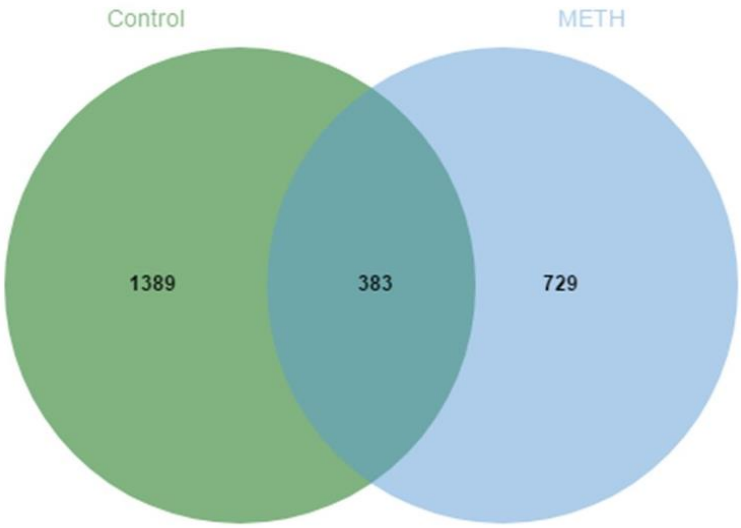

Figure 1 Venn diagram of protein expression with different intensity between control group and METH group of rat testis

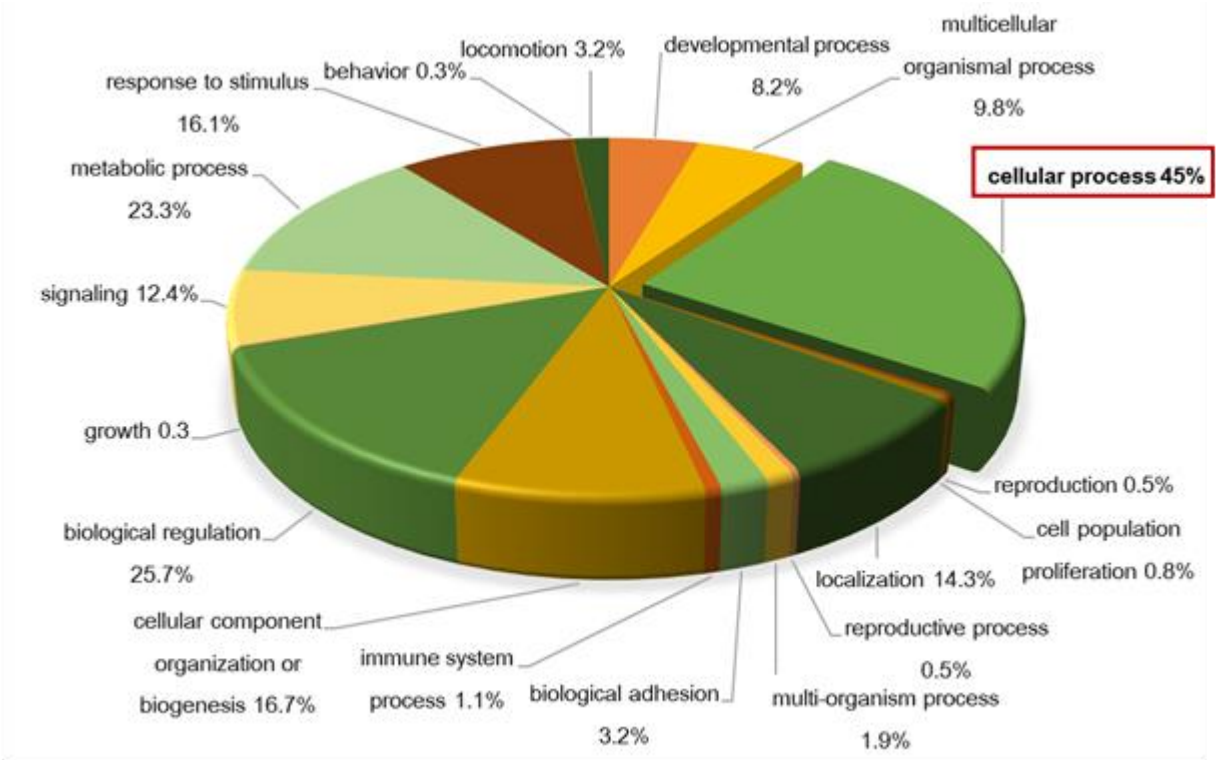

Figure 2 Percentage of Gene Ontology (GO) terms in biological process by PANTHER classification system.

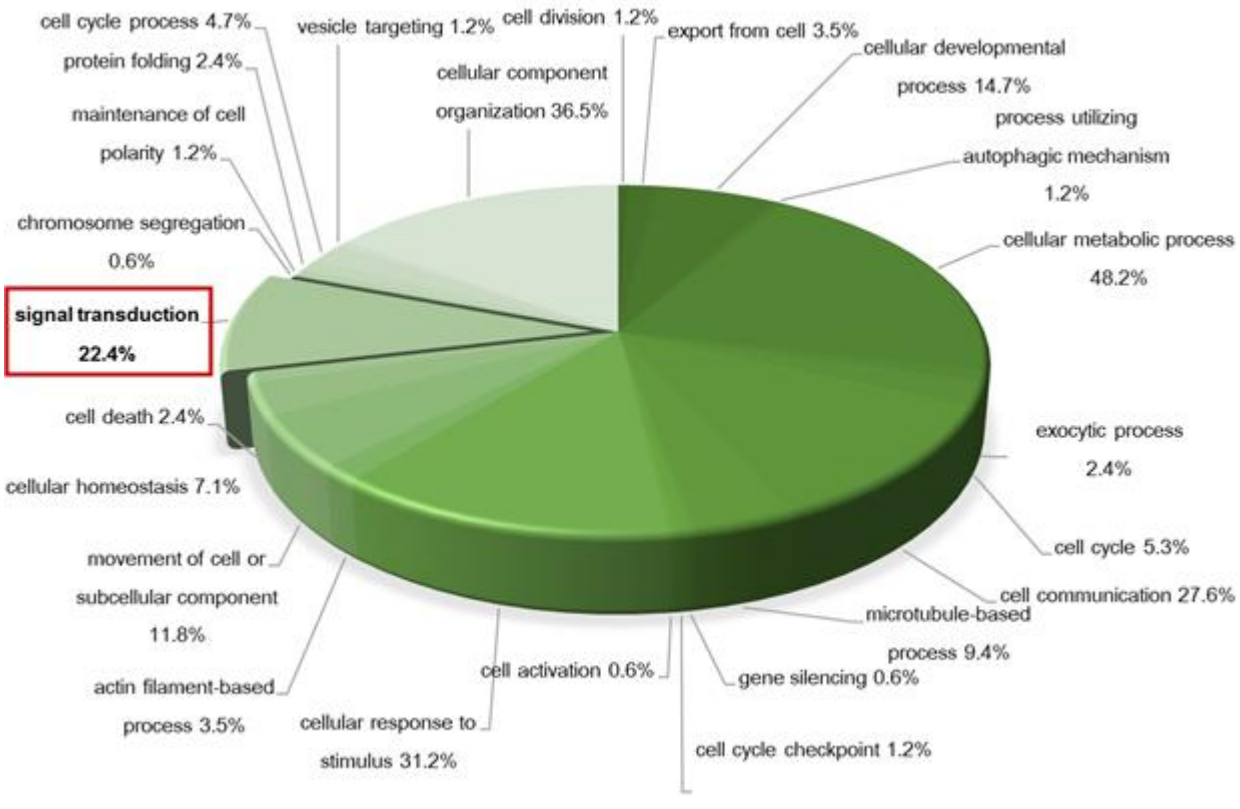

Figure 3 Percentage of sub-class in cellular process by PANTHER classification system.

Thirty-eight expressed proteins in the signal transduction sub-class were focused. An expression pattern of these proteins was analyzed by using the MultiExperiment Viewer (MeV, Version 4.9) software, as shown in **Figure 4**. We found that 19 proteins were downregulated, while 19 proteins were upregulated in the ED-binge METH group compared with the control group (**Table 1**). The results of the interaction network from STITCH 4.0 database in **Figure 5** represents the association between METH and 8 proteins including Histamine H2 receptor (Hrh2), Serine/threonine-protein kinase mTOR (Mtor), 1-phosphatidylinositol 4, 5-bisphosphate phosphodiesterase delta-4 (Plcd4), Leukotriene B4 receptor 2 (Ltb4r2), Alpha-1B adrenergic receptor (Adra1b), Thyrotropin-releasing hormone receptor (Trhr), Metabotropic glutamate receptor 5 (Grm5), and SH3 and multiple ankyrin repeat domains protein 1 (Shank1).

Table 1 Lists of differential expressed proteins after METH exposure associated with the signal transduction in rat testis.

Gene Symbol	Protein name	Accession no.	Fold change (log 2)*	Function
Down-regulation				
Gsk3a	Glycogen synthase kinase-3 alpha	P18265	-0.6	Cell growth and proliferation
Pkn2	Serine/threonine-protein kinase N2	O08874	-1.1	Apoptosis, Cell adhesion, Cell division
Smo	Smoothened homolog	P97698	-4.0	Regulation of signal transduction
Wnk1	Serine/threonine-protein kinase WNK1	Q9JIH7	-2.0	Intracellular signal transduction
Itgb1	Integrin beta-1	P49134	-0.3	Cell adhesion
Adgrl1	Adhesion G protein-coupled receptor L1	O88917	-0.03	Regulation of adenylate cyclase activity, Regulation of cAMP-mediated signaling
Apc	Adenomatous polyposis coli protein	P70478	-2.0	Regulation of cell differentiation
Ric8b	Synembryn-B	Q80ZG0	-3.4	Regulation of G protein-coupled receptor signaling pathway
Itgbl1	Integrin beta-like protein 1	Q5PQQ8	-0.5	Cell adhesion
Rita1	RBPJ-interacting and tubulin-associated protein 1	Q2KJ10	-0.2	Cell-cell communication
Pik3r3	Phosphatidylinositol 3-kinase regulatory subunit gamma	Q63789	-1.3	Regulation of kinase activity
Adgrl2	Adhesion G protein-coupled receptor L2	O88923	-1.7	Regulation of adenylate cyclase activity, Regulation of cAMP-mediated signaling
Prkce	Protein kinase C epsilon type	P09216	-1.0	Intracellular signal transduction
Hyou1	Hypoxia up-regulated protein 1	Q63617	-2.7	Regulation of cellular response to stress
Grm5	Metabotropic glutamate receptor 5	P31424	-3.8	Glutamate receptor signaling pathway

Gene Symbol	Protein name	Accession no.	Fold change (log 2)*	Function
Down-regulation				
Plcd4	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-4	Q62711	-2.5	Intracellular signal transduction, Acrosome reaction
Ccn3	CCN family member 3	Q9QZQ5	-0.8	Cell adhesion
Pak3	Serine/threonine-protein kinase PAK 3	Q62829	-2.6	Signal transduction by protein phosphorylation
Adra1b	Alpha-1B adrenergic receptor	P15823	-0.1	Cell-cell signaling
Up-regulation				
Card9	Caspase recruitment domain-containing protein 9	Q9EPY0	0.6	NF-kappaB signaling, transcription
Erbb2	Receptor tyrosine-protein kinase erbB-2	P06494	3.2	Transcription regulation
Plcb4	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-4	Q9QW07	2.4	Lipid degradation, Lipid metabolism
Ikbkb	Inhibitor of nuclear factor kappa-B kinase subunit beta	Q9QY78	3.2	Transcription regulation
Shank1	SH3 and multiple ankyrin repeat domains protein 1	Q9WV48	4.0	Regulation of signal transduction
Ltb4r2	Leukotriene B4 receptor 2	Q924U0	1.8	Inflammatory response
Hrh2	Histamine H2 receptor	P25102	0.5	Cell-cell signaling
Csnk1g3	Casein kinase I isoform gamma-3	Q62763	1.0	Cell-cell signaling
Trhr	Thyrotropin-releasing hormone receptor	Q01717	1.3	Regulation of cellular process
Itgb6	Integrin beta-6	Q6AYF4	4.1	Cell adhesion
Cblb	E3 ubiquitin-protein ligase CBL-B	Q8K4S7	2.4	Cell-cell communication
Btnl2	Butyrophilin-like protein 2	Q6MG97	1.0	Immune response
Ptk2b	Protein-tyrosine kinase 2-beta	P70600	0.1	Adaptive immunity
Ntrk2	BDNF/NT-3 growth factors receptor	Q63604	3.8	Differentiation
Mtor	Serine/threonine-protein kinase mTOR	P42346	3.5	Intracellular signal transduction
Plce1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1	Q99P84	1.5	Lipid degradation, Lipid metabolism
Acvr1b	Activin receptor type-1B	P80202	3.3	Protein phosphorylation
Prl6a1	Prolactin-6A1	P24800	0.6	Regulation of cell proliferation
Nrg2	Pro-neuregulin-2, membrane-bound isoform	O35569	0.3	Intracellular signal transduction

(*) Fold change in ED-binge METH group vs control group. A value > 0 represents up-regulation, whereas a value < 0 represents down-regulation.

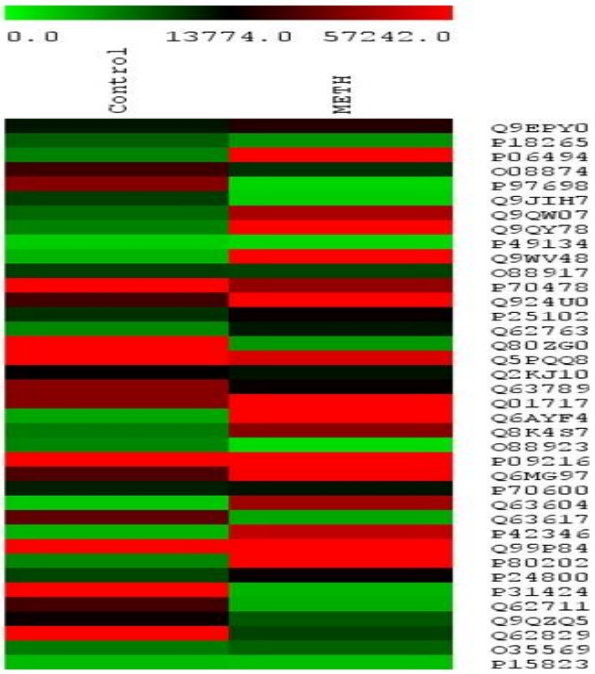

Figure 4 Heat map of differentially expressed proteins between control and METH-treated rats. (Absent in green, lowest in dark green, and highest in red)

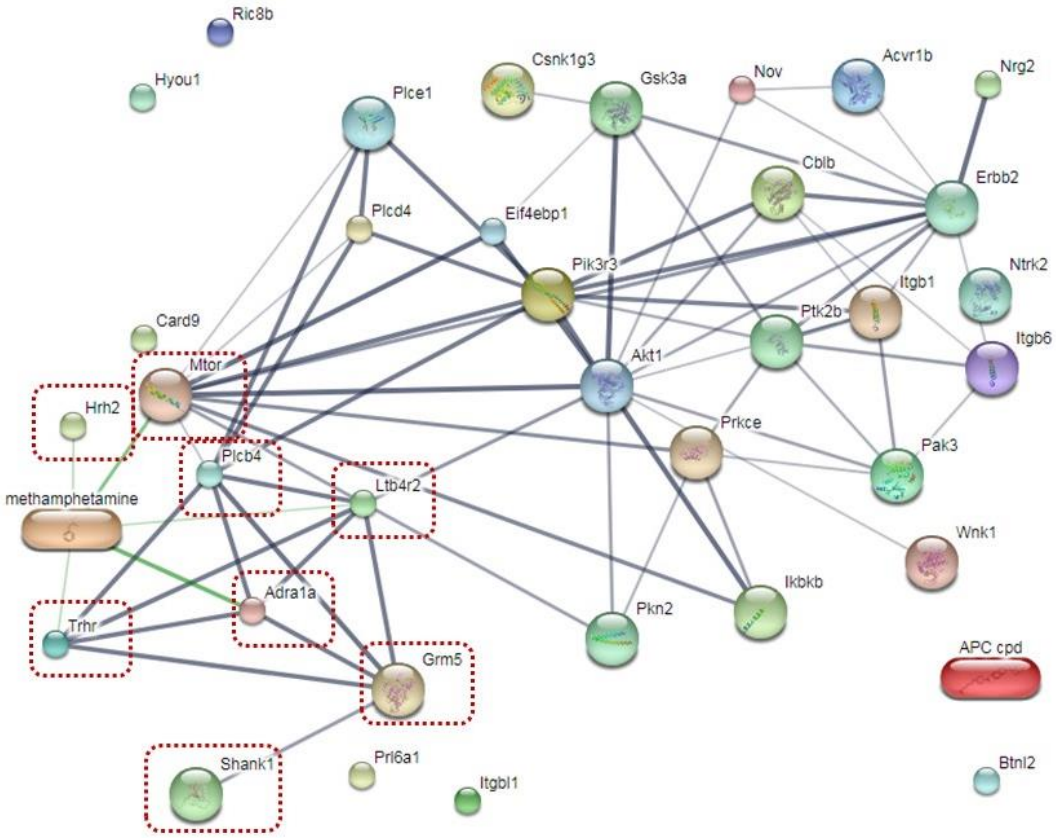

Figure 5 Predicted interactions between METH and expressed protein on signal transduction in rat testis.

Discussion and conclusion

In this study, the results of protein profiles in rat testis demonstrating through shotgun proteomics indicate the change of protein expression in METH-administrated rats compared to control. There were 383 differentially proteins expressing both in the control and the ED-binge METH groups. Most of these proteins were found in the category of the cellular process (45%) which consists of 38 proteins involving the signal transduction pathways. The results from quantitative proteomics analysis indicate that METH-administrated rats had lower levels of metabotropic glutamate receptor 5 (Grm5, log 2-fold change = -3.8) and higher levels of Serine/threonine-protein kinase mTOR (mTOR, log 2 fold change = 3.5) than that of control animals. Moreover, both Grm5 and mTOR had the association with METH.

The previous study has reported that metabotropic glutamate receptor (Grm) abundantly expressed in the testis and spermatozoa [22]. The expressions of Grm5 were localized in germinal cells of the seminiferous tubule in rat testis and in the mid-piece and tail of human sperm. This receptor plays a role in the regulation of the maturation of testis germinal cells and sperm motility [23]. Interestingly, our result showed that Grm5 alteration in testis was expressed in response to METH exposure. Moreover, in the present study, alpha-1B adrenergic receptor protein (Adra1b) also showed the interaction with METH; however, its expression in METH-administrated rats was slightly different from controls. Our results support the findings of the previous studies that METH can lead to the decrease of norepinephrine and its metabolite concentrations as well as alpha 1 adrenergic receptor in spermatogonia in rat testis [24]. Adrenergic receptors have been found in Leydig, Sertoli, and spermatogenic cells in testis [25]. Alpha-1B adrenergic receptor in knockout mice also showed the abnormal Sertoli cells morphology and spermatogenesis arrest [26]. Additionally, norepinephrine and adrenergic receptors have been involved in sperm functions such as sperm capacity, acrosome reaction and motility [27], [28], [29]. Therefore, the down-regulation of Grm5 and Adra1b in this study might be in response to METH exposure leading to sperm function abnormalities.

As mentioned earlier, METH affects not only the down-regulation of those proteins but also the up-regulation of mTOR, Trhr and Hrh2 protein. mTOR is an important signal transduction molecule interacting with growth factors, nutrition molecules, hormones, and other proteins to control protein synthesis. It can regulate the cell proliferation, growth, differentiation, and apoptosis [30]. Several studies have reported that mTOR plays an important role in spermatogonial cells and blood-testis barrier maintenance [31], [32]. Trhr is a small neuropeptide, which binds with thyrotropin-releasing hormone (TRH) to promote secretion of Thyroid-stimulating hormone (TSH) [33]. In addition, Trhr is distributed in rat testis, especially in the Leydig cells that are important in testosterone production [34], [35]. In this study, Hrh2 was up-regulated after METH exposure. Hrh2 is linked to an increase in cAMP and other intracellular signals. Besides, it has been found in germinal and interstitial cells of the testis, especially Leydig cells and macrophages. Previous studies also have been reported that Hrh2 plays a role in Leydig cell steroidogenesis and immune responses of testicular macrophages [36], [37]. The up-regulation of mTOR, Trhr, and Hrh2 might have occurred to compensate the impairment of testis because of METH exposure. However, the role of

mTOR, Trhr and Hrh2 on the reproductive system in the METH addiction is still unclear, further studies are needed.

In conclusion, ED-binge METH exposure leads to the changes in protein expression involving the signal transduction pathways in rat testis. These changes might affect an abnormality of sperm during spermatogenesis after METH exposure. However, our findings will be useful for further study in the validation of these proteins in testis for better understanding of METH mechanism on testicular function such as spermatogenesis.

Acknowledgements

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